Diabetic nephropathy: a disorder of oxygen metabolism?

Toshio Miyata and Charles van Ypersele de Strihou

Abstract | Chronic hypoxia induces sequential abnormalities in oxygen metabolism (for example, oxidative stress, nitrosative stress, advanced glycation, carbonyl stress, endoplasmic reticulum stress) in the kidneys of individuals with diabetes. Identification of these abnormalities improves our understanding of therapeutic benefits that can be achieved with antihypertensive agents, the control of hyperglycemia and/or hyperinsulinemia and the dietary correction of obesity. Key to the body's defense against hypoxia is hypoxia-inducible factor, the activity of which is modulated by prolyl hydroxylases (PHDs)—oxygen sensors whose inhibition may prove therapeutic. Renal benefits of small-molecule PHD inhibitors have been documented in several animal models, including those of diabetic nephropathy. Three different PHD isoforms have been identified (PHD1, PHD2 and PHD3) and their respective roles have been delineated in knockout mouse studies. Unfortunately, none of the current inhibitors is specific for a distinct PHD isoform. Nonspecific inhibition of PHDs might induce adverse effects, such as those associated with PHD2 inhibition. Specific disruption of PHD1 induces hypoxic tolerance, without angiogenesis and erythrocytosis, through the reprogramming of basal oxygen metabolism and decreased generation of oxidative stress in hypoxic mitochondria. A specific PHD1 inhibitor might, therefore, offer a novel therapy for abnormal oxygen metabolism not only in the diabetic kidney, but also in other diseases for which hypoxia is a final, common pathway.

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Introduction

The poor outcome associated with diabetic nephropathy has stimulated the development of numerous therapeutic options for this disorder.[1] Prevention of this disorder and of its associated severe complications undoubtedly relies on a multipronged approach that targets factors such as blood pressure, and serum levels of glucose, insulin and lipids. Despite notable advances in the treatment of diabetes mellitus, current therapies do not fully prevent the associated renal complications. Identification of additional causative factors that lead to adverse renal effects and the development of novel agents to prevent or treat them are urgently needed.

In this Review, the roles of oxygen metabolism in the development and progression of diabetic nephropathy are explored and the various abnormalities of oxygen metabolism (that is, hypoxia, oxidative stress, nitrosative stress and advanced glycation and/or carbonyl stress) and innovative therapeutic targets are discussed.

Abnormal oxygen metabolism

Oxidative stress

Generalized versus local oxidative stress

Oxidative stress is a state of accumulation of reactive oxygen species (ROS), which can cause disruption of normal cell functions. The presence and localization of oxidative stress in diabetes have been disputed. Williamson *et al.* demonstrated an increased cellular NADH to NAD^+ ratio in diabetes and suggested that the disease is associated with a state of reductive stress and pseudo-hypoxia rather than a state of oxidative stress.[2] Subsequently, the presence of oxidative stress in diabetes was postulated on the basis of indirect evidence, including an increased ratio of $NADP^+$ to NADPH and of oxidized to reduced glutathione.[3–5] Such a redox imbalance may however rely possibly on nonoxidative mechanisms, such as the polyol pathway, so that their presence does not necessarily indicate oxidative stress. Baynes and colleagues revisited this issue with a new methodology centered on oxidative protein modifications and argued against generalized oxidative stress in diabetes: after adjustment for age, they showed that levels of oxidized amino acids—*ortho*-tyrosine and methionine sulfoxide—in skin collagen were virtually identical in individuals with and without diabetes.[6]

In contrast with generalized oxidative stress, our group focused on local oxidative stress in the kidneys of individuals with diabetes.[7] Advanced glycation end products (AGEs), generated nonenzymatically through the bonding of sugars with proteins, include two different classes of structures: those whose production is dependent on oxidative stress (pentosidine and *N*ε-[carboxymethyl]lysine [CML]) and those produced independently of oxidative stress (pyrraline). If tissue AGE formation depends solely on hyperglycemia, all AGE structures should be detected in diabetic kidneys. We

Competing interests
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Key points

- Chronic hypoxia induces sequential abnormalities in oxygen metabolism in the diabetic kidney, leading to oxidative stress, nitrosative stress, advanced glycation, carbonyl stress and endoplasmic reticulum stress
- Understanding the key features of abnormal oxygen metabolism improves the interpretation of the therapeutic benefits achieved by antihypertensive therapy, the control of hyperglycemia and/or hyperinsulinemia and the dietary correction of obesity
- Activity of hypoxia-inducible factor—central to the defense against hypoxia—is modulated by prolyl hydroxylases (PHDs), which act as oxygen sensors
- Three PHD isoforms have been identified and their respective roles have been elucidated, but none of the current PHD inhibitors exhibits absolute specificity for any subtype
- Disruption of PHD1 induces hypoxic tolerance by reducing oxidative stress in hypoxic mitochondria, indicating that a specific PHD1 inhibitor could be an innovative treatment for abnormal oxygen metabolism in the diabetic kidney
- Treatment of chronic hypoxia might apply to other chronic diseases that share a final common pathway, including a wide variety of kidney disorders, ischemic heart disease, and stroke

established that only those that were dependent on oxidative stress were present in diabetic glomerular lesions, whereas pyrraline was absent. We also saw other protein modifications derived from the oxidation of lipids (for example, malondialdehyde-lysine). Thus, we postulated the presence of local, rather than generalized, oxidative stress,[7,8] which has been subsequently confirmed in diabetic vascular lesions.[9] Substantial supporting evidence for this feature has since been gathered *in vitro* and *in vivo* in animal and human studies.[10,11] Altogether, diabetes is associated not with generalized but rather with local oxidative stress.

Causes of oxidative stress

The primary cause of local oxidative stress in the diabetic kidney remains debated, as numerous enzymatic and nonenzymatic mechanisms lead to production of ROS. Activation of the renin–angiotensin system (RAS),[12,13] NADPH oxidase activation,[14–16] nitric oxide synthase (NOS) and its metabolites (for example, reactive nitrogen species),[17] mitochondrial respiratory chain reaction abnormalities,[18,19] the polyol pathway,[11] increased concentrations of AGEs and reactive carbonyl compounds,[7,8,20] auto-oxidation of glucose and lipids, Fenton reactions catalyzed by transition-metal ions,[21] and depletion of glutathione and other sulfhydryls[22] are all recognized factors.

More recently, hypoxia, detailed in the latter part of this Review, has been strongly implicated as a cause of oxidative stress in diabetic nephropathy. Aragones *et al.*[23] have demonstrated in mice that disruption of the gene encoding prolyl hydroxylase-1 (PHD1), an intracellular oxygen sensor, lowers oxygen consumption in the mitochondria of skeletal muscle, with an attendant reduction in oxidative stress and, eventually, enhances cellular survival during hypoxia. Thus, hypoxia induces energy depletion and generates oxidative stress. This hypothesis is supported by *in vitro* studies, which have shown that the activation of hypoxia-inducible factor (HIF)-1α reduces ROS generation,[24] whereas inhibition of this transcriptional regulator worsens oxidative stress by increased ROS generation.[25]

The existence of both an increase and a decrease in oxidative stress during hypoxia seems paradoxical. Still, both situations in oxygen tension are known to lead to oxidative stress. During hypoxia, the cell relies on anaerobic glycolysis to generate ATP, although the residual low oxygen supply supports some level of oxidative ATP production through the tricarboxylic acid cycle and electron transport chain. In hypoxic cells, electron leakage from the mitochondrial electron transport chain occurs and results in excessive ROS formation (that is, oxidative stress). Reoxygenation or high oxygen levels following ischemia further exaggerates ROS generation. This concept suggests a role for agents that scavenge ROS or prevent their formation in ischemic diseases.[26] Concurrently, oxidative stress exacerbates the status of hypoxia. *In vitro* studies in rat proximal tubular cells and *in vivo* studies of streptozotocin-induced diabetes in rats show that high glucose levels blunts the activation of HIF, an effect that is fully reversed by treatment with antioxidants, such as α-tocopherol or tempol.[27,28] Activation of NADPH oxidase also aggravates renal hypoxia.[29] Hypoxia and oxidative stress are thus closely linked in the diabetic kidney.

Hypoxia

Local hypoxia

Oxygen is essential to various biometabolic processes, including oxidative phosphorylation during mitochondrial respiration. All organs, including the kidney, depend on a sufficient and consistent supply of oxygen. The role of chronic hypoxia in the progression of chronic kidney disease was originally proposed by Norman and Fine[30] and has been validated in a variety of human and experimental kidney diseases, including diabetic nephropathy.[31,32] Ries *et al.* first visualized tissue hypoxia in the kidneys of rats with streptozotocin-induced diabetes by use of blood oxygen level-dependent imaging.[33] Presence of chronic hypoxia was subsequently confirmed in the same model by Rosenberger *et al.* by means of pimonidazole staining for hypoxia and HIF induction.[28] Tissue hypoxia was also demonstrated in a rat model of type 2 diabetes.[13]

Adaptation to hypoxia differs according to the type of renal cell affected. During normal nephrogenesis, oxygen tension regulates the developing kidney in a cell-specific manner; HIF-1α is primarily involved in tubulogenesis, HIF-2α (also known as EPAS1) in renal vasculogenesis, and both isoforms in glomerulogenesis.[34] In *ex vivo* isolated perfused rat kidneys, differences in responses to HIF between collecting ducts and medullary thick ascending limbs correlate with cell viability under hypoxic stress caused by radiocontrast-induced acute renal failure.[35]

The localization of hypoxia has not been precisely determined in the kidney because few methods are able to identify and quantify tissue oxygenation at the cellular level. Tanaka *et al.* used a hypoxia-responsive reporter

vector to generate a novel hypoxia-sensing transgenic rat.[36] The researchers identified diffuse cortical hypoxia in rats with puromycin aminonucleoside-induced nephrotic syndrome and focal and segmental hypoxia in a remnant kidney model of kidney damage. In both models the degree of hypoxia was positively correlated with microscopic tubulointerstitial injury. Localization of tissue hypoxia may, therefore, differ according to the type of renal disease, including diabetic nephropathy but this hypothesis remains to be proven.

Causes of hypoxia

Causes of chronic hypoxia in the diabetic kidney are depicted in Figure 1. Glomerular efferent arterioles enter the peritubular capillary plexus, enabling provision of oxygen to tubular and interstitial cells. In individuals with diabetes, glomerular and vascular lesions damage efferent arterioles and reduce the number of peritubular capillaries, which in turn causes a reduction in oxygen diffusion to tubulointerstitial cells, eventually leading to tubular dysfunction and fibrosis.[31] The postglomerular peritubular blood flow is further decreased by vasoactive substances generated in the diabetic kidney, such as angiotensin II and nitric oxide (NO).[37] Palm *et al.* showed that the latter regulates oxygen availability.[38] NO concentrations are reduced in individuals with diabetes, which causes hypoxia in the renal medulla. Anemia associated with chronic kidney disease also hinders oxygen supply.[39,40]

In addition to the notable decrease in oxygen supply, oxygen demand is increased in the outer medulla tubule of diabetic kidneys. Remnant nephrons compensate for tubular nephron loss with an attendant increase in tubular transport and, hence, more energy consumption.[41]

Effects of abnormal oxygen metabolism

Hypoxia is not only a powerful cause of local oxidative stress in diabetic the kidney, but also has an impact on various biological reactions linked to oxygen metabolism (Figure 2).

Nitrosative stress

NO regulates numerous kidney functions, including renal hemodynamics, renin release, and extracellular fluid volume.[42] Deficiency or excess of NO can contribute to disease. Animal models of NO deficiency show development of hypertension, proteinuria, and glomerulosclerosis.[43] In rat diabetic kidneys, NO production and bioavailability have been shown to progressively decline,[44] influencing both the use of and supply of oxygen.[45] Overproduction of superoxide and other related ROS, resulting in oxidative stress, blunts the biological effects of NO. Superoxide combined with NO forms peroxynitrite, a cytotoxic oxidant,[17] which activates the nuclear enzyme, poly (ADP-ribose) polymerase (PARP).[46] In turn, PARP inhibits the activity of the glycolytic enzyme glyceraldehyde-3-phoshate dehydrogenase,[47] eventually activating the polyol pathway, the formation of AGEs, protein kinase C and the hexosamine pathway,[18] all of which have been implicated in the genesis of diabetic nephropathy. Thus, ROS and reactive nitrogen species (that is, nitrosative stress), trigger subsequent cellular dysfunction in diabetes through a multitude of mechanisms.[42]

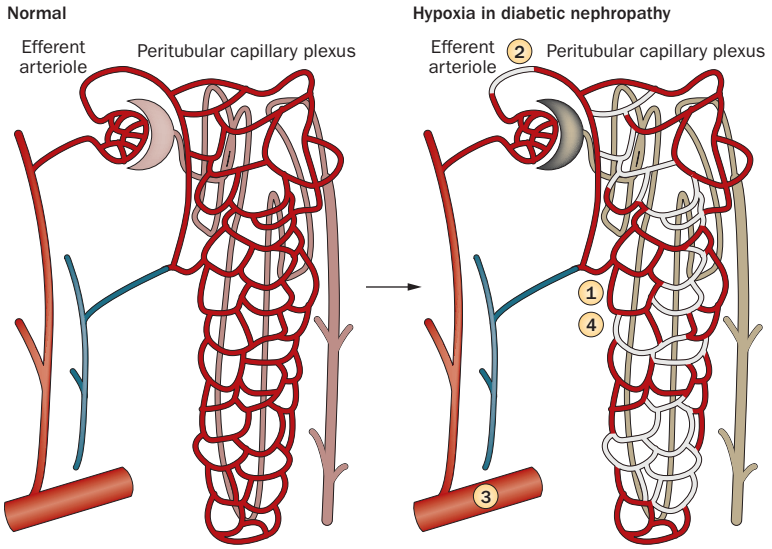

Figure 1 | Causes of hypoxia in the diabetic kidney. During hypoxia, oxygen supply is substantially decreased, especially in tubular segments of the inner medulla, through a multitude of mechanisms. (1) The number of peritubular capillaries is decreased. The postglomerular peritubular blood flow is reduced (2) by angiotensin II and nitric oxide, and (3) by anemia. (4) Oxygen demand is also increased in the tubule of the outer medulla of the diabetic kidney due to compensation for the tubular loss of nephrons.

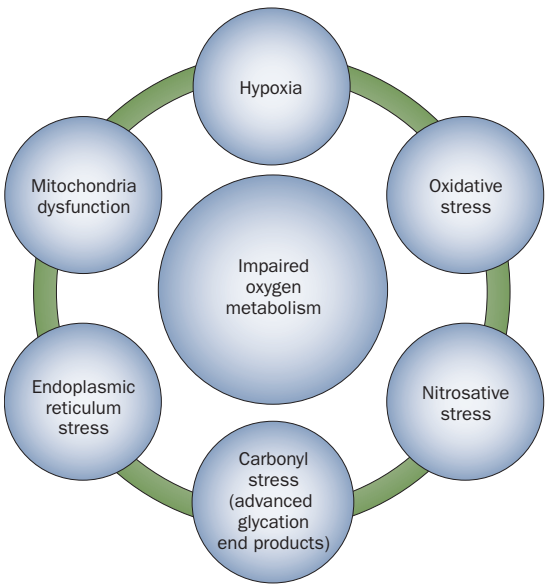

Figure 2 | The impact of impaired oxygen metabolism in the diabetic kidney on various biological reactions linked to oxygen metabolism.

Advanced glycation and carbonyl stress

Oxidative stress modifies proteins either directly through the oxidation of amino acids by ROS or indirectly by an increased generation of reactive carbonyl compounds from carbohydrates and lipids (that is, carbonyl stress).[48]

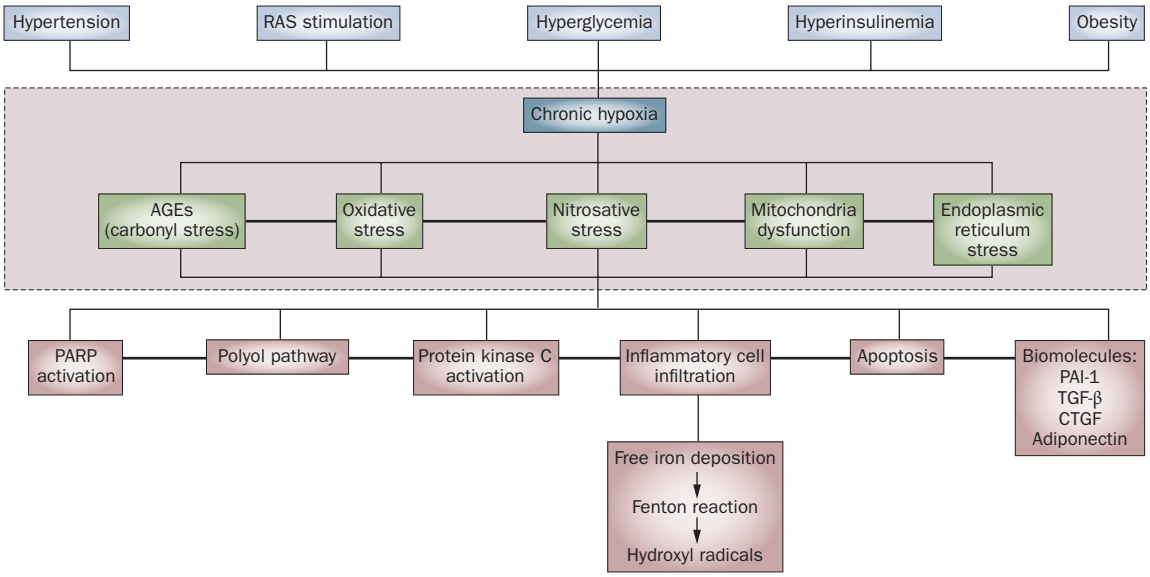

Figure 3 | Interplay between hemodynamic or metabolic abnormalities and impaired oxygen metabolism in the diabetic kidney. Abbreviations: AGE, advanced glycation end product; CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor 1; PARP, poly (ADP-ribose) polymerase; RAS, renin–angiotensin system; TGF-β, transforming growth factor β.

In turn, the latter process stimulates the production of AGEs. A causal role of oxidative stress in the formation of AGEs is supported by the correlation observed in diabetic and uremic serum between pentosidine (an AGE) and markers of oxidation, such as dehydroascorbate and advanced oxidation protein products,[49,50] as well as by the co-localization of oxidation-dependent AGE structures and lipid peroxidation products in diabetic glomerular lesions.[7,8]

Reactive carbonyl compounds also interfere with various cellular functions, independently of the effect of AGE modification of proteins, and influence intracellular signaling by multiple pathways,[51] for example, by interacting with the receptor for AGEs.[52]

Mitochondrial dysfunction

Impaired oxygen metabolism affects various intracellular organelles. The function of mitochondria is influenced by several factors, including hypoxia, oxidative stress, and NO.[53] During oxidative production of ATP through the tricarboxylic acid cycle and electron transport chain, ROS generation increases substantially when cells are partially deprived of oxygen.

Endoplasmic reticulum stress

The function of the endoplasmic reticulum (ER) is also modified by hypoxia and oxidative stress.[54] The ER has a critical role in the processing, folding, and transport of newly synthesized proteins. All cells are able to regulate the capacity of the ER to process synthesized proteins and can adapt to an imbalance between protein load and folding capacity—ER stress—which has been implicated in the pathogenesis of diabetic nephropathy.[55] Defenses against ER stress include the unfolded protein response, which includes a transient attenuation of new protein synthesis, the degradation of misfolded proteins, and the expression of a variety of antistress proteins. Excessive ER stress tips the balance beyond the limit of the cellular unfolded protein response, and occasionally leads to apoptosis.

Impaired oxygen metabolism

The phenomena stemming from hypoxia and its consequences are tentatively integrated in a hypothetical scheme depicted in Figure 3. The inter-relationship between these harmful chain reactions is so complex that a single culprit might not account for the alterations seen in diabetic nephropathy. Furthermore, prevention of renal consequences (for example, tubulointerstitial fibrosis, podocyte injury, mesangial activation, and macrophage infiltration) might, in theory, rely on many more intermediates whose actual contribution to renal alterations remains to be determined. Whatever the sequential events of diabetic renal injury, the consequences of hypoxia and the subsequent impairment in oxygen metabolism have a pivotal role in the genesis and progression of the diabetic kidney. Therapies that interfere with impaired oxygen metabolism may, therefore, prove clinically useful.

Actions of current therapies

Understanding the key features of abnormal oxygen metabolism in the diabetic kidney assists in the interpretation of current therapeutic benefits.

Antihypertensive agents (RAS inhibition)

Inhibition of the RAS with antihypertensive agents, such as angiotensin-converting-enzyme inhibitors, angiotensin-II-receptor antagonists, or direct renin inhibitors, achieves better renoprotection regardless of diabetes status than other antihypertensive drugs.[56–61] Angiotensin-II-receptor antagonists and angiotensin-converting-enzyme

inhibitors are used as standard treatments for diabetic nephropathy, whether systemic hypertension is present or not, as RAS inhibitors provide renoprotection independently of lowering blood pressure.[56–61] This dissociation is probably a result of the substantially higher angiotensin II concentrations within the kidney than in the systemic circulation.[62]

Some studies suggest that RAS-independent effects of angiotensin-II-receptor antagonists include the improvement of oxygen metabolism in the kidney independently of lowering blood pressure. In addition to the protective benefits conferred by angiotensin II type 1 receptor (AT_1) blockade and lowering blood pressure, angiotensin-II-receptor antagonists (or angiotensin-converting-enzyme inhibitors) have the unique ability to correct not only tissue hypoxia (by an increase in postglomerular peritubular blood flow[63] or through receptor activation of angiotensin II type 2 receptor and NO production[64]) but also to correct oxidative stress and nitrosative stress,[65,66] carbonyl stress and advanced glycation,[67,68] redox imbalance[69] and ER stress.[70]

To investigate the mechanisms by which angiotensin-II-receptor antagonists confer these protective benefits, we synthesized a novel, nontoxic angiotensin-II-receptor antagonist derivative, R-147,176, characterized by a weak affinity for the AT_1 (6,700 times less effective than olmesartan in AT_1-binding inhibition), but with an ability to confer striking inhibition of oxidative stress and advanced glycation.[71] Despite a minimal effect on blood pressure, R-147,176 provided notable renoprotection in two different rat models of type 2 diabetes—(SHR/NDmcr-cp and Zucker diabetic fatty rats).[71] The renal benefit of angiotensin-II-receptor antagonist therapy seems, therefore, to depend partly on the potent inhibition of oxidative stress and advanced glycation. R-147,176, like angiotensin-II-receptor antagonists, protects not only the kidney but also brain cells in an experimental stroke model in rats. This finding suggests that inhibition of oxidative stress and advanced glycation provides benefits in a broad spectrum of renal cardiovascular disorders.[72] Whether this compound corrects tissue hypoxia remains undetermined.

Control of hyperglycemia and hyperinsulinemia

In addition to the critical role of hyperglycemia,[73] insulin resistance or hyperinsulinemia have important roles in the genesis of diabetic renal injury.[74] Insulin sensitizers are, therefore, recommended for patients with diabetes and nephropathy who are also obese.

The renal benefits of insulin and pioglitazone (an insulin sensitizer) are associated with a reduction in hypoxia,[75] oxidative stress,[76] nitrosative stress[77] and advanced glycation.[76] Katavetin *et al.* have shown in a rat model of streptozotocin-induced diabetes that hyperglycemia blunts HIF activation, and that this effect is fully reversed by insulin treatment.[27] In a rat model of hypertensive type 2 diabetes (SHR/NDmcr-cp), we demonstrated that insulin and pioglitazone equally reduce renal accumulation of AGEs and markers of oxidative stress.[76] In contrast with insulin, pioglitazone caused a substantial decrease in plasma insulin levels, and afforded a markedly better renoprotection. The explanation for this puzzling fact rests on the ability of pioglitazone to reduce the renal expression of transforming growth factor β. The latter together with hyperinsulinemia might, therefore, prove to be a useful therapeutic target, independent of glycemic control and an impaired oxygen metabolism.[78]

Dietary correction of obesity

Restriction of energy intake reduces oxidative stress in experimental animals.[79,80] Evidence supports a link between obesity and hypoxia.[81,82] Crujeiras *et al.* demonstrated that energy restriction in obese individuals improves mitochondrial function through the reduction of oxidative stress.[83]

In an obese rat model of type 2 diabetes (SHR/NDmcr-cp), restriction of caloric intake by 30% for 20 weeks corrected obesity.[84] Unlike in human studies, caloric restriction was associated with a mild rather than a substantial fall in levels of hemoglobin A_{1c}. Nevertheless, despite unchanged blood pressure, hyperglycemia and hyperinsulinemia, proteinuria and histological abnormalities of the kidney were prevented.[84] Renal damage was impressively correlated not only with body weight but also with the renal content of AGEs and the degree of oxidative stress.[84] Renoprotection in this model is dependent, therefore, upon a reduction in oxidative stress but remains independant of hypertension and hyperglycemia.

Improvement of abnormal oxygen metabolism

Targets of current therapies are numerous and heterogeneous (for example, blood pressure, glucose, insulin and obesity). In experimental models of diabetes, including ours in hypertensive, obese, type 2 diabetic rats,[85] renoprotection is not necessarily linked to blood pressure or glycemic control but, interestingly, seems to be associated with an improved oxygen metabolism (Table 1).

Potential future therapeutic targets

Several mechanisms include potential targets for defense against abnormal oxygen metabolism in diabetic nephropathy.

Hypoxia-inducible factor

Defense against hypoxia hinges upon HIF,[86,87] the activation of which induces the expression of a broad range of genes that participate in erythrocytosis, angiogenesis, glucose metabolism, or cell proliferation and survival, with the eventual protection of hypoxic tissues (Figure 4).

HIF-α is constitutively transcribed and translated, and its levels are primarily regulated by the rate of degradation. Oxygen affects the stability of HIF-α through enzymatic hydroxylation by PHDs, which only occurs under normoxic conditions.[88,89] The hydroxylated HIF-α is recognized by von Hippel–Lindau tumor suppressor protein,[90,91] which functions as an E3 ubiquitin ligase, and is rapidly degraded by the proteasome.[92,93] Nonhydroxylated HIF-α cannot interact with

Table 1 | Summary of animal experiments of renoprotection, oxygen metabolism, hemodynamics and metabolism

Outcome	Treatments							
	Caloric restriction	Antihypertensive agents			Pioglitazone	Insulin	Cobalt	AGE inhibitor (R147176)
		ARB	CCB	β-blocker				
Renoprotection	+	+	–	–	+	±	+	+
Oxygen metabolism								
AGE inhibition	+	+	–	–	+	+	+	+
Antioxidative stress	+	+	–	–	+	+	+	+
Hypoxia correction	ND	+	–	–	+	+	+	ND
Hemodynamics								
BP lowering	–	+	+	+	–	–	–	±
RAS inhibition	–	+	–	–	–	–	–	±
Metabolism								
Obesity correction	+	–	–	–	Worsening	–	–	–
Glycemic control	+	–	–	–	+	+	–	–
Lipid lowering	+	+	–	–	+	+	–	–
Hyperinsulinemia correction	–	–	–	–	+	Worsening	–	–
References*	84	13, 68	13	13	75, 76	27, 76	110	71

*In hypertensive, type 2 diabetic rats, SHR/NDmcr-cp;[13,68,71,76,84,110] streptozotocin-induced diabetic rats;[27] db/db mice.[75] Abbreviations: AGE, advanced glycation end product; ARB, angiotensin receptor blocker; BP, blood pressure; CCB, calcium channel blocker; ND, not determined; RAS, renin–angiotensin system.

von Hippel–Lindau tumor suppressor protein and is, therefore, stabilized. Nonhydroxylated HIF-α binds to the heterodimeric partner HIF-β, localized mainly in the nucleus,[94] and transactivates genes involved in the adaptation to hypoxic–ischemic stress.

Three isoforms of the HIF-α subunit have been identified—HIF-1α, HIF-2α (also known as EPAS1) and HIF-3α.[95] HIF-1α and HIF-2α are structurally and functionally similar. By contrast, HIF-3α lacks the structures for transactivation that are found in the C-termini of HIF-1α and HIF-2α, suggesting an alternative role as a negative regulator of hypoxia-inducible gene expression.

The distribution of HIF-1α is rather ubiquitous, whereas HIF-2α is localized in certain cell types:[34] for example, in the kidney, HIF-1α is expressed in tubules, but HIF-2α is confined to endothelial and interstitial cells. A study that disrupted the genes that encode HIF-1α or HIF-2α in mice revealed that HIF-2α functions as a physiological regulator of erythropoietin.[96] HIF-2α is indeed responsible for familial erythrocytosis in humans[97] and for raised hemoglobin concentrations in polycystic kidney disease (pericystic hypoxia leading to HIF-2α induction).[98] In addition, as described below, HIF-2α has a crucial role in defense against oxidative stress.[23,99]

Therapeutic approaches that target HIF would be clinically of little benefit if this transcription factor were maximally activated under pathological conditions. Fortunately, HIF activation is suboptimal, as illustrated by the increase in its levels with antioxidant therapy in rats with diabetic kidneys.[27,28] Data obtained in a rat model of rhabdomyolysis also suggest that cellular adaptation to hypoxia is limited to certain cells within a relatively short period of time, supporting the potential therapeutic usefulness of efforts to extend these adaptational responses.[100] Finally, the amount of HIF-1α detected in acute ischemia is substantially lower than that seen in animals exposed to carbon monoxide, which acts as a HIF activator that supports the suboptimal activation of HIFs in experimental renal ischemia.[101] Further, HIF activation might prove beneficial for renoprotection. In agreement with this contention, Hill *et al.* induced renal ischemia reperfusion injury in knockout mice (for genes encoding either HIF-1α or HIF-2α) and found that renal injury was more severe in the knockout mice than in wild-type controls from the same litter.[101]

Various potential options for increasing HIF activity are available for exploration. As mentioned above, HIF-α is constitutively transcribed but degraded through the oxygen-dependent hydroxylation of specific proline residues by PHDs. Inhibition of PHDs should, therefore, be an efficient approach to increase HIF activation. Asparagine-β hydroxylase hydroxylates the specific asparagine residue within the C-terminal activation domain of HIF-1α and blocks binding to the transcriptional co-activator P300, thus preventing the subsequent transcription of downstream genes.[102] Pharmacological inhibition of this one enzyme, however, might affect the full range of HIF-activating genes.[103] Furthermore, asparagine-β hydroxylase remains active at lower oxygen concentrations than PHDs and, therefore, might suppress the activity of HIF-1α proteins that escape destruction in moderate hypoxia.[103] Thus, current studies of HIF activity are focusing on the inhibition of PHDs by small-molecule compounds to modulate HIF activity.

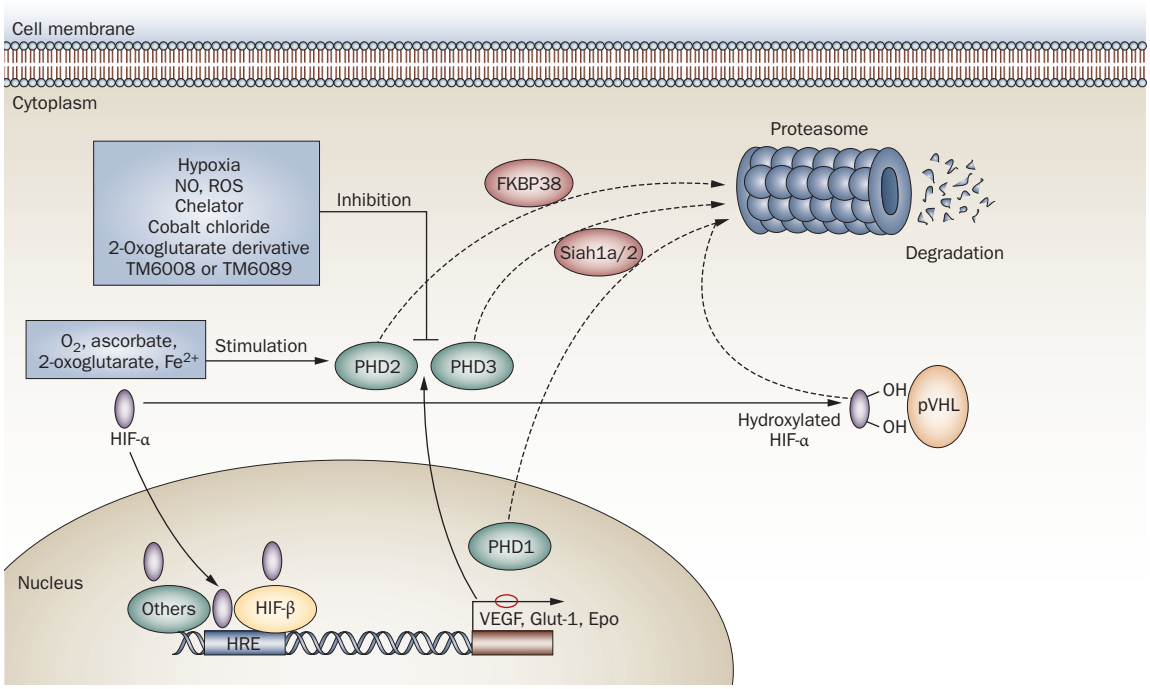

Figure 4 | The HIF–PHD pathway. HIF-α is constitutively transcribed and translated and its level is primarily regulated by its rate of degradation. Oxygen determines stability of HIF-α through enzymatic hydroxylation by PHDs. Hydroxylated HIF-α is recognized by pVHL, which functions as an E3 ubiquitin ligase, and is rapidly degraded by the proteasome. Nonhydroxylated HIF-α cannot interact with pVHL and is thus stabilized. Nonhydroxylated HIF-α binds to its heterodimeric partner HIF-β, mainly in the nucleus, and transactivates genes involved in the adaptation to hypoxic–ischemic stress, such as *VEGF*, *Glut-1*, and *Epo*. Expression of 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 interaction with NO, ROS, transition metal chelators, cobalt chloride, 2-oxoglutarate analogs, or TM6008 or TM6089. Abbreviations: HIF, hypoxia-inducible factor; HRE, hormone response element; NO, nitrogen oxide; PHD, prolyl hydroxylase; pVHL, von Hippel–Lindau tumor suppressor protein; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

Prolyl hydroxylases

Nonspecific inhibitors

PHDs belong to the Fe^{2+} and 2-oxoglutarate-dependent dioxygenase superfamily, which incorporate both atoms of molecular oxygen into their substrates.[95] One oxygen atom is used in the oxidative decarboxylation of 2-oxoglutarate and yields succinate and carbon dioxide, whereas the other atom is incorporated directly into the oxidized proline residue of HIF-α. PHDs are called oxygen sensors because their activity rigorously depends on oxygen tension.[104]

Iron is essential for PHD activity, so use of transition-metal chelators could potentially inhibit PHD activity. Cobalt chloride also inhibits PHD activity through intracellular depletion of ascorbate, which is necessary for iron activity.[105] Chemical preconditioning with cobalt chloride has been shown to protect kidneys in a variety of experimental models, including ischemic reperfusion,[106] progressive uninephrectomized anti-Thy1 nephritis,[107] remnant kidney,[108] cisplatin nephropathy,[109] and a hypertensive rat model of type 2 diabetes (SHR/NDmcr-cp).[110] In the latter model, cobalt chloride administered for 20 weeks lessened proteinuria and histological kidney injury, despite sustained hypertension and metabolic abnormalities. Renal improvement is paralleled by a marked reduction in the renal expression of HIF-regulated gene products, including erythropoietin, vascular endothelial growth factor (VEGF), and heme oxygenase 1, and by reduced renal production of transforming growth factor β and AGEs.

The erythropoietic effect of cobalt has been established in humans since the 1940s.[111,112] In the 1970s, cobalt chloride was used in the treatment of anemia associated with chronic renal failure.[113] Unfortunately, this treatment proved toxic and its clinical use was discontinued.

Less cumbersome, nontoxic, small-molecule inhibitors for PHDs have been investigated.[104] Binding of the substrate 2-oxoglutarate to the catalytic domain of PHDs seems to be essential for enzymatic PHD activity. Thus, chemical compounds with structures that mimic 2-oxoglutarate (for example, *N*-oxalylglycine [dimethyloxalylglycine],[114,115] *N*-oxalyl-D-phenylalanine,[116] and L-mimosine[101]) inhibit PHD activity.

We synthesized two novel inhibitors of PHDs (TM6008 and TM6089) to test a docking simulation strategy based on the three-dimensional protein structure of human PHD2.[117] Both compounds compete with HIF and bind to the active site within the PHD2 molecule where HIF binds (Figure 5). As anticipated, given orally, the compounds stimulated HIF activity in various organs of transgenic rats that expressed a hypoxia-responsive reporter vector. Given locally, the compounds induced angiogenesis in a mouse sponge assay.[117]

Another small-molecule inhibitor of PHD, FG4487, also offers renal benefits. Given intraperitoneally in a rat model of ischemic acute renal failure, the compound activated HIF-1α and HIF-2α, induced the expression of HIF target genes, ameliorated tubular injury and eventually improved renal function.[118] A similar renoprotective effect has been demonstrated for other PHD inhibitors, such as L-mimosine and dimethyloxalylglycine,[101] both of which increase expression of HIF-1α and HIF-2α and protect against renal ischemic injury by decreasing the number of apoptotic cells in the absence of angiogenesis.

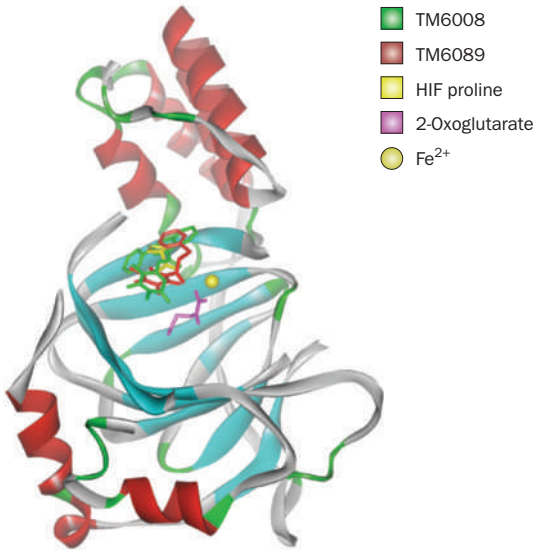

Figure 5 | Predicted binding modes of the PHD inhibitors to human PHD2. Compounds might compete with HIF and bind to the active site within the PHD2 molecule where HIF binds, thus stimulating HIF activity. Abbreviations: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase.

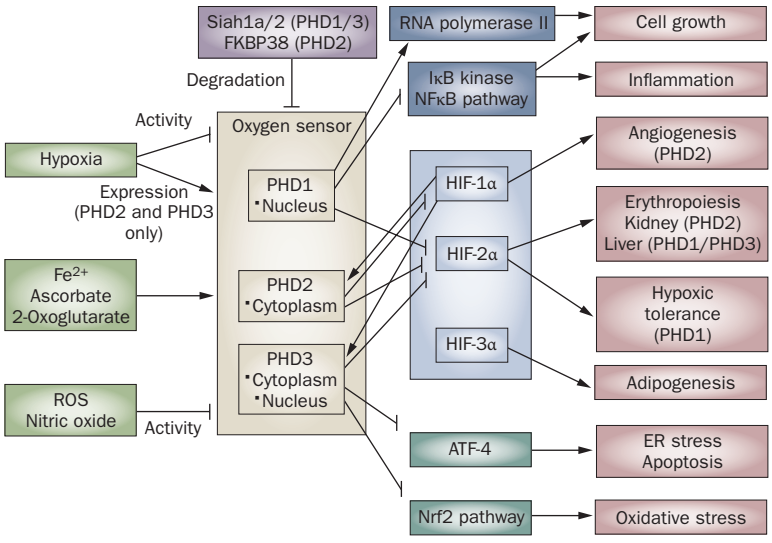

Figure 6 | Respective role of each PHD isoform in response to hypoxia. PHD2 acts as a decisive oxygen sensor in the HIF-degradation pathway. Hypoxia decreases PHD activities but induces expression of PHD2 and PHD3 through upregulation of HIF-1α. NO and ROS reduce PHD activity. The PHD2–HIF-1α pathway regulates angiogenesis and PHD2–HIF-2α dose erythrocytosis in the kidney. PHD1–HIF-2α is involved in hypoxic tolerance through reprogramming basal oxygen metabolism and by substantially reducing oxidative stress generated in mitochondria. PHDs also have targets other than the hydroxylation of HIF-α, for example the NFκB pathway (inflammation), RNA polymerase II (cell growth), ATF-4 (ER stress), the Nrf2 pathway (oxidative stress). Abbreviations: ATF-4, activating transcription factor 4; ER, endoplasmic reticulum; HIF, hypoxia-inducible factor; IκB, κB kinase-β inhibitor; NFκB, nuclear factor κB; NO, nitrogen oxide; Nrf2, nuclear factor erythroid 2-related factor 2; PHD, prolyl hydroxylase; ROS, reactive oxygen species.

Specific inhibitors

Nonspecific inhibition of HIF degradation can augment production of VEGF and erythropoietin, both of which have proven detrimental effects in human diabetic retinopathy.[119] Dissociation of the benefits of HIF activation from its effects on VEGF and erythropoietin should prove helpful.

Three different PHD isoforms have been identified—PHD1, PHD2, PHD3[95] (encoded by *EGLN2*, *EGLN1* and *EGLN3*, respectively)—each of which has its own tissue and subcellular distribution:[120] PHD1 is exclusively nuclear; PHD2 is mainly cytoplasmic but shuttles between the nucleus and cytoplasm;[121] and PHD3 is present in both cytoplasm and nucleus. PHD2 acts as a decisive oxygen sensor in the HIF degradation pathway.[122] In rat kidneys, all three isoforms of PHDs are expressed but PHD2 is the most abundant.[123] PHDs are especially abundant in tubular segments of the inner medulla where oxygen tension is physiologically low.[124] Despite causing a reduction in PHD activity, hypoxia induces the expression of PHD2 and PHD3 through upregulation of HIF-1α.[123] This effect ensures rapid removal of HIF-α after reoxygenation. Raised concentrations of NO and ROS also reduce PHD activity,[122,124] but again result in a feedback loop that causes an upregulation of PHD expression because of an accumulation of HIF-α. Different levels of hypoxia signaling may be associated with the triggering of different feedback loops.[125] PHD activity changes according to the type of renal injury: for example, cisplatin-induced renal injury in rats causes notable reductions in PHD2 and PHD3 activities, but no change is seen in a rat model of contrast-media-induced nephropathy.[126]

The roles of the three PHD isoforms have been delineated by the specific disruption of each PHD gene (Figure 6). The angiogenic phenotypes of mice with targeted disruptions of these genes reveals that knockout of the genes encoding PHD1 and PHD3 does not yield apparent angiogenic defects.[127] By contrast, broad-spectrum, conditional knockout of the gene encoding PHD2 leads to increased production of VEGF and hyperactive angiogenesis, with the formation of mature and perfused blood vessels. In agreement with these observations, TM6008 potentially binding to human PHD2 in a docking simulation study induces angiogenesis in mice.[117] PHD3 is also involved in angiogenesis and silencing of the gene encoding PHD3 provided a better therapeutic revascularization than silencing of the gene encoding PHD2 in mice with hindlimb ischemia.[128]

Data demonstrating that upregulation of HIF results in tumor progression might caution against the long-term

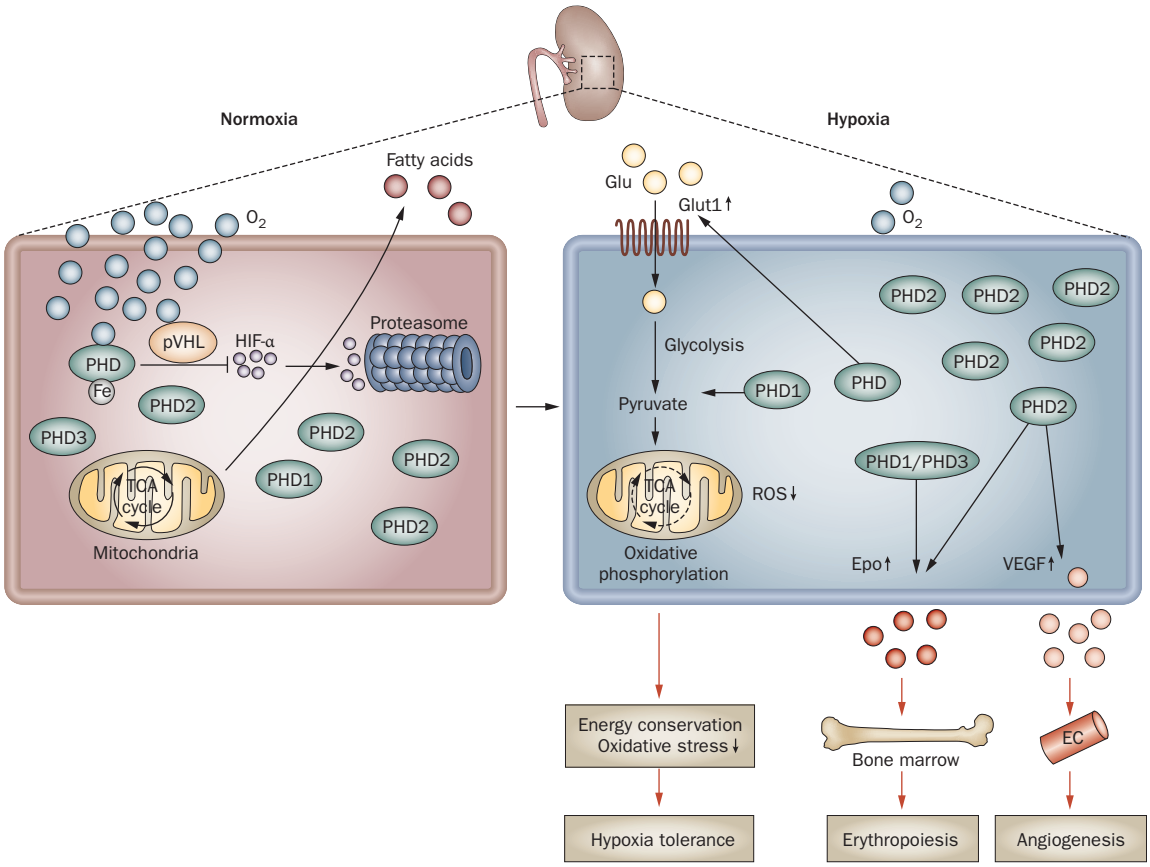

Figure 7 | Mechanisms of PHD inhibition, increased oxygen supply, and decreased oxygen demand (hypoxia tolerance). During hypoxia, expression of erythropoietin, VEGF, and GLUT-1 are augmented to increase the supply of oxygen and nutrients, eventually leading to erythropoiesis (PHD2, PHD1/PHD3) and angiogenesis (PHD2). PHD1-induced reactions reduce oxygen demand, conserve energy, reduce oxidative damage, and protect the cell from hypoxic damage (hypoxia tolerance). Those reactions also stimulate ATP production through enhanced glycolysis, and by restricting the entry of glycolytic intermediates (for example, pyruvate) into the oxidative phosphorylation pathway. Abbreviations: EC, endothelial cell; Epo, erythropoietis; GLUT-1, glucose transporter 1; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; pVHL, von Hippel–Lindau tumor suppressor protein; ROS, reactive oxygen species; TCA, tricarboxylic acid; VEGF, vascular endothelial growth factor.

use of PHD inhibitors.[129] A study, however, has demonstrated that inhibition of PHD2 prompts endothelial cells to readjust their shape and phenotype to restore oxygen supply, improves tumor perfusion and oxygenation and inhibits tumor-cell invasion, intravasation and metastasis.[130]

Knockout of the genes encoding either PHD1 or PHD3 in mice has no apparent effect on erythropoiesis.[96] Knockout of both genes was however associated with an accumulation of HIF-2α in the liver and the development of moderate erythrocytosis, due partly to activation of the hepatic HIF-2α–erythropoietin pathway. Adult mice deficient in PHD2 developed severe erythrocytosis by activation of the renal HIF-2α–erythropoietin pathway, with a dramatic increase in erythropoietin serum levels and in erythropoietin renal messenger RNA.[96]

Evidence suggests that PHDs, especially PHD1 and PHD3, have targets other than the hydroxylation of HIF-α. The nuclear factor κB pathway is suppressed under normoxic conditions, but activated by hypoxia through a modification by PHD1 of IκB kinase-β.[131] PHD1 also interacts with RNA polymerase II, which regulates tumor growth.[132] PHD3 is involved in the apoptosis of neuronal cells after nerve growth factor withdrawal, a phenomenon that is not prevented by simultaneous activation of HIF-α.[133] PHD3 binds to and regulates the stability of activating transcription factor 4, which is involved in unfolded protein responses under ER stress.[134] Of interest, a nonspecific PHD inhibitor, dimethyloxalylglycine, activates antioxidant gene expression through the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway.[135] Nrf2 induces the expression of various antioxidant proteins with critical roles in the adaptive responses to oxidative stress (for example, heme oxygenase 1 or glutathione peroxidase 2).[136] These PHD-dependent but HIF-independent pathways might offer additional therapeutic targets for protection against hypoxic tissue injury.

Dissociation between the benefits of HIF activation and the effects on angiogenesis and erythropoiesis has been illustrated by Aragonés *et al.*[23] The specific disruption of PHD1 induces hypoxic tolerance in muscle cells, without induction of angiogenesis and erythrocytosis. This unexpected effect is caused, at least in part, by the activation of

HIF-2α. Basal oxygen metabolism is reprogrammed and the generation of oxidative stress is reduced in hypoxic mitochondria (Figure 7). Inhibition of PHD1 probably induces various protective mechanisms, including increased ATP production through an increased rate of glycolysis and a restricted entry of glycolytic intermediates into the oxidative phosphorylation pathway by the induction of pyruvate dehydrogenase kinase, which attenuates entry of electrons into the electron transport chain. These reactions conserve energy, reduce oxidative damage, and protect the cell from hypoxic damage. Such findings explain in part why hibernating or hypoxia-tolerant animals are resistant to ischemic insults.[137,138]

Inhibition of PHD1 also protects cultured rat neuronal cells: knockdown of PHD1, but not PHD2 and PHD3, prevents neuronal death induced by oxidative stress.[139] Specific inhibition of PHD1 may, therefore, mediate tissue protection by reducing oxidative stress.

Unfortunately, inhibitors specific for a PHD subtype have not yet been developed. A specific PHD1 inhibitor may provide a novel therapy without adverse effects associated with PHD2 inhibition (for example, polycythemia[96,140,141] congestive heart failure,[139] and placental defects during pregnancy[142]).

Other targets

The PHD–HIF pathway is linked to a variety of biological reactions. Agents interfering with some of these processes may be of potential benefit. For example, an increase in angiopoietin 1 activity notably inhibits PHD2 and activates HIF-1α.[143] Hypoxia also increases the expression of plasminogen activator inhibitor 1[144,145] and connective tissue growth factor,[138] both of which have pivotal roles in the development of diabetic nephropathy. Inhibitors of these factors (for example, plasminogen activator inhibitor 1 inhibitors[146,147] or monoclonal antibodies against connective tissue growth factor[148]) may interfere indirectly with the detrimental consequences of abnormal oxygen metabolism.

Conclusions

Chronic hypoxia is a key factor in diabetic nephropathy and various other chronic conditions, including a wide variety of kidney disorders, ischemic heart disease and stroke. Advances in the treatment or prevention of diabetic nephropathy delineated in the present Review may thus herald newer concepts in the management of a broad spectrum of chronic illnesses.

Review criteria

We searched PubMed for English-language articles published up to August 2009, using the following search terms: “diabetic nephropathy”, “oxygen sensor”, “hypoxia”, “oxidative stress”, “reactive oxygen species”, “nitric oxide”, “nitrosative stress”, “advanced glycation”, “carbonyl stress”, “hypoxia inducible factor”, “prolyl hydroxylase”, “mitochondria”, “endoplasmic reticulum stress”, “angiotensin receptor blocker” and “renin-angiotensin”.

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