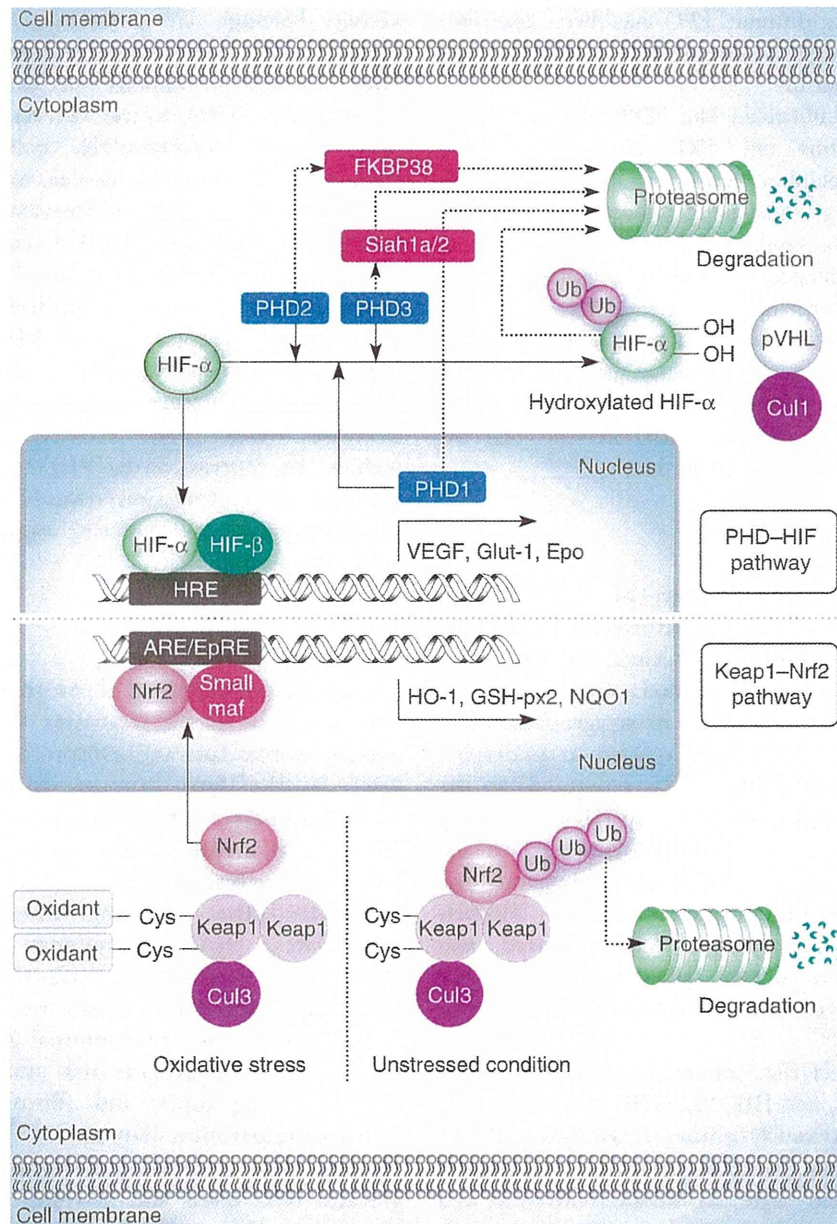

Figure 1 | Cellular defense mechanisms against hypoxia and oxidative stress (OS). (Upper panel) Prolylhydroxylase–hypoxia-inducible factor (PHD–HIF) pathway under hypoxia. HIF-α is constitutively transcribed and translated. Its level is primarily regulated by its rate of degradation. Oxygen determines 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 ensuying 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]

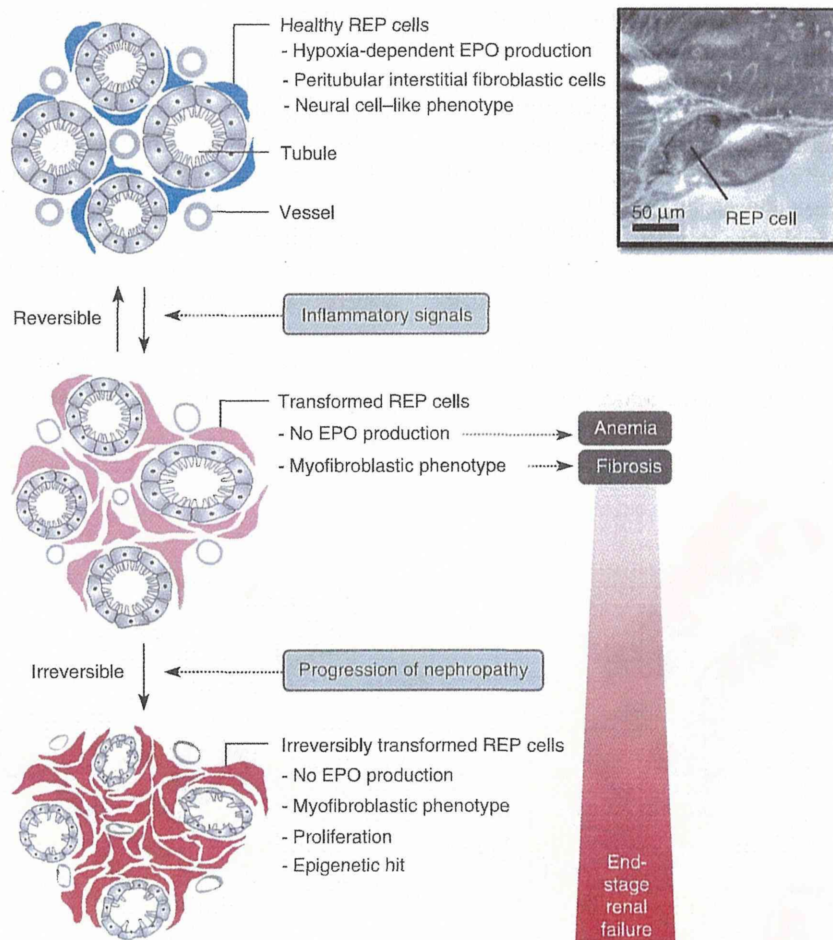

Figure 2 | Relevance of REP cells to renal fibrosis. REP (renal erythropoietin (EPO)-producing) cells are peritubular interstitial cells distributing over all the renal cortex (top). An electron microscopic image of the interstitium of renal cortex is shown in the inlet picture: REP cells localized in 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, irrespective of whether they received dialysis or not. Surprisingly, total cholesterol levels decreased in the FG-4592 group after

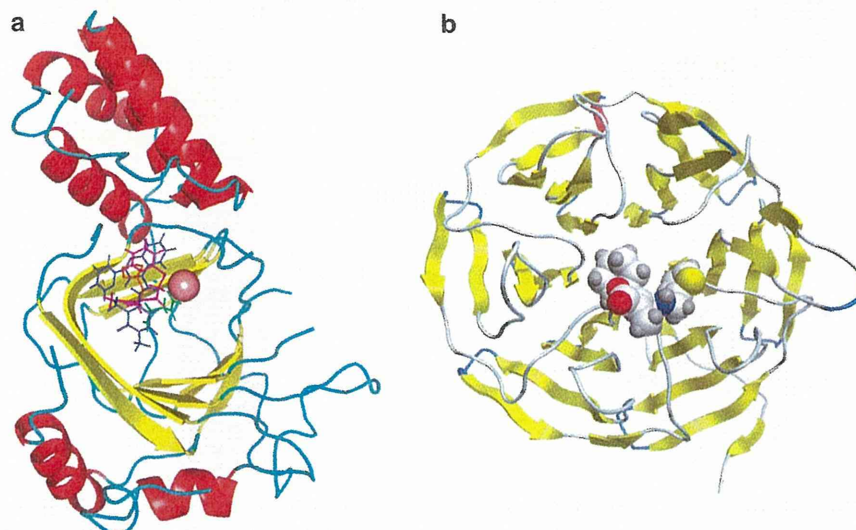

Figure 3 | Predicted binding modes by docking simulation computer study. (**a**) Oxygen sensor (human prolylhydroxylase-2 (PHD2)). TM6008 (blue), TM6089 (magenta), hypoxia-inducible factor (HIF) proline (orange), 2-oxoglutarate (light green), and Fe(II)(pink sphere) are shown. (**b**) Keap1 is depicted 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

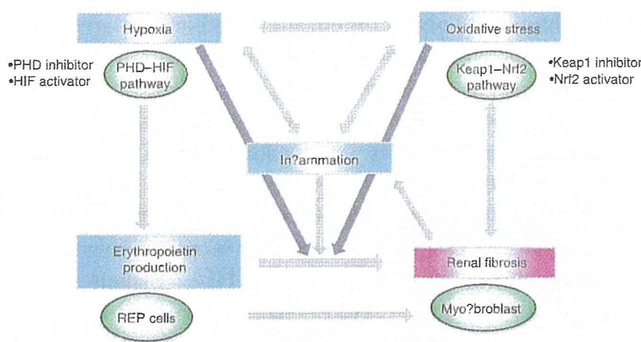

Figure 4 | A broad range of anomalies associated with oxygen biology. Hypoxia, oxidative stress, and dyserythropoiesis have been implicated in chronic kidney disease (CKD). The prolylhydroxylase–hypoxia-inducible factor (PHD–HIF) system mitigates hypoxia whereas the Keap1–Nrf2 system does the same for 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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REFERENCES

1. Kulkarni AC, Kuppusamy P, Parinandi N. Oxygen, the lead actor in the pathophysiologic drama: enactment of the trinity of normoxia, hypoxia, and hyperoxia in disease and therapy. *Antioxid Redox Signal* 2007; **9**: 1717–1730.
2. Wu H, Liu X, Jaenisch R *et al.* Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 1995; **83**: 59–67.
3. Jelkmann W. Regulation of erythropoietin production. *J Physiol* 2011; **589**: 1251–1258.
4. Obara N, Suzuki N, Kim K *et al.* Repression via the GATA box is essential for tissue-specific erythropoietin gene expression. *Blood* 2008; **111**: 5223–5232.
5. Maxwell PH, Ferguson DJ, Nicholls LG *et al.* The interstitial response to renal injury: fibroblast-like cells show phenotypic changes and have reduced potential for erythropoietin gene expression. *Kidney Int* 1997; **52**: 715–724.
6. Asada N, Takase M, Nakamura J *et al.* Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. *J Clin Invest* 2011; **121**: 3981–3990.
7. Williamson JR, Chang K, Frangos M *et al.* Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 1993; **42**: 801–813.
8. Jennings PE, Chirico S, Jones AF *et al.* Vitamin C metabolites and microangiopathy in diabetes mellitus. *Diabetes Res* 1987; **6**: 151–154.
9. Suzuki E, Yasuda K, Takeda N *et al.* Increased oxidized form of human serum albumin in patients with diabetes mellitus. *Diabetes Res Clin Pract* 1992; **18**: 153–158.
10. Bravi MC, Pietrangeli P, Laurenti O *et al.* Polyol pathway activation and glutathione redox status in non-insulin-dependent diabetic patients. *Metabolism* 1997; **46**: 1194–1198.
11. Wells-Knecht MC, Lyons TJ, McCance DR *et al.* Age-dependent increase in ortho-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes. Evidence against a generalized increase in oxidative stress in diabetes. *J Clin Invest* 1997; **100**: 839–846.
12. Horie K, Miyata T, Maeda K *et al.* Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. *J Clin Invest* 1997; **100**: 2995–3004.
13. Suzuki D, Miyata T, Saotome N *et al.* Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions. *J Am Soc Nephrol* 1999; **10**: 822–832.
14. Uchida K, Kanematsu M, Sakai K *et al.* Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci USA* 1998; **95**: 4882–4887.
15. Pennathur S, Wagner JD, Leeuwenburgh C *et al.* A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease. *J Clin Invest* 2001; **107**: 853–860.
16. Shah SV, Baliga R, Rajapurkar M *et al.* Oxidants in chronic kidney disease. *J Am Soc Nephrol* 2007; **18**: 16–28.

17. Forbes JM, Coughlan MT, Cooper ME. Oxidative stress as a major culprit in kidney disease in diabetes. *Diabetes* 2008; **57**: 1446–1454.
18. Jaimes EA, Galceran JM, Raij L. Angiotensin II induces superoxide anion production by mesangial cells. *Kidney Int* 1998; **54**: 775–784.
19. Nishikawa T, Edelstein D, Du XL *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; **404**: 787–790.
20. Monnier VM. Transition metals redox: reviving an old plot for diabetic vascular disease. *J Clin Invest* 2001; **107**: 799–801.
21. Gorin Y, Block K, Hernandez J *et al.* Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. *J Biol Chem* 2005; **280**: 39616–39626.
22. Tan AL, Forbes JM, Cooper ME. AGE, RAGE, and ROS in diabetic nephropathy. *Semin Nephrol* 2007; **27**: 130–143.
23. Aragonés J, Schneider M, Van Geyte K *et al.* Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nat Genet* 2008; **40**: 170–180.
24. Miyata T, Kikuchi K, Kiyomoto H *et al.* New era for drug discovery and development in renal disease. *Nat Rev Nephrol* 2011; **7**: 469–477.
25. Ries M, Basseau F, Tyndal B *et al.* Renal diffusion and BOLD MRI in experimental diabetic nephropathy. Blood oxygen level-dependent. *J Magn Reson Imaging* 2003; **17**: 104–113.
26. Rosenberger C, Rosen S, Shina A *et al.* Hypoxia-inducible factors and tubular cell survival in isolated perfused kidneys. *Kidney Int* 2006; **70**: 60–70.
27. Tanaka T, Miyata T, Inagi R *et al.* Hypoxia in renal disease with proteinuria and/or glomerular hypertension. *Am J Pathol* 2004; **165**: 1979–1992.
28. Nangaku M. Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *J Am Soc Nephrol* 2006; **17**: 17–25.
29. Palm F, Friederich M, Carlsson PO *et al.* Reduced nitric oxide in diabetic kidneys due to increased hepatic arginine metabolism: implications for renomedullary oxygen availability. *Am J Physiol Renal Physiol* 2008; **294**: F30–F37.
30. Nangaku M, Eckardt KU. Pathogenesis of renal anemia. *Semin Nephrol* 2006; **26**: 261–268.
31. Bahlmann FH, Fliser D. Erythropoietin and renoprotection. *Curr Opin Nephrol Hypertens* 2009; **18**: 15–20.
32. Miyata T, de Strihou CY. Diabetic nephropathy: a disorder of oxygen metabolism? *Nat Rev Nephrol* 2010; **6**: 83–95.
33. Garcia Soriano F, Virág L, Jagtap P *et al.* Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. *Nat Med* 2001; **7**: 108–113.
34. Du X, Matsumura T, Edelstein D *et al.* Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 2003; **112**: 1049–1057.
35. Prabhakar S, Starnes J, Shi S *et al.* Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. *J Am Soc Nephrol* 2007; **18**: 2945–2952.
36. Yan SD, Schmidt AM, Anderson GM *et al.* Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994; **269**: 9889–9897.
37. Miyata T, Kurokawa K, Van Ypersele De Strihou C. Advanced glycation and lipoxidation end products: Role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. *J Am Soc Nephrol* 2000; **11**: 1744–1752.
38. Miyata T, Kurokawa K, van Ypersele de Strihou C. From molecular footprints of disease to new therapeutic interventions in diabetic nephropathy: a detective story (review). *Curr Drug Target* 2005; **5**: 323–329.
39. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005; **115**: 2656–2664.
40. Lindenmeyer MT, Rastaldi MP, Ikehata M *et al.* Proteinuria and hyperglycemia induce endoplasmic reticulum stress. *J Am Soc Nephrol* 2008; **19**: 2225–2236.
41. Zhang F, Gagnon RF, Richards GK *et al.* Anemia of chronic renal failure: characterization in the mouse and correction with human recombinant erythropoietin. *Nephron* 1996; **72**: 654–661.
42. Ataka K, Maruyama H, Neichi T *et al.* Effects of erythropoietin-gene electrotransfer in rats with adenine-induced renal failure. *Am J Nephrol* 2003; **23**: 315–323.
43. Tanaka T, Nangaku M. Recent advances and clinical application of erythropoietin and erythropoiesis-stimulating agents. *Exp Cell Res* 2012; **318**: 1068–1073.
44. Righetti M. Cardio-renal-anemia syndrome: a link between erythropoietin, dimethylarginine and homocysteine. *Curr Med Chem* 2012; **19**: 3502–3507.
45. Suzuki N, Ohneda O, Takahashi S *et al.* Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. *Blood* 2002; **100**: 2279–2288.
46. Suzuki N, Obara N, Yamamoto M. Use of gene-manipulated mice in the study of erythropoietin gene expression. *Methods Enzymol* 2007; **435**: 157–177.
47. Noguchi CT, Wang L, Rogers HM *et al.* Survival and proliferative roles of erythropoietin beyond the erythroid lineage. *Expert Rev Mol Med* 2008; **10**: e36.
48. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**: 721–732.
49. Marx J. How cells endure low oxygen. *Science* 2004; **303**: 1454–1456.
50. Epstein AC, Gleadle JM, McNeill LA *et al.* C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001; **107**: 43–54.
51. Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 2004; **5**: 343–354.
52. Ivan M, Kondo K, Yang H *et al.* HIFa targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 2001; **292**: 464–468.
53. Hon WC, Wilson MI, Harlos K *et al.* Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. *Nature* 2002; **417**: 975–978.
54. Maxwell PH, Wiesener MS, Chang GW *et al.* The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999; **399**: 271–275.
55. Ohh M, Park CW, Ivan M *et al.* Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2000; **2**: 423–427.
56. Eguchi H, Ikuta T, Tachibana T *et al.* A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J Biol Chem* 1997; **272**: 17640–17647.
57. Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 2008; **30**: 393–402.
58. Takeda K, Aguila HL, Parikh NS *et al.* Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. *Blood* 2008; **111**: 3229–3235.
59. Percy MJ, Furlow PW, Lucas GS *et al.* A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med* 2008; **358**: 162–168.
60. Bernhardt WM, Wiesener MS, Weidemann A *et al.* Involvement of hypoxia-inducible transcription factors in polycystic kidney disease. *Am J Pathol* 2007; **170**: 830–842.
61. Kojima I, Tanaka T, Inagi R *et al.* Protective role of hypoxia-inducible factor-2alpha against ischemic damage and oxidative stress in the kidney. *J Am Soc Nephrol* 2007; **18**: 1218–1226.
62. Fraisl P, Aragonés J, Carmeliet P. Inhibition of oxygen sensors as a therapeutic strategy for ischaemic and inflammatory disease. *Nat Rev Drug Discov* 2009; **8**: 139–152.
63. Salnikow K, Donald SP, Bruick RK *et al.* Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* 2004; **279**: 40337–40344.
64. Smith EL. Presence of cobalt in the anti-pernicious anaemia factor. *Nature* 1948; **162**: 144.
65. Berk L, Burchenal JH, Castle WB. Erythropoietic effect of cobalt in patients with or without anemia. *N Engl J Med* 1949; **240**: 754–761.
66. Edwards MS, Curtis JR. Use of cobaltous chloride in anaemia of maintenance hemodialysis patients. *Lancet* 1971; **2**: 582–583.
67. Metzen E, Berchner-Pfannschmidt U, Stengel P *et al.* Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. *J Cell Sci* 2003; **116**: 1319–1326.
68. Steinhoff A, Pientka FK, Möckel S *et al.* Cellular oxygen sensing: importins and exportins are mediators of intracellular localisation of prolyl-4-hydroxylases PHD1 and PHD2. *Biochem Biophys Res Commun* 2009; **387**: 705–711.
69. Berchner-Pfannschmidt U, Tug S, Trinidad B *et al.* Nuclear oxygen sensing: induction of endogenous prolyl-hydroxylase 2 activity by hypoxia and nitric oxide. *J Biol Chem* 2008; **283**: 31745–31753.
70. Fong GH, Takeda K. Role and regulation of prolyl hydroxylase domain proteins. *Cell Death Differ* 2008; **15**: 635–641.
71. Berchner-Pfannschmidt U, Yamac H, Trinidad B *et al.* Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. *J Biol Chem* 2007; **282**: 1788–1796.

72. Tug S, Delos Reyes B, Fandrey J *et al.* Non-hypoxic activation of the negative regulatory feedback loop of prolyl-hydroxylase oxygen sensors. *Biochem Biophys Res Commun* 2009; **384**: 519–523.
73. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol* 2003; **43**: 233–260.
74. Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2 Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 2005; **7**: 385–394.
75. Cho HY, Jedlicka AE, Reddy SP *et al.* Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 2002; **26**: 175–182.
76. Cho HY, Reddy SP, Yamamoto M *et al.* The transcription factor NRF2 protects against pulmonary fibrosis. *FASEB J* 2004; **18**: 1258–1260.
77. Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J Neurosci* 2005; **25**: 10321–10335.
78. Leonard MO, Kieran NE, Howell K *et al.* Reoxygenation-specific activation of the antioxidant transcription factor Nrf2 mediates cytoprotective gene expression in ischemia-reperfusion injury. *FASEB J* 2006; **20**: 2624–2626.
79. Liu M, Grigoryev DN, Crow MT *et al.* Transcription factor Nrf2 is protective during ischemic and nephrotoxic acute kidney injury in mice. *Kidney Int* 2009; **76**: 277–285.
80. Yoh K, Hirayama A, Ishizaki K *et al.* Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in Nrf2-deficient mice. *Genes Cells* 2008; **13**: 1159–1170.
81. Itoh K, Wakabayashi N, Katoh Y *et al.* Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999; **13**: 76–86.
82. Wakabayashi N, Itoh K, Wakabayashi J *et al.* Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* 2003; **35**: 238–245.
83. Miyata T, Takizawa S, van Ypersele de Strihou C. Hypoxia. 1. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets. *Am J Physiol Cell Physiol* 2011; **300**: C226–C231.
84. Okada K, Shoda J, Taguchi K *et al.* Nrf2 counteracts cholestatic liver injury via stimulation of hepatic defense systems. *Biochem Biophys Res Commun* 2009; **389**: 431–436.
85. Pan X, Suzuki N, Hirano I *et al.* Isolation and characterization of renal erythropoietin-producing cells from genetically produced anemia mice. *PLoS One* 2011; **6**: e25839.
86. Maxwell PH, Osmond MK, Pugh CW *et al.* Identification of the renal erythropoietin-producing cells using transgenic mice. *Kidney Int* 1993; **44**: 1149–1162.
87. Koury ST, Koury MJ, Bondurant MC *et al.* Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. *Blood* 1989; **74**: 645–651.
88. Anders HJ, Ryu M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int* 2011; **80**: 915–925.
89. Inoue T, Takenaka T, Hayashi M *et al.* Fibroblast expression of an IκB dominant-negative transgene attenuates renal fibrosis. *J Am Soc Nephrol* 2010; **21**: 2047–2052.
90. Ivan M, Haberberger T, Gervasi DC *et al.* Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci USA* 2002; **99**: 13459–13464.
91. McDonough MA, McNeill LA, Tilliet M *et al.* Selective inhibition of factor inhibiting hypoxia-inducible factor. *J Am Chem Soc* 2005; **127**: 7680–7681.
92. Hill P, Shukla D, Tran MG *et al.* Inhibition of hypoxia inducible factor hydroxylases protects against renal ischemia-reperfusion injury. *J Am Soc Nephrol* 2008; **19**: 39–46.
93. Nangaku M, Izuhara Y, Takizawa S *et al.* A novel class of prolyl hydroxylase inhibitors induces angiogenesis and exerts organ protection against ischemia. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2548–2554.
94. Watanabe D, Suzuma K, Matsui S *et al.* Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. *N Engl J Med* 2005; **353**: 782–792.
95. Takeda K, Cowan A, Fong GH. Essential role for prolyl hydroxylase domain protein 2 in oxygen homeostasis of the adult vascular system. *Circulation* 2007; **116**: 774–781.
96. Mazzone M, Dettori D, Leite de Oliveira R *et al.* Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 2009; **136**: 839–851.
97. Loinard C, Ginouvès A, Vilar J *et al.* Inhibition of prolyl hydroxylase domain proteins promotes therapeutic revascularization. *Circulation* 2009; **120**: 50–59.
98. Beuck S, Schänzer W, Thevis M. Hypoxia-inducible factor stabilizers and other small-molecule erythropoiesis-stimulating agents in current and preventive doping analysis. *Drug Test Anal* 2012; **4**: 830–845.
99. Minamishima YA, Moslehi J, Bardeesy N *et al.* Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure. *Blood* 2008; **111**: 3236–3244.
100. Ladroue C, Carcenac R, Leporrier M *et al.* PHD2 mutation and congenital erythrocytosis with paraganglioma. *N Engl J Med* 2008; **359**: 2685–2692.
101. Siddiq A, Aminova LR, Troy CM *et al.* Selective inhibition of hypoxia-inducible factor (HIF) prolyl-hydroxylase 1 mediates neuroprotection against normoxic oxidative death via HIF-α and CREB-independent pathways. *J Neurosci* 2009; **29**: 8828–8838.
102. Zhou F, Zhu X, Castellani RJ *et al.* Hibernation, a model of neuroprotection. *Am J Pathol* 2001; **158**: 2145–2151.
103. Drew KL, Harris MB, LaManna JC *et al.* Hypoxia tolerance in mammalian heterotherms. *J Exp Biol* 2004; **207**: 3155–3162.
104. Fylaktakidou KC, Lehn JM, Greferath R *et al.* Inositol tripyrophosphate: a new membrane permeant allosteric effector of haemoglobin. *Bioorg Med Chem Lett* 2005; **15**: 1605–1608.
105. Bunn HF, Jandl JH. Control of hemoglobin function within the red cell. *N Engl J Med* 1970; **282**: 1414–1421.
106. Bersin RM, Kwasman M, Lau D *et al.* Importance of oxygen-hemoglobin binding to oxygen transport in congestive heart failure. *Br Heart J* 1993; **70**: 443–447.
107. Teisseire B, Ropars C, Villeréal MC *et al.* Long-term physiological effects of enhanced O2 release by inositol hexaphosphate-loaded erythrocytes. *Proc Natl Acad Sci USA* 1987; **84**: 6894–6898.
108. Biolo A, Greferath R, Siwik DA *et al.* Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, myo-inositol trispyrophosphate. *Proc Natl Acad Sci USA* 2009; **106**: 1926–1929.
109. Aprahamian M, Bour G, Akladios CY *et al.* Myo-InositolTrisPyroPhosphate treatment leads to HIF-1α suppression and eradication of early hepatoma tumors in rats. *Chembiochem* 2011; **12**: 777–783.
110. Sihn G, Walter T, Klein JC *et al.* Anti-angiogenic properties of myo-inositol trispyrophosphate in ovo and growth reduction of implanted glioma. *FEBS Lett* 2007; **581**: 962–966.
111. Shuaib A, Lees KR, Lyden P *et al.* NXY-059 for the treatment of acute ischemic stroke. *N Engl J Med* 2007; **357**: 562–571.
112. Bath PM, Gray LJ, Bath AJ *et al.* Effects of NXY-059 in experimental stroke: an individual animal meta-analysis. *Br J Pharmacol* 2009; **157**: 1157–1171.
113. Dinkova-Kostova AT, Liby KT, Stephenson KK *et al.* Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci USA* 2005; **102**: 4584–4589.
114. Yoh K, Itoh K, Enomoto A *et al.* Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int* 2001; **60**: 1343–1353.
115. Pergola PE, Raskin P, Toto RD *et al.* Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N Engl J Med* 2011; **365**: 327–336.
116. Ping Z, Liu W, Kang Z *et al.* Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme. *Brain Res* 2010; **1343**: 178–185.
117. Zheng H, Whitman SA, Wu W *et al.* Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes* 2011; **60**: 3055–3066.
118. Tong KI, Kobayashi A, Katsuoka F *et al.* Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol Chem* 2006; **387**: 1311–1320.
119. Suzuki N, Obara N, Pan X *et al.* Specific contribution of the erythropoietin gene 3′ enhancer to hepatic erythropoiesis after late embryonic stages. *Mol Cell Biol* 2011; **31**: 3896–3905.
120. Minamishima YA, Kaelin WG Jr. Reactivation of hepatic EPO synthesis in mice after PHD loss. *Science* 2010; **329**: 407.

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Plasminogen Activator Inhibitor-1 Antagonist TM5441 Attenuates N$^{\omega}$-Nitro-L-Arginine Methyl Ester–Induced Hypertension and Vascular Senescence

Amanda E. Boe, BA; Mesut Eren, PhD; Sheila B. Murphy, BS; Christine E. Kamide, BS; Atsuhiko Ichimura, PhD; David Terry, PhD; Danielle McAnally, MS; Layton H. Smith, PhD; Toshio Miyata, MD; Douglas E. Vaughan, MD

Background**—**Long-term inhibition of nitric oxide synthase by L-arginine analogues such as N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) has been shown to induce senescence in vitro and systemic hypertension and arteriosclerosis in vivo. We previously reported that plasminogen activator inhibitor-1 (PAI-1)–deficient mice (PAI-1$^{-/-}$) are protected against L-NAME-induced pathologies. In this study, we investigated whether a novel, orally active PAI-1 antagonist (TM5441) has a similar protective effect against L-NAME treatment. Additionally, we studied whether L-NAME can induce vascular senescence in vivo and investigated the role of PAI-1 in this process.

Methods and Results**—**Wild-type mice received either L-NAME or L-NAME and TM5441 for 8 weeks. Systolic blood pressure was measured every 2 weeks. We found that TM5441 attenuated the development of hypertension and cardiac hypertrophy compared with animals that had received L-NAME alone. Additionally, TM5441-treated mice had a 34% reduction in periaortic fibrosis relative to animals on L-NAME alone. Finally, we investigated the development of vascular senescence by measuring p16^{Ink4a} expression and telomere length in aortic tissue. We found that L-NAME increased p16^{Ink4a} expression levels and decreased telomere length, both of which were prevented with TM5441 cotreatment.

Conclusions**—**Pharmacological inhibition of PAI-1 is protective against the development of hypertension, cardiac hypertrophy, and periaortic fibrosis in mice treated with L-NAME. Furthermore, PAI-1 inhibition attenuates the arterial expression of p16^{Ink4a} and maintains telomere length. PAI-1 appears to play a pivotal role in vascular senescence, and these findings suggest that PAI-1 antagonists may provide a novel approach in preventing vascular aging and hypertension. **(*Circulation.* 2013;128:2318-2324.)**

Key Words: aging ■ hypertension ■ nitric oxide synthase

Endothelial nitric oxide (NO) synthase is an enzyme that catalyzes the formation of NO from L-arginine. NO is an important signaling molecule that is involved in a variety of physiological processes,[1] most notably the regulation of vascular tone and structure. By stimulating the production of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells surrounding blood vessels, NO causes muscle relaxation and a decrease in blood pressure.[2] Additionally, NO has atheroprotective, antithrombotic, and anti-inflammatory properties through its ability to inhibit platelet aggregation, expression of adhesion molecules, and lipid oxidation.[2] Mice lacking expression of endothelial NO synthase lose the ability to produce vascular NO, and as a result develop hypertension.[3,4] Similar results are also seen when NO synthase activity is blocked by the competitive inhibitor N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME).[5–7] NO also has important biological functions outside of the vasculature, including roles in the gastrointestinal, respiratory, nervous, and immune systems.[2]

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Clinical Perspective on p 2324

It has been reported that NO suppresses the expression of plasminogen activator inhibitor-1 (PAI-1) in vascular smooth muscle cells.[8] Similarly, long-term inhibition of NOS in rats by L-NAME treatment resulted in increased vascular PAI-1 expression.[9] PAI-1 is the primary physiological inhibitor of plasminogen activation and is a member of the SERPIN superfamily of serine protease inhibitors.[10] In plasma, PAI-1 has a critical role in regulating endogenous fibrinolytic activity and resistance to thrombolysis. In vascular tissues, PAI-1 mediates the response

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to injury by inhibiting cellular migration[11] and matrix degradation.[12] Additionally, substantial evidence exists showing that PAI-1 may contribute to the development of fibrosis and thrombosis as a result of chemical[13] or ionizing injury.[14] In the absence of vascular injury or hyperlipidemia, our group has reported that transgenic mice overexpressing a stable form of human PAI-1 develop spontaneous coronary arterial thrombosis.[15]

We have also previously reported that PAI-I deficiency prevents the development of perivascular fibrosis associated with long-term NOS inhibition by L-NAME.[16,17] In the present study, we demonstrate that a novel, orally active small molecule inhibitor of PAI-1, TM5441, is as effective as complete deficiency of PAI-1 in protecting against L-NAME-induced pathologies. TM5441 is a derivative of the previously reported PAI-1 inhibitor TM5275,[18] which was generated by optimizing the structure-activity relationships of the lead compound TM5007.[19] TM5007 was originally identified as a PAI-1 inhibitor by virtual, structure-based drug design, which used a docking simulation to select candidates that fit within a cleft in the 3-dimensional structure of human PAI-1.

Beyond examining PAI-1 in L-NAME-induced arteriosclerosis, the present study focuses on the roles of NO and PAI-1 in vascular senescence. Senescent endothelial cells exhibit reduced endothelial NO synthase activity and NO production,[20,21] and NO has been shown to be protective against the development of senescence, an effect that is abrogated by L-NAME treatment.[22,23] However, the role of NO and L-NAME in vascular senescence in vivo is uncertain. PAI-1 is recognized as a marker of senescence and is a key member of a group of proteins collectively known as the senescence-messaging secretome.[24] However, it is likely that PAI-1 is not just a biomarker of senescence, but instead may be a critical driver of this process. Evidence supporting this hypothesis has already been shown in vitro. PAI-1 expression is both necessary and sufficient to drive senescence in vitro downstream of p53, and PAI-1–deficient murine embryonic fibroblasts are resistant to replicative senescence.[25,26] However, very little is known about the role of PAI-1 in senescence in vivo.

In this study, we show that L-NAME treatment and the subsequent loss of NO production induces vascular senescence in wild-type (WT) mice, and that treatment with the PAI-1 antagonist TM5441 is protective against this senescence. Therefore, in addition to validating TM5441 as a potential therapeutic, we also have demonstrated a role for L-NAME, NO, and PAI-1 in vascular senescence in vivo.

Methods

TM5441 Activity and Specificity Assays

The inhibitory activity and specificity of TM5441 (developed at the United Centers for Advanced Research and Translational Medicine (ART), Tohoku University Graduate School of Medicine, Miyagi, Japan) was assessed using recombinant PAI-1, antithrombin III, and α2-antiplasmin by chromogenic assay as previously described.[27,28] The reaction mixture includes 0.15 mol/L NaCl, 50 mmol/L Tris-HCl pH 8, 0.2mmol/L CHAPS, 0.1% PEG-6000, 1% dimethylsulfoxide, 5 nmol/L of either human active PAI-1 (Molecular Innovations, Southfield, MI), human antithrombin III (Sigma-Aldrich, Saint Louis, MO), or human α2-antiplasmin (Sigma-Aldrich), 2 nmol/L of either human 2-chain tPA (American Diagnostica Inc, Stanford, CT), thrombin (Sigma-Aldrich), or plasmin (Sigma-Aldrich), and 0.2 mmol/L of either Spectrozyme tPA (Chromogenix, Milano, Italy), chromogenic substrate S-2238 (Sekisui medical, Tokyo, Japan), or chromogenic substrate S-2251 (Sekisui medical) at a final concentration. Tested compounds were added at various concentrations and the IC50 was calculated by the logit-log analysis.

TM5441 Pharmacokinetics and Toxicity

TM5441, suspended in a 0.5% carboxymethyl cellulose sodium salt solution, was administered orally by gavage feeding to male Wistar rats (5 mg/kg; CLEA Japan Inc.). Heparinized blood samples were collected from the vein before (0 h) and 1, 2, 6, and 24 h after oral drug administration. Plasma drug concentration was determined on a reverse-phase high-performance liquid chromatography. Maximum drug concentration time (T_{max}), maximum drug concentration (C_{max}), and drug half-life ($T_{1/2}$) were then calculated.

All toxicity studies followed the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Harmonized Tripartite Guidelines at the non-GLP conditions. A repeated-dose toxicity study of TM5441 was assessed for 2 weeks in 5 Crl:CD (SD) rats per sex per group and no observed adverse effect level was concluded at 30 mg/kg in female rats and 100 mg/kg in male rats. As for the reverse mutation Ames test, TM5441 was negative. The effect of TM5441 on the human ether-a-go-go-related gene (hERG) electric current was investigated in HEL293 cells, which were transfected with the hERG gene, and TM5441 does not affect on hERG electric current in a concentration of up to 10 mmol/L.

Experimental Animals

Studies were performed on littermate 6- to 8-week-old C57BL/6J mice of both sexes purchased from Jackson Laboratories (Bar Harbor, ME). L-NAME (Sigma Aldrich, St. Louis, MO) was administered in the drinking water at 1 mg/mL (approximately 100–120 mg/kg/day). TM5441 was mixed in the chow at a concentration of 20 mg/kg/day. This dose was based on both preliminary studies conducted in our laboratory feeding mice with TM5441 and on personal communication with Dr Miyata. The weight of chow consumed by the mice and their body weight were monitored. Mice remained in the study for 8 weeks before undergoing final measurements and tissue harvest. All experimental protocols were approved by the IACUC of Northwestern University.

Blood Pressure

Systolic and diastolic blood pressures were measured in conscious mice (n=12–13/group) at baseline and every 2 weeks thereafter using a noninvasive tail-cuff device (Volume Pressure Recording, CODA, Kent Scientific Corp, Torrington, CT). Mice were placed in the specialized holder for 10–15 minutes before the measurement to acclimate to their surroundings. The animals underwent 3 training sessions before initial baseline measurements. This method has been validated against classic tail plethysmography.

Echocardiograms

Left ventricular function at diastole was determined in the mice (n=12–13/group) with the use of 2-dimensional (2D), M, and Doppler modes of echocardiography (Vevo 770, Visualsonics Inc, Toronto, Ontario, Canada). Mice were imaged at both baseline and after 8 weeks of treatment. The animals were anesthetized and placed supine on a warming platform. Parasternal long- and short-axis views were obtained in each mode to assess function.

Histology and Morphometry

Hearts and aortas were harvested from the animals after 8 weeks of treatment. The tissues were formalin fixed, paraffin embedded, and sectioned at 6 microns. Morphometric analysis was performed on left ventricular myocytes stained with hematoxylin and eosin (H & E) to calculate myocyte cross-sectional area using ImagePro Plus 6.3. Myoyctes that had a clear, unbroken cellular membrane and a visible nucleus were

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