NOVEL THERAPEUTIC APPROACH TARGETING THE HIF-HRE SYSTEM IN THE KIDNEY

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Abstract: Recent studies emphasize the role of chronic hypoxia in the tubulo-interstitium as a final common pathway to end-stage renal disease. Therefore, therapeutic approaches which target the chronic hypoxia should prove effective against a broad range of renal diseases.

Many of hypoxia-triggered protective mechanisms are hypoxia inducible factor (HIF)-dependent. Although HIF-1α and HIF-2α share both structural and functional similarity, they have different localization and can contribute in a non-redundant manner. While gene transfer of constitutively active HIF has been shown effective, pharmacological approaches to activate HIF are more desirable. Oxygen-dependent activation of prolyl hydroxylases (PHD) regulates the amount of HIF by degradation of this transcription factor. Therefore, PHD inhibitors have been the focus of recent studies on novel strategies to stabilize HIF. Cobalt is one of the inhibitors of PHD, and stimulation of HIF with cobalt is effective in a variety of kidney disease models. Furthermore, crystal structures of the catalytic domain of human prolyl hydroxylase 2 have been clarified recently. The structure aids in the design of PHD selective inhibitors for the treatment of hypoxic tissue injury.

Current advance has elucidated the detailed mechanism of hypoxia-induced transcription, giving hope for the development of novel therapeutic approaches against hypoxia.

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1. INTRODUCTION

Recent studies emphasize the role of chronic hypoxia in the tubulointerstitium as a final common pathway to end-stage renal failure[1]. Hypoxia in the kidney has been demonstrated in a variety of disease models utilizing pimonidazole staining, a Clark-type electrode, blood oxygen level dependent (BOLD)-MRI, and transgenic animals expressing a hypoxia-sensing reporter vector[2].

Chronic hypoxia of the kidney occurs via several mechanisms acting in concert. When advanced, tubulointerstitial damage is associated with the loss of peritubular capillaries. Associated interstitial fibrosis impairs oxygen diffusion and supply to tubular and interstitial cells. In addition, a number of mechanisms that induce tubulointerstitial hypoxia at an early stage have been identified. Glomerular injury and vasoconstriction of efferent arterioles due to imbalances in vasoactive substances decrease post-glomerular peritubular capillary blood flow. Oxidative stress also hampers the efficient utilization of oxygen in tubular cells, leading to reduced renal oxygen tension[3]. Relative hypoxia in the kidney also results from increased metabolic demand in tubular cells. Further, renal anemia hinders oxygen delivery. These factors can affect the kidney before the appearance of significant pathological changes in the vasculature and predispose the kidney to tubulointerstitial injury.

Therefore, therapeutic approaches which target the chronic hypoxia should prove effective against a broad range of renal diseases. At the center of the cellular response to hypoxia is hypoxia-inducible factor, HIF, and activation of this “master gene” switch results in a broad and coordinated downstream reaction to protect organs against hypoxia.

2. HIF IN THE KIDNEY

HIF is composed of two subunits, an oxygen-sensitive HIF-α subunit and a constitutively expressed HIF-β subunit (also known as ARNT, the aryl hydrocarbon receptor nuclear translocator). Both HIF-1α and HIF-1β are members of the basic helix-loop-helix PER/ARNT/SIM (HLH-PAS) family of transcription factors. HIF binds to the HRE in the cis-regulatory regions of its target genes, and transcriptionally activates various genes encoding proteins that mediate adaptive responses to reduced oxygen availability. Under normoxic conditions, two conserved proline residues within the central oxygen-dependent degradation domains of the HIF proteins are hydroxylated by the protein products “*p*rolyl *h*ydroxylase *d*omain containing” (PHDs). This promotes binding of the von Hippel Lindau tumor suppressor protein (pVHL), part of a ubiquitin ligase complex, resulting in polyubiquitylation and rapid degradation. Similarly, a conserved asparagine residue in the carboxyl-terminal transactivation domain (CAD) of the HIF proteins is hydroxylated in normoxia by factor inhibiting HIF (FIH), preventing recruitment of the p300/CBP transcriptional co-activators and thus leading to transcriptional repression. Under hypoxia, oxygen is lacking as an essential substrate for the hydroxylation reaction, and the unmodified HIF proteins avoid degradation but rather heterodimerize with HIF-β and up-regulate the transcription of target genes.

HIF-α subunits have different isoforms, and a biological role of each isoform remains to be elucidated. HIF-1α is expressed in most cell types, whereas HIF-2α shows a more restricted pattern of expression. In the adult kidney, HIF-2α is expressed in peritubular endothelial cells and fibroblasts as well as glomerular endothelial cells,

whereas HIF-1α is predominantly localized in tubular cells[4,5]. Up-regulation of the two HIF-α isoforms in the kidney by hypoxia was demonstrated in models of segmental renal infarction and radio contrast nephropathy[6,7]. While cell-type specificity of HIF isoforms in these models was consistent with previous findings, temporal and spatial profiles of HIF activation were relatively complex, suggesting an important but complicated role of HIF in tissue preservation as a response to regional renal hypoxia.

Because mice with complete deficiency of HIF are embryonic or perinatal lethal, analysis of a biologic function of a HIF isoform after birth has been hampered. One possible way to overcome this problem is to utilize heterozygous knockout mice. Studies using *Hif1a*$^{+/-}$ mice showed no benefit of preconditioning of the heart by hypoxia, demonstrating that cardiac protection against ischemia-reperfusion injury by preconditioning is critically dependent on *Hif1a* gene dosage[8]. We employed HIF-2α knockdown mice and showed that these mice were more susceptible to ischemia-reperfusion injury model of the kidney[9]. Our studies utilizing the Cre-loxP system to rescue HIF-2α specifically in the endothelium demonstrated that HIF-2α in the kidney endothelium is responsible for regulation of oxidative stress and subsequent tubulointerstitial injury.

3. PHD IN THE KIDNEY

Under normoxia, hydroxylation of HIF-α-subunits by HIF prolyl hydroxylases (PHD) is required for binding to the pVHL-E3-ubiquitin ligase complex. After polyubiquitination, HIF-α is degraded by the proteasome. The enzymatic activity of PHD depends on iron as the activating metal, 2-oxoglutarate as a co-substrate, and ascorbic acid as a cofactor. Three PHD with the potential to catalyze this reaction have been identified, and these proteins, termed PHD1, PHD2, and PHD3, appear to have arisen by gene duplication. In the kidney all three isoforms of PHD are expressed, and all PHD exhibited much higher levels in renal medulla than cortex[10]. PHD2 is the most abundant isoform in various organs including the kidney[11]. The contribution of each to the physiological regulation of HIF remains uncertain.

Recent experiments using suppression by small interference RNA showed that each of the three PHD isoforms contributes in a non-redundant manner to the regulation of both HIF-1α and HIF-2α subunits, and that the contribution of each PHD is strongly dependent on its relative abundance[12]. Although Phd1(-/-) and Phd3(-/-) mice did not display apparent angiogenic defects, conditional knockout of Phd2 led to hyperactive angiogenesis and angiectasia, demonstrating a major role of PHD2 as a negative regulator for vascular growth in adult mice[13].

4. GENE TRANSFER TO ACTIVATE HIF IN THE KIDNEY

We previously showed that *in vivo* gene transfer of DNA expressing constitutively active fusion protein of HIF protects the kidney against ischemic injury[14]. Recently phase I trial of adenoviral delivery of a constitutively active form of HIF was conducted in patients with critical limb ischemia. HIF gene transfer therapy was well tolerated in the patients, and at 1 year, limb status observations in HIF-1α patients included complete rest pain resolution in 14 of 32 patients and complete ulcer healing in 5 of 18 patients[15].

5. PHD INHIBITORS

PHD inhibitors have been the focus of recent studies on novel strategies to stabilize HIF. More than half a century ago, oral administration of cobaltous chloride was employed to treat anemia associated with chronic renal disease and led to a transient but significant erythropoietic response[16]. Although the mechanism of erythropoiesis was unknown at that time, cobalt is now recognized as an inhibitor of PHD, and thereby serves as a stimulator of HIF. We demonstrated the renoprotective effects of chemical pre-conditioning with cobaltous chloride in an ischemic model of renal injury[17]. Administration of cobalt induced up-regulation of HIF-regulated genes, such as VEGF and EPO, and subsequently protected the kidney against the tubulointerstitial damage induced by hypoxia. Pretreatment of experimental animals with cobalt also ameliorated disease manifestations of a new model of glomerulonephritis induced by co-administration of angiotensin II and Habu snake venom[18]. Furthermore, we showed that cobalt treatment was effective when given after the initial insult in a chronic progressive glomerulonephritis model, a model of cyclosporin nephrotoxicity, and a model of chronic renal failure with glomerular hypertension, demonstrating not only its preventive but also its therapeutic potential[19-21]. HIF activation in these models restored the peritubular capillary network, reduced the number of apoptotic cells, and improved renal functions as well as histological damages.

Although cobalt administration has been somewhat effective in experimental animals, long-term administration to humans is hindered by various side effects. Less toxic and more potent PHD inhibitors are desirable, and a variety of new candidates are now under development. Three distinct PHD inhibitors, l-mimosine (L-Mim), ethyl 3,4-dihydroxybenzoate (3,4-DHB), and 6-chlor-3-hydroxychinolin-2-carbonic acid-N-carboxymethylamid (S956711), induced HIF-α protein in human and rodent cells and enhanced angiogenesis in the sponge assays[22]. Systemic administration of L-Mim and S956711 in rats led to HIF-α induction in the kidney. Systemic treatment of mice with 3,4-DHB displayed significantly increased viability and enhanced exercise performance in severe hypoxia[23]. Pretreatment with the novel PHD inhibitor FG-4487 strongly induced the accumulation of HIF-1α and HIF-2α in tubular and peritubular cells, respectively, with significant amelioration of ischemic renal injury[24].

Technological advances in computer-based drug design will be useful to develop specific PHD inhibitors. Recent studies by Schofield's group clarified crystal structures of the catalytic domain of human PHD2[25], and this information enables us to perform docking simulation based on the three-dimensional structure of PHD2 for development of novel compounds.

6. OTHER TARGETS OF HIF ACTIVATION THERAPY

FIH hydroxylates regulates HIF activation via hydroxylation of an asparagine residue in the COOH-terminal transactivation domain of HIF-α. During hypoxia, asparagine hydroxylation is blocked and CBP/p300 recruitment is facilitated, enabling increased levels of transcription of the target genes. In the kidney, FIH is expressed in tubular epithelial cells and glomeruli, as shown by immunohistochemical methods[26]. Thus, inhibition of FIH can lead to increased HIF target gene expression.

The phosphoinositide 3-kinase (PI3K)/Akt pathway and the protein kinase C signaling have also been implicated in the regulation of HIF-α. While the dogma proposes that stability is the rate-limiting factor to determine the protein levels of HIF-α, some inputs including activation of these pathways function at the level of transcription and translation.

Other proteins that govern HIF activity include Siah2, OS-9, ING4 (inhibitor of growth 4), Hsp90 (heat-shock protein 90), ARD1 (arrest at start of cell cycle defective 1), and p53 among others. Whether these pathways can be a good target for therapeutic approaches in kidney disease *in vivo* remains to be elucidated.

7. CONCLUSION

Chronic hypoxia is the final common pathway to end-stage renal failure, and accumulating evidence suggests the pathogenic role of hypoxia from an early stage of kidney disease. Therapeutic approaches against this final common pathway should be effective in a broad range of renal diseases. Although a fine line between therapeutic benefit and harmful side effects must be drawn, HIF activation is promising, as it facilitates a variety of defensive mechanisms in a coordinated manner.

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