Diabetic nephropathy: are there new and potentially promising therapies targeting oxygen biology?

Toshio Miyata[1], Norio Suzuki[1] and Charles van Ypersele de Strihou[2]

[1]United Centers for Advanced Research and Translational Medicine (ART), Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan and [2]Service de Nephrologie, Universite Catholique de Louvain, Brussels, Belgium

The multipronged drug approach targeting blood pressure and serum levels of glucose, insulin, and lipids fails to fully prevent diabetic nephropathy (DN). Recently, a broad range of anomalies associated with oxygen biology, such as hypoxia, oxidative stress (OS), and dyserythropoiesis, have been implicated in DN. This review delineates the cellular mechanisms of these anomalies to pinpoint novel therapeutic approaches. The PHD–HIF system mitigates hypoxia: HIF activates a broad range of reactions against hypoxia whereas PHD is an intracellular oxygen sensor negatively regulating HIF. The Keap1–Nrf2 system mitigates OS: Nrf2 activates cellular reactions against OS whereas Keap1 negatively regulates Nrf2. Clinical trials of PHD inhibitors to correct anemia in patients with CKD as well as of a Nrf2 activator, bardoxolone methyl, for DN are under way, even if the latter has been recently interrupted. A specific PHD1 inhibitor, a Keap1 inhibitor, and an allosteric effector of hemoglobin may offer alternative, novel therapies. Erythropoietin (EPO) is critical for the development of erythroid progenitors and thus for tissue oxygen supply. Renal EPO-producing (REP) cells, originating from neural crests, but not fibroblasts from injured tubular epithelial cells, transdifferentiate into myofibroblasts and contribute to renal fibrosis. Agents restoring the initial function of REP cells might retard renal fibrosis. These newer approaches targeting oxygen biology may offer new treatments not only for DN but also for several diseases in which hypoxia and/or OS is a final, common pathway.

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Correspondence: *Toshio Miyata, United Centers for Advanced Research and Translational Medicine (ART), Tohoku University Graduate School of Medicine, 2-1 Seiryo-Machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan. E-mail: miyata@med.tohoku.ac.jp*

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Numerous factors have been implicated in the development of diabetic nephropathy (DN). Their actual significance has been documented in several animal and human studies by the demonstration that their inhibition slowed the progression of DN. Still, despite a multipronged drug approach targeting blood pressure, serum levels of glucose, insulin, lipids, obesity, and so on, full prevention of DN remains elusive. Newer culprits thus remain to be identified. Besides hemodynamic and metabolic abnormalities, a broad range of abnormalities associated with oxygen biology, such as hypoxia, oxidative stress (OS), and dyserythropoiesis, have emerged in our understanding of DN.

All mammalian organs require a supply of oxygen to fuel various biometabolic processes. A decreased oxygen supply, that is, hypoxia, induces not only acute disorders such as ischemic heart disease but also chronic disorders such as renal fibrosis. OS during hypoxia may sound paradoxical. Yet, it may be induced not only by a rise but also a fall in oxygen tension. Hypoxic cells rely on anaerobic glycolysis to generate adenosine-5′-triphosphate but their residual low oxygen supply supports some level of oxidative production of adenosine-5′-triphosphate through the tricarboxylic acid cycle and electron transport chain. Electrons leaking from the mitochondrial electron transport chain generate an excess of reactive oxygen species (ROS), that is, OS. Thus, hypoxia and OS are closely linked. Reoxygenation or high oxygen levels following severe hypoxia further exaggerate ROS generation, a concept validated by the clinical benefits accruing from the use of agents able to scavenge ROS or the prevention of their formation in hypoxic lesions.[1]

Erythropoietin (EPO) is essential for the proliferation and differentiation of erythroid progenitors and hence of tissue oxygen supply.[2] Recent studies have unraveled the cellular mechanism of renal EPO production and the sequential events leading to renal fibrosis, both of which are closely linked to each other.[3–6] In contrast to previous knowledge, fibroblasts originating from injured tubular epithelial cells do not play a major role in renal fibrosis, but renal EPO-producing (REP) cells, stemming from neural crests, do transdifferentiate into myofibroblasts upon long-term exposure to inflammatory conditions and

contribute to renal fibrosis.[6] Fortunately, to some extent, REP cells retain their plasticity: in experimental animals, some agents restore their initial function and retard renal fibrosis.[6] These observations provide the missing link in chronic kidney disease (CKD) between anemia and renal fibrosis.

We review the cellular mechanisms of various abnormalities associated with oxygen biology, such as hypoxia, OS, and dyserythropoiesis, with an emphasis on the genesis of DN. Eventually, we propose novel and potentially promising therapeutic approaches for DN.

OXIDATIVE STRESS

OS results from the accumulation of ROS and disrupts cellular function. Its existence and its possible localization in diabetes have been disputed. Williamson *et al.*[7] demonstrated an increased cellular nicotinamide adenine dinucleotide ratio ($NADH/NAD^+$) and suggested that diabetes is a state of 'reductive stress' and 'pseudo-hypoxia' rather than OS. On the contrary, OS was postulated in diabetes on the basis of indirect evidence including increased nicotinamide adenine dinucleotide phosphate ratio ($NADP^+/NADPH$) and of oxidized to reduced glutathione.[8–10] Still, Wells-Knecht *et al.*[11] argued against a 'generalized' OS in diabetes: the age-adjusted levels in skin collagen of two oxidized amino acids, *ortho*-tyrosine and methionine sulfoxide, proved virtually identical in diabetics and nondiabetics.

In contrast, we demonstrated a 'local' OS in the human diabetic kidney.[12,13] Advanced glycation end products (AGEs), generated nonenzymatically with sugars on proteins, include two different classes of structures: OS-dependent molecules (pentosidine and N^{ε}-(carboxymethyl)lysine) and OS-independent molecules (pyrraline). Should tissue AGE formation depend solely on hyperglycemia, all AGE structures should be detected in the diabetic kidney. The identification of individual AGE structures established that this is not the case. Both pentosidine and N^{ε}-(carboxymethyl)lysine were present in diabetic glomerular lesions, together with other protein modifications derived from the oxidation of lipids (for example, malondialdehyde–lysine), whereas pyrraline was absent. The contention of a 'local' OS in DN was subsequently confirmed in diabetic vascular lesions by us and others[14,15] and is now supported by a large body of evidence gathered in *in vitro* experiments as well as in *in vivo* animal and human studies.[16,17]

The primary cause of local OS in DN remains debated as ROS are generated by numerous enzymatic and nonenzymatic sources,[18–22] for example, the activation of the renin–angiotensin system, of NADPH oxidase, of nitric oxide synthase, and so on.

A newer pathway for OS has recently emerged: the prolylhydroxylase-1 (PHD1)–hypoxia-inducible factor (HIF) system. Aragonés *et al.*[23] demonstrated in mice that the genetic disruption of PHD1, an intracellular oxygen sensor, lowers oxygen consumption in mitochondria of skeletal muscle, mitigates the OS, and enhances cellular survival during hypoxia.

HYPOXIA

Renal tissue hypoxia remains difficult to document directly from blood or urine analyses; however, recently, molecular imaging technologies have allowed an evaluation of renal oxygen levels. For instance, blood oxygen level–dependent magnetic resonance imaging performed in a healthy subject given 1 l water load after an 8-h water restriction documents a significant increase in the oxygen level of the renal outer medulla.[24] In addition, inhibition of sodium reabsorption in the outer medulla by furosemide should reduce oxygen consumption and, indeed, renal blood oxygen level–dependent magnetic resonance imaging reveals a rise in medullary oxygen level within 15 min after furosemide administration to a healthy subject.[24]

Tissue hypoxia in the streptozotocin-induced diabetic rat kidney has been visualized by Ries *et al.*[25] by blood oxygen level–dependent imaging, a finding confirmed later by Rosenberger *et al.*[26] by pimonidazole staining (a probe to detect hypoxia) and HIF.

Localization of hypoxia within the kidney is hampered by the scarcity of methods that are able to identify and quantify tissue oxygenation at the cellular level. Tanaka *et al.*[27] relied on a new hypoxia-responsive reporter vector to generate a novel hypoxia-sensing transgenic rat. In this model, they identified 'diffuse cortical' hypoxia in the puromycin aminonucleoside-induced nephrotic syndrome and 'focal and segmental' hypoxia in the remnant kidney model. In both models, the degree of hypoxia was positively correlated with microscopic tubulointerstitial injury. Localization of tissue hypoxia may thus differ according to the type of renal disease, but remains precisely elusive in DN.

The causes of chronic hypoxia in DN are heterogeneous.[28–31] Glomerular efferent arterioles enter the peritubular capillary plexus to provide oxygen to tubular and interstitial cells. Lesions in efferent arterioles decrease the number of peritubular capillaries, which in turn impair oxygen diffusion to tubulointerstitial cells and lead eventually to tubular dysfunction and fibrosis. Dyserythropoiesis and anemia associated with chronic kidney disease further hinder oxygen supply.

Hypoxia not only causes local OS in DN, but also affects various biological reactions linked to oxygen metabolism,[32] including nitrosative stress,[33–35] advanced glycation and carbonyl stress,[36–38] and endoplasmin reticulum stress.[39,40] The interrelationship between these detrimental chain reactions is so complex that a single culprit unlikely accounts for the alterations of DN. Whatever the sequential events of diabetic renal injury, the consequences of hypoxia and the attendant impairment of oxygen metabolism is pivotal in the genesis and progression of DN. Therapies interfering with it may prove clinically useful.

DYSERYTHROPOIESIS

EPO production occurs mainly in the kidney and is reduced in CKD patients with an eventual anemia.[28] Plasma EPO concentration is dramatically reduced in a uremic animal

model.[41,42] Recombinant human EPO has been used for more than 20 years in CKD to compensate for the reduced endogenous EPO production.[3]

Recent studies have indicated that EPO administration improves kidney functions in CKD either directly or indirectly.[43] Low hemoglobin levels are associated with adverse outcomes such as renal and cardiac failure, the so-called cardio–renal anemia syndrome.[43,44] A broad array of cellular processes is modulated not only by the mitigation of hypoxia but also by the development of progenitor stem cell, cellular integrity, and angiogenesis.[43,44] The therapeutic benefits of EPO beyond the correction of anemia are still debated. It is noteworthy that recently evidence has been published on the pleiotropic effects of EPO on the central nervous and the cardiovascular systems as well as on the kidney.[45–47]

CELLULAR MECHANISMS

PHD–HIF pathway

Defense against hypoxia hinges upon the HIF[48,49] that activates a broad range of genes that stimulate erythrocytosis, angiogenesis, glucose metabolism, or cell proliferation/survival, and eventually protect hypoxic tissues. The level of HIF-α is determined by its oxygen-dependent degradation rate. In the presence of oxygen, it undergoes enzymatic hydroxylation by PHDs,[50,51] is recognized by the Hippel–Lindau tumor-suppressor protein (pVHL),[52,53] acting as an E3 ubiquitin ligase, and is rapidly degraded by the proteasome (Figure 1, upper panel).[54,55] During hypoxia, the nonhydroxylated HIF-α escapes interaction with Hippel–Lindau tumor-suppressor protein, is thus stabilized, and binds to its heterodimeric partner HIF-1β, mainly in the nucleus, to transactivate genes involved in the adaptation to hypoxic–ischemic stress.[56]

Three isoforms of the HIF-α subunit have been identified (that is, HIF-1α, HIF-2α, and HIF-3α).[57] HIF-1α and HIF-2α are structurally and functionally similar. In contrast, HIF-3α lacks the structures for transactivation present in the C-termini of HIF-1α and HIF-2α and might play an alternative role as a negative regulator of hypoxia-inducible gene expression.

Recent studies in mice, utilizing gene disruption of either HIF-1α or HIF-2α, disclosed that HIF-2α acts as a physiological regulator of EPO.[58] In humans, the *HIF2A* gene is responsible for familial erythrocytosis[59] and for comparatively high hemoglobin concentrations in polycystic kidney disease[60] (pericystic hypoxia leading to HIF-2 induction). In addition, it plays a crucial role in the defense against OS.[23,61]

PHDs belong to the Fe(II) and 2-oxoglutarate-dependent dioxygenase superfamily, which incorporates two atoms of molecular oxygen into their substrates:[57] 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.[62]

PHD activity critically requires iron and is thus inhibited by transition metal chelators.[62] Cobalt chloride inhibits PHD activity through an intracellular depletion of ascorbate necessary for iron (reduced) activity.[63] Its erythropoietic effect is known in humans since the 1940s[64,65] and has been utilized in the 1970s to treat anemia associated with chronic renal failure.[66] Unfortunately, cobalt chloride proved too toxic and is no longer in clinical use.

Three different PHD isoforms have been identified (that is, PHD1, PHD2, and PHD3),[57] each of which has its own tissue and subcellular distribution.[67,68] PHD1 is exclusively nuclear, PHD2 is mainly cytoplasmic (but shuttles between nucleus and cytoplasm), and PHD3 is present in both cytoplasm and nucleus. PHD2 acts as a decisive oxygen sensor in the HIF degradation pathway.[69] Although hypoxia decreases overall PHD activity, upregulation of HIF-1α induces the expression of PHD2 and PHD3.[70] This HIF-induced PHD expression ensures rapid removal of HIF-α after reoxygenation. Feedback loops may thus exist during hypoxia signaling.[71,72]

Keap1–Nrf2 pathway

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), a transcriptional factor, regulates the expression of several cellular antioxidant and cytoprotective genes[73,74] (Figure 1, lower panel). Upon exposure to OS and/or electrophiles, Nrf2 translocates into nuclei, heterodimerizes with a small Maf protein, eventually binds to the antioxidant/electrophile-responsive element, and activates the transcription of antioxidant genes, including heme oxygenase-1, glutathione peroxidase-2, NAD(P)H-quinone oxidoreductase 1, and glutathione *S*-transferase. Nrf2 thus causes a broad and coordinated set of downstream reactions against OS.

Nrf2-mediated transcriptional responses are protective in a variety of experimental animals models including oxidative lung injury and fibrosis, asthma, and brain ischemia–reperfusion damage.[75–77] For example, induction of renal ischemia followed by reperfusion in wild-type mice elevates Nrf2 levels and activates their downstream target genes in the kidney.[78] In contrast, Nrf2 deficiency enhances their susceptibility to both ischemic and nephrotoxic acute kidney injury.[79] 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 levels of nitric oxide metabolites (an indirect evidence of OS) and develop renal injury.[80] Upregulation of Nrf2 is thus a potential therapeutic target in order to mitigate OS-induced tissue injury.

The regulation of Nrf2 has been recently elucidated (Figure 1, lower panel). Nrf2 is ubiquitinated continuously through the Keap1–Cul3 system and degraded within the proteasome.[81,82] Its level depends on its rate of destruction. Keap1 is a sensor of OS and acts as a negative regulator of Nrf2.[83] Under OS, reactive cysteines within the Keap1 moiety undergo conformational changes, eventually leading to the detachment of Nrf2 from Keap1 and the inhibition of its

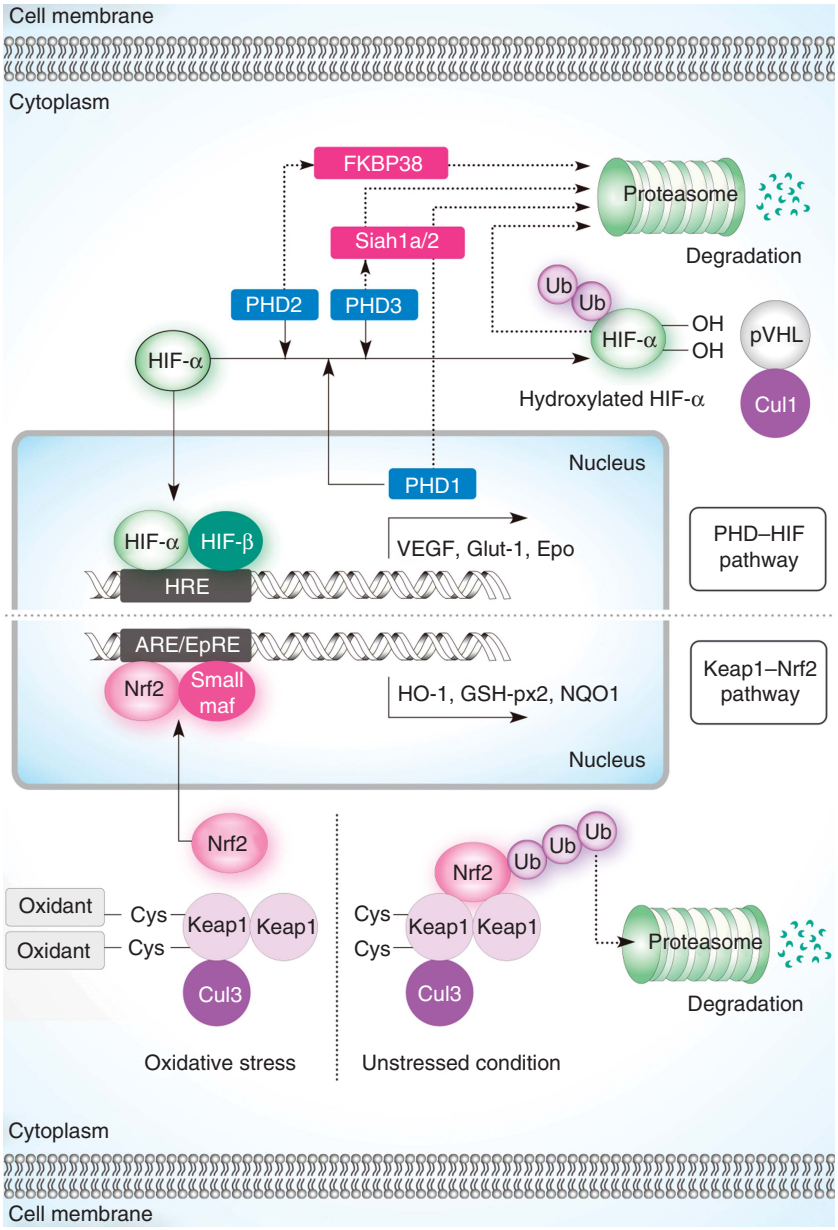

Figure 1 | Cellular defense mechanisms against hypoxia and oxidative stress (OS). (Upper panel) Prolylhydroxylase–hypoxia-inducible factor (PHD–HIF) pathway under hypoxia. HIF-α is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation. Oxygen determines its stability through its enzymatic hydroxylation by PHDs. Hydroxylated HIF-α is recognized by Hippel–Lindau tumor-suppressor protein (pVHL) and rapidly degraded by the proteasome. Nonhydroxylated HIF-α does not interact with pVHL and is thus stable. It binds to its heterodimeric partner HIF-α 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. (Lower panel) Keap1–Nrf2 pathway under OS. Nrf2 is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation by Keap1. Under OS, reactive cysteines within the Keap1 moiety undergo conformational changes, eventually leading to detachment of Nrf2 from Keap1 and to inhibition of its ubiquitination. OS 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. GSH-Px2, glutathione peroxidase-2; HO-1, heme oxygenase-1; NQO1, NAD(P)H-quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; VEGF, vascular endothelial growth factor.

ubiquitination. OS 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 obstructive cholestasis.[84] Inhibition of Keap1 might thus afford tissue protection against hypoxia through an increased nuclear translocation of Nrf2 and the ensuing activation of antioxidant genes.

REP cells

EPO is produced in the liver by hepatocytes as well as in the kidney by a specific cell lineage located within the peritubular interstitium.[3,4,85] The latter cells, referred to as REP cells, exhibit a fibroblastic phenotype with several projections extending between tubular and endothelial cells (Figure 2).[85,86] REP cells likely originate from the neural crest[6] as they express some neural cell markers. They are widely distributed in the interstitium of cortex and outer medulla.[85,86] Under normal conditions, only a very small population of the REP cells, mainly located in the outer medulla (corresponding to a lower oxygen concentration, 10–15 mm Hg), produce EPO.[46] Under moderate anemia, for example, induced in mice by bleeding, the REP cells located in the inner cortex are stimulated to produce EPO. Under severe chronic anemia, almost all REP cells including those in the outer cortex contribute to EPO production. Renal EPO production thus appears regulated by an ON/OFF mode, that is, by the number of EPO-producing REP cells (ON-REP cells) rather than by the gradual regulation of the expression levels in each REP cell.[4,87]

Erythropoiesis and renal fibrosis

During CKD progression, myofibroblasts emerge in the peritubular interstitium, and their expansion eventually leads to the end-stage renal failure.[28] The myofibroblasts in renal fibrosis were initially thought to originate from a variety of cell types including tubular epithelial cells and vascular smooth muscle cells.[28,88] However, recent studies have shown that this is not the case. A gene-modified mouse line meant to trace the fate of REP cells has demonstrated that the REP cells transform to myofibroblasts in an experimental CKD model generated by unilateral ureteral obstruction.[6] Almost all myofibroblasts expressing α-smooth muscle actin are derived from the REP cells, which are innately peritubular interstitial fibroblastic cells expressing neural cell marker genes but not α-smooth muscle actin (Figure 2). No myofibroblastic cell derived from the tubular epithelium or the vasculature was found, at least in the unilateral ureteral obstruction–treated CKD model mice.

Ureteral obstruction immediately suppresses the EPO-producing ability of REP cells, and induces their transformation[6]

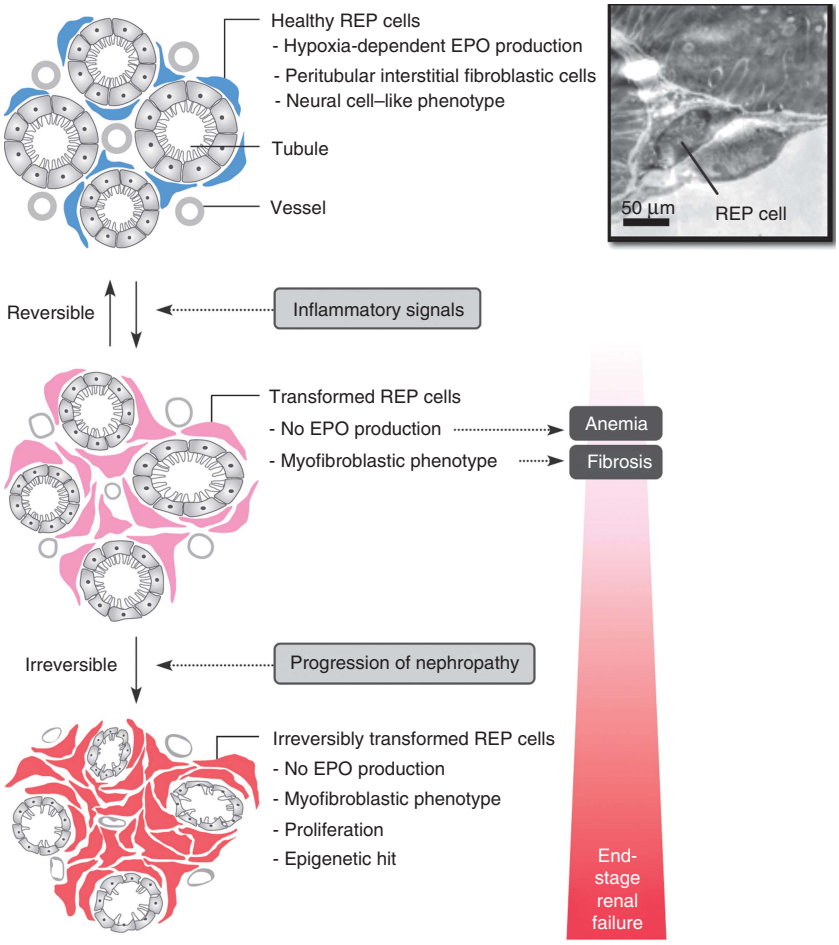

Figure 2 | Relevance of REP cells to renal fibrosis. REP (renal erythropoietin (EPO)-producing) cells are peritubular interstitial cells distributing over all the renal cortex (top). An electron microscopic image of the interstitium of renal cortex is shown in the inlet picture: REP cells localized in a transgenic mouse between tubular epithelial cells (TECs) and vascular endothelial cells (ECs). Inflammatory signals in chronic kidney disease (CKD) transform REP cells into the myofibroblasts and deteriorate their EPO-producing ability (middle). In the early phase of renal fibrosis, REP cells may recover their initial nature through the correction of the inflammatory milieu. However, during prolonged CKD progression, the transformed REP cells are no longer able to regain their EPO-producing ability (bottom).

(Figure 2). The loss of renal EPO production eventually leads to anemia. Myofibroblastic transformation in tissue fibrosis is mainly mediated by inflammatory signals such as those of the nuclear factor-κB pathway.[89] Forced activation of nuclear factor-κB signaling in the REP cells suffices to induce fibrosis in healthy mouse kidneys. Anti-inflammatory drugs may therefore block the transformation of REP cells and prevent a negative spiral between renal fibrosis and anemia.[6]

POTENTIAL FUTURE THERAPIES

The therapeutic perspectives in this section rest on recent findings in the fields of basic biology and of clinical medicine of diseases other than DN. These hypothetical approaches require further testing in DN.

PHD inhibitor

HIF activation potentially corrects tissue hypoxia and provides pleiotropic effects, such as anti-inflammation, antioxidative stress, and oxygen-independent energy production. 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.[62] Binding of the substrate 2-oxoglutarate to the catalytic domain of PHDs appears essential for the PHD enzymatic activity. Chemical compounds whose structure mimic 2-oxoglutarate (for example, *N*-oxalylglycine,[90] *N*-oxalyl-D-phenylalanine,[91] and L-Minosine[92]) are therefore able to inhibit PHD activity.

Relying on a strategy including docking simulation based on the three-dimensional protein structure of human PHD2 (Figure 3a), we synthetized two novel inhibitors of PHDs (TM6008 and TM6089).[93] Both compounds bind to the same active site as HIF. Orally, they stimulate HIF activity in various organs of transgenic rats expressing a hypoxia-responsive reporter vector. Locally, they induce angiogenesis in a mouse sponge assay.

Unfortunately, nonspecific inhibition of HIF-α degradation also augments vascular endothelial growth factor and EPO production, both of which have proven detrimental for proliferative diabetic retinopathy in humans.[94]

The role of the three PHD isoforms has been recently delineated by the specific disruption of their gene. Broad-spectrum conditional knockout of PHD2 induces vascular endothelial growth factor and hyperactive angiogenesis, with the formation of mature and perfused blood vessels.[95,96] PHD3 is also involved in angiogenesis: in mice with hindlimb ischemia, therapeutic revascularization is better after PHD3 than after PHD2 gene silencing.[97]

In mice, both PHD1 and PHD3 gene knockout does not affect erythropoiesis but double PHD1 and PHD3 knockout induces the accumulation of HIF-2α in the liver with a moderate erythrocytosis.[58] Adult PHD2-deficient mice develop a prominant erythrocytosis with a dramatic increase in the serum levels of EPO and EPO mRNA in kidney. These results are taken to indicate that PHD1/3 double deficiency leads to erythrocytosis partly through the activation of the hepatic HIF-2α/EPO pathway, whereas PHD2 deficiency acts by activating the renal pathway.[58]

Unfortunately, none of the present PHD inhibitors is specific for a distinct PHD subtype.[62] A Phase II clinical trial of a PHD inhibitor, FG-4592, is currently underway in patients with stage 3–4 CKD to alleviate anemia, hypertension, and hyperlipidemia, all of which are independent risk factors not only for cardiovascular events but also CKD.[98] FG-4592 corrects and maintains stable hemoglobin levels without intravenous supplementation with iron in patients, irespective of whether they received dialysis or not. Surprisingly, total cholesterol levels decreased in the FG-4592 group after

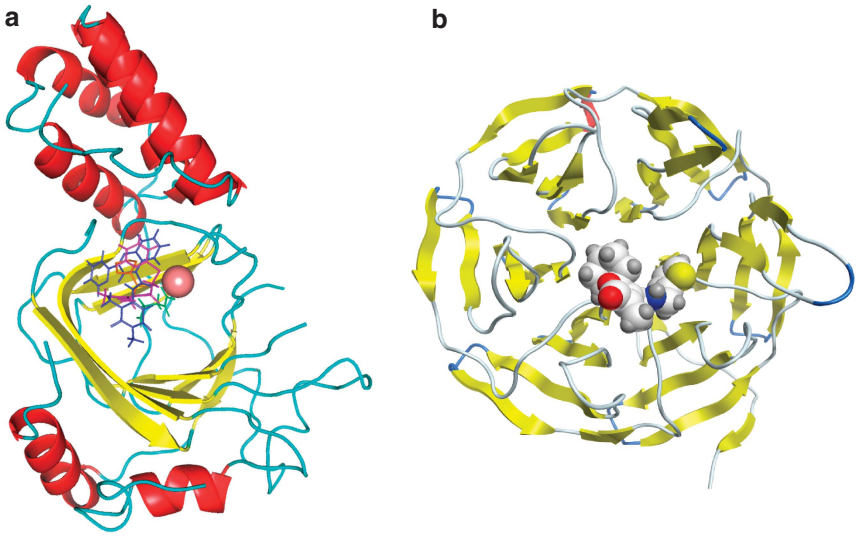

Figure 3 | Predicted binding modes by docking simulation computer study. (**a**) Oxygen sensor (human prolylhydroxylase-2 (PHD2)). TM6008 (blue), TM6089 (magenta), hypoxia-inducible factor (HIF) proline (orange), 2-oxoglutarate (light green), and Fe(II)(pink sphere) are shown. (**b**) Keap1 is depicted as a colored cartoon mode and an inhibitor molecule bound in the center of the concavity is shown by a space-filling model. Reprinted with permission from Miyata *et al.*[24]

16 weeks of treatment. The fall was similar irrespective of the concomitant intake of lipid-lowering agents (primarily statins and fibrates). Levels returned to control values after completion of the FG-4592 treatment. The high-density lipoprotein/low-density lipoprotein ratio also increased. During the 24-week observational period, FG-4592 treatment did not raise the risk of cardiovascular events, polycythemia, and thrombosis, or elevate blood pressure requiring initiation or intensification of antihypertensive medications. None of the adverse effects seen in experimental animals on long-term PHD2 inhibition (for example, polycythemia[58,99,100] and congestive heart failure,[101]) were reported.

Although clinically available PHD inhibitors such as FG-4592 are not specific for a distinct PHD subtype, they mainly inhibit PHD2. Dissociation between the benefits of HIF activation and the effects on angiogenesis and erythropoiesis has been recently examined by the Aragonés *et al.*[23] The specific disruption of PHD1 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 OS generation is decreased in hypoxic mitochondria. Inhibition of PHD1 further stimulates various protective mechanisms: adenosine-5′-triphosphate is produced through enhanced glycolysis and substrate for oxidative phosphorylation is restricted through the induction of pyruvate dehydrogenase kinase, with the eventual attenuation of electron entry into electron transport chain. Energy is thus conserved, oxidative damage reduced, and cells 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.[102,103]

A specific PHD1 inhibitor has not yet been reported but it should protect hypoxic tissues through a reduced OS without affecting angiogenesis and/or erythropoiesis. It might be suitable for the treatment of DN and other types of CKD where chronic hypoxic renal injury is concomitant.

Allosteric effector of hemoglobin

Recently, unique compounds have been reported that also increase oxygen supply and lead to the suppression of HIF activity.[104]

At physiological oxygen partial pressure levels, normal red blood cells release up to 25% of the oxygen bound by hemoglobin (Hb). The organic phosphate 2,3-bisphosphoglycerate,[105] a natural allosteric effector, decreases the oxygen-binding affinity of human Hb: increases in its level play a compensatory role in a variety of circumstances including high altitude, chronic pulmonary disease, and in patients with low-output heart failure.[106] Interventions to further decrease Hb oxygen-binding affinity might prove to be of clinical value.

Myo-Inositol hexakisphosphate is a powerful allosteric effector of Hb but is unable to cross the red blood cell membrane.[107] More recently, *myo*-inositol trispyrophosphate (ITPP) hexasodium salt, a synthetic derivative of *myo*-inositol hexakisphosphate, has been developed.[104] It crosses the red blood cell plasma membrane and acts as an allosteric effector of Hb, shifting the oxyhemoglobin dissociation curve to higher oxygen pressures. ITPP given in mice with severe exercise limitation due to a reduced cardiac output enhances exercise capacity.[108] It is noteworthy that ITPP suppresses HIF-1α and downstream hypoxia-inducible genes such as vascular endothelial growth factor in rats.[109] This mechanism is in contrast to PHD2 inhibitors that increase oxygen supply by augmenting the activity of HIF. Because of its antiangiogenic effect, ITPP has been tested for its anticancer potential in animals.[109,110] Its clinical benefits in DN and CKD remain to be demonstrated.

Nrf2 activator/Keap1 inhibitor

Recent demonstration that the radical scavenger NXY-059 eventually proved ineffective for acute ischemic stroke in humans should call for caution.[111] Although radical scavengers are effective in experimental animals including those with kidney disease,[112] this may not be the case in humans. Strategies to reduce OS intended to alleviate various diseases have been widely explored in experimental animals, but clinical success in humans is yet to be shown.

An alternative, novel approach to reduce OS has been devised and tested. Bardoxolone methyl,[113] derived from a natural product oleanolic acid, is a potent inducer of Nrf2. Originally developed as an anticancer drug, it produced unexpected benefits on the kidney during a clinical trial and was further developed as a renal drug.[114] A Phase II clinical trial, known as BEAM, has thus been undertaken in patients with advanced CKD and type 2 diabetes.[115] Bardoxolone improved renal function with only mild side effects, such as muscle spasm, weight loss, and hypomagnesemia. Unfortunately, a subsequent Phase III BEACON trial in patients with stage 4 CKD and type 2 diabetes had to be terminated on 18 November 2012 because of serious adverse events (www.clinicaltrials.gov/ct2/show/NCT01351675).

No effective Keap1 inhibitor is currently available. Sulforaphane, a natural product present in broccoli sprouts, modulates Keap1.[116] Given to a mouse model of streptozotocin-induced DN, sulforaphane ameliorated renal injury.[117] Recent informations on the X-ray crystal structure of Keap1[118] and on the molecular interaction between Nrf2 and Keap1 led us to search, by computer-based virtual screening, for a compound binding the active site of Keap1 and able to inhibit the interaction between Nrf2 and Keap1 (Figure 3b). Should its benefits be confirmed in experimental animals, a specific Keap1 inhibitor might offer an alternative approach to blunt OS injury.

REP modulating agent

EPO production in the liver is significantly larger in the fetus than in the adult.[119] Hence, the idea to treat renal anemia through the induction of EPO production in the liver. As already stated, under hypoxic conditions, EPO production is activated through the PHD–HIF pathway in the liver as well as in the kidney.[3] Hopefully, the development of PHD

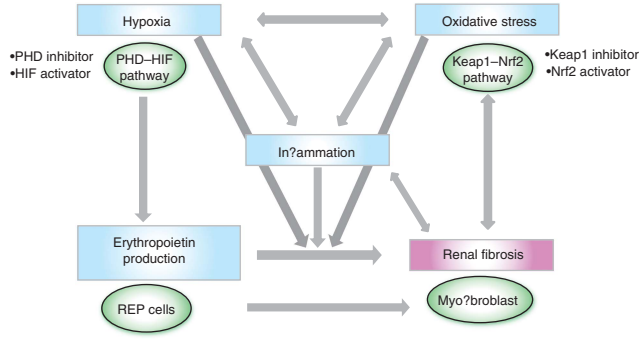

Figure 4 | A broad range of anomalies associated with oxygen biology. Hypoxia, oxidative stress, and dyserythropoiesis have been implicated in chronic kidney disease (CKD). The prolylhydroxylase–hypoxia-inducible factor (PHD–HIF) system mitigates hypoxia whereas the Keap1–Nrf2 system does the same for oxidative stress. Under hypoxia, renal erythropoietin (EPO)-producing (REP) cells, originating from neural crests, transdifferentiate into myofibroblasts and contribute to renal fibrosis. The interrelationship between these pathways or factors may preclude the identification of a single culprit in the progression of CKD. Besides these oxygen-associated anomalies, many more pathways or factors involve and exacerbate renal injury. Recent findings in the fields of basic biology and of clinical medicine of diseases other than CKD suggest that agents interfering with the PHD-HIF system (e.g., PHD inhibitor, HIF activator) or the Keap1–Nrf2 system (e.g., Keap1 inhibitor, Nrf2 activator), or restoring the initial function of REP cells might retard renal fibrosis and progression of CKD. These hypothetical approaches require further testing in CKD.

inhibitors (mainly PHD2) might stimulate EPO production in the liver instead of the damaged kidneys.[28,119,120]

The kidney structure is dramatically changed by the influx of REP cell–derived myofibroblasts filling the peritubular interstitium within 2 days after unilateral ureteral obstruction, whereas the controlateral kidney (nontreated side) remains normal.[6] REP cells retain cellular plasticity for a while after their transformation. Release of the obstruction within a week returns the transformed myofibroblasts to their original status, including their hypoxia-dependent EPO production, but the myofibroblastic transformation becomes irreversible after a more prolonged obstruction and inflammatory stimulations.

Reverse transformation of the myofibroblasts in CKD may be expected. A previous paper demonstrated that the attenuated EPO production by transdifferentiated REP cells was restored and the prevention of renal fibrosis was achieved by the administration of neuroprotective agents, dexamethasone and neurotrophins, in agreement with the neural crest origin of REP cells.[6]

CONCLUSION

The concern of DN prevention remains shared by all physicians as the meticulous correction of obesity, blood pressure, serum glucose, or lipid level is still unable to fully avoid the renal consequences of diabetes mellitus. This failure points to the limits of the present hypotheses to unravel the various mechanisms of DN and requires the consideration of newer pathophysiologic culprits.

The roles of defective oxygen delivery, of ROS generation, and of impaired erythropoiesis are scrutinized. How these pathways interact, how these pathways contribute to the progression of CKD, and promising therapeutic targets are summarized in Figure 4. Their diverse steps and their compensation are considered: the PHD–HIF pathway for hypoxia, the Keap1–Nrf2 pathway for OS, and the altered production of EPO by REP cells. Diverse agonists and antagonists are to be considered and their usefulness to reach the ultimate goal, that is, full prevention, discussed and tested. These novel prospects justify renewed efforts and suggest that full prevention might be in sight.

DISCLOSURE

All the authors declared no competing interests.

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