Oxidative stress in early diabetic nephropathy: fueling the fire

Dhruv K. Singh, Peter Winocour and Ken Farrington

Abstract | Diabetic nephropathy is a major microvascular complication of diabetes mellitus and the most common cause of end-stage renal disease worldwide. The treatment costs of diabetes mellitus and its complications represent a huge burden on health-care expenditures, creating a major need to identify modifiable factors concerned in the pathogenesis and progression of diabetic nephropathy. Chronic hyperglycemia remains the primary cause of the metabolic, biochemical and vascular abnormalities in diabetic nephropathy. Promotion of excessive oxidative stress in the vascular and cellular milieu results in endothelial cell dysfunction, which is one of the earliest and most pivotal metabolic consequences of chronic hyperglycemia. These derangements are caused by excessive production of advanced glycation end products and free radicals and by the subjugation of antioxidants and antioxidant mechanisms. An increased understanding of the role of oxidative stress in diabetic nephropathy has lead to the exploration of a number of therapeutic strategies, the success of which has so far been limited. However, judicious and timely use of current therapies to maintain good glycemic control, adequate blood pressure and lipid levels, along with lifestyle measures such as regular exercise, optimization of diet and smoking cessation, may help to reduce oxidative stress and endothelial cell dysfunction and retard the progression of diabetic nephropathy until more definitive therapies become available.

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Introduction

Diabetic nephropathy has surpassed other major diseases to become the most common cause of end-stage renal disease; patients with diabetes mellitus constitute about 20–40% of individuals who require renal replacement therapy worldwide.[1] Diabetic nephropathy is a major risk factor for cardiovascular disease, and the majority of individuals with diabetic nephropathy die of cardiovascular disease-related causes even before end-stage renal disease develops.[2] Traditionally, diabetic nephropathy has been described as a glomerular disease with five different stages: glomerular hyperfiltration, incipient nephropathy, microalbuminuria, overt proteinuria and end-stage renal disease.[3] The aim of this Review is to summarize the role of oxidative stress in diabetes mellitus, as it constitutes a major factor in the pathogenesis of endothelial cell dysfunction, which leads to diabetic nephropathy, and to explore possible therapeutic interventions.

Endothelial physiology and dysfunction

The endothelium of blood vessels comprises a layer of endothelial cells that serves as a biological barrier between the blood and vascular smooth muscle cells in the arterial wall. This membrane is not quiescent, as previously thought, but plays an important part in the regulation of modulators of vascular tone, hemostasis, growth and differentiation of vascular smooth muscle cells and inflammation.[4] Endothelial cells modulate vascular tone via the release of vasodilators, such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor, or vasoconstrictors, such as endothelin-1, prostaglandin H_2, reactive oxygen species (ROS), angiotensin 2 and thromboxane A_2 (Figure 1).[5]

The term endothelial cell dysfunction was initially introduced to describe the inability of endothelial cells to maintain vascular tone as a result of loss of nitric oxide-mediated vasodilatation. However, as understanding of the functioning of endothelial cells has grown, endothelial cell dysfunction now encompasses impairment of various other properties that manifest as a disequilibrium of vascular relaxing and contracting factors, procoagulant and anticoagulant mediators and vascular-growth-inhibiting and growth-promoting substances.[6]

Endothelial cell dysfunction in diabetes

Endothelial cell dysfunction precedes and predicts the manifestation of microalbuminuria, the first clinical marker of diabetic nephropathy.[7] Microalbuminuria is also considered a marker of generalized endothelial cell dysfunction and an independent risk factor for atherosclerosis, cardiovascular disease and diabetic nephropathy.[8]

The pathogenesis of endothelial cell dysfunction in type 1 diabetes mellitus is incompletely understood. In type 2 diabetes mellitus, endothelial cell dysfunction can develop as a result of various risk factors of the metabolic syndrome, such as obesity, glucose intolerance, hypertension, dyslipidemia, hyperinsulinemia, insulin resistance and elevated inflammatory markers.[9] Insulin has a vasodilatory effect on

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the skeletal muscle vasculature, as it promotes synthesis and/or release of nitric oxide from the endothelial cell.[10] Absolute insulin deficiency in type 1 diabetes mellitus and functional insulin deficiency or insulin resistance in type 2 diabetes mellitus can thus contribute to endothelial cell dysfunction (Figure 1).

Oxidant injury as a result of excessive oxidative stress has been implicated in the etiology of endothelial cell dysfunction in the glomerulus and tubules, before the manifestation of microalbuminuria.[11] Chronic hyperglycemia associated with diabetes mellitus is considered a state of increased oxidative stress related to the excess generation of ROS and an impaired antioxidant response.[12] In addition, several other factors contribute to oxidative stress in the renal milieu (Figure 2), for example, impaired release of nitric oxide from endothelial cells, excessive production of advanced glycation end products (AGEs) and enhanced cytokine activation.[6]

Key points

- Oxidative stress, which precedes the development of endothelial cell dysfunction, plays a key part in the pathogenesis of diabetic nephropathy
- Chronic hyperglycemia is central to excessive generation of reactive oxygen species and the resultant impairment of the antioxidant response
- No target-specific antioxidant agent is currently available, although several potential agents are under evaluation
- Judicious use of current therapies such as antihyperglycemics, antihypertensives and statins, along with lifestyle modification, may help to contain oxidative stress in diabetes mellitus, pending the arrival of definitive therapies

Reactive oxygen species

ROS, such as superoxide or hydroxyl, are natural byproducts of oxygen metabolism (Figure 3) that play a major part in cell signaling, ageing and degenerative disease processes.[13] In healthy individuals, the amount of ROS produced is finely balanced with the antioxidant activity required to neutralize its adverse effects. Any metabolic alteration that increases the generation of ROS or diminishes the production of antioxidants can lead to increased oxidant-derived tissue injury or oxidative stress.[13]

Sources of ROS in diabetes mellitus

ROS are generated through a number of enzymatic and nonenzymatic sources in the body. The main sources of ROS in the vessel wall include oxidative phosphorylation of glucose, the polyol pathway (also known as the sorbitol–aldose reductase pathway), advanced glycation, mitochondrial respiratory processes and uncoupling of NADPH oxidases (Figure 4), among others.[14]

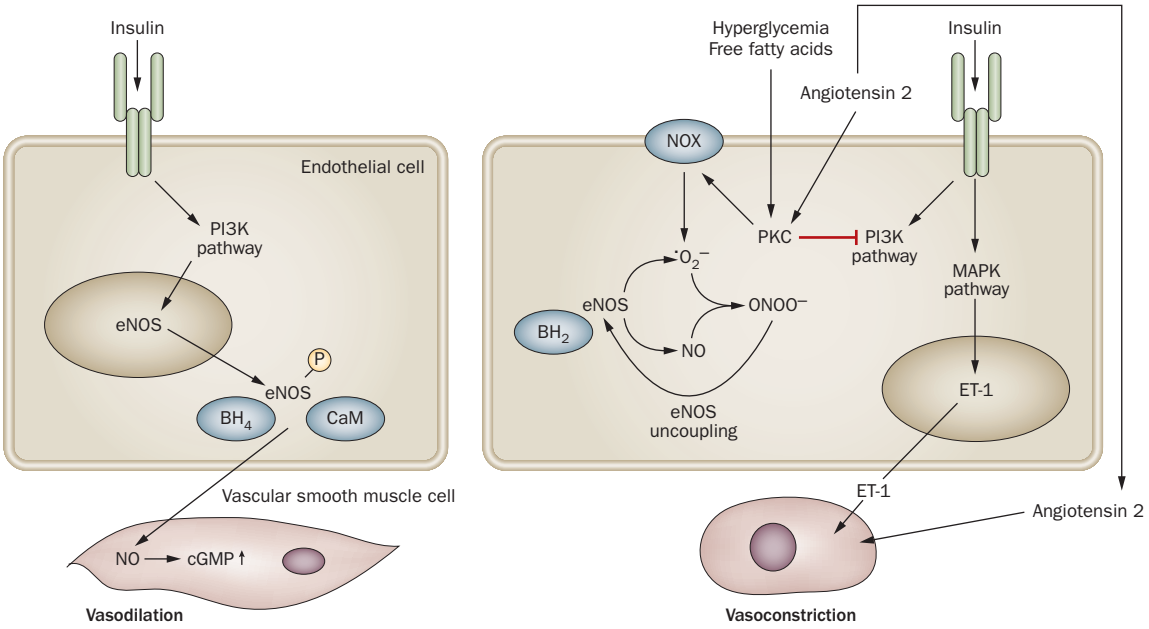

Figure 1 | Endothelium-derived vasodilators and vasoconstrictors in health (left) and in diabetes mellitus (right). Insulin has a vasodilatory effect on the skeletal muscle vasculature, as it promotes synthesis and/or release of nitric oxide from the endothelial cell.[10] The synthesis of nitric oxide occurs from amino acid L-arginine by enzymatic action of endothelial NOS (eNOS) in the presence of cofactors such as flavin dinucleotide, flavin mononucleotide, tetrahydrobiopterin (BH_4) and calmodulin (CaM).[99] Once released into the vascular lumen, nitric oxide diffuses to the surrounding tissues and cells, including smooth muscle,[100] and binds with the heme moiety of soluble guanylate cyclase, resulting in increased production of intracellular cyclic GMP, which mediates smooth muscle cell relaxation.[30] Insulin deficiency in type 1 and type 2 diabetes mellitus contribute to endothelial cell dysfunction, as enhanced production of superoxide ($^{\cdot}O_2^-$) causes excessive consumption of nitric oxide to generate peroxynitrite ($ONOO^-$).[32] Low levels of nitric oxide in endothelial cells may potentially result in ineffective suppression of ROS and could indirectly lead to enhanced vasoconstriction. These alterations of nitric oxide metabolism promote oxidative stress, especially in the renal milieu.[33] Abbreviations: BH_2, dihydrobiopterin; ET-1, endothelin 1; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NOX, NADPH oxidase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C. Adapted with permission from Macmillan Publishers © Rask-Madsen, C. & King, G. L. *Nat. Clin. Pract. Endocrinol. Metab.* **3**, 46–56 (2007).

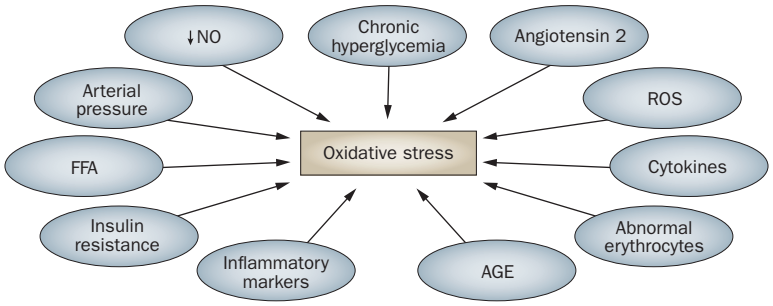

Figure 2 | Pathogenesis of oxidative stress in diabetes mellitus.

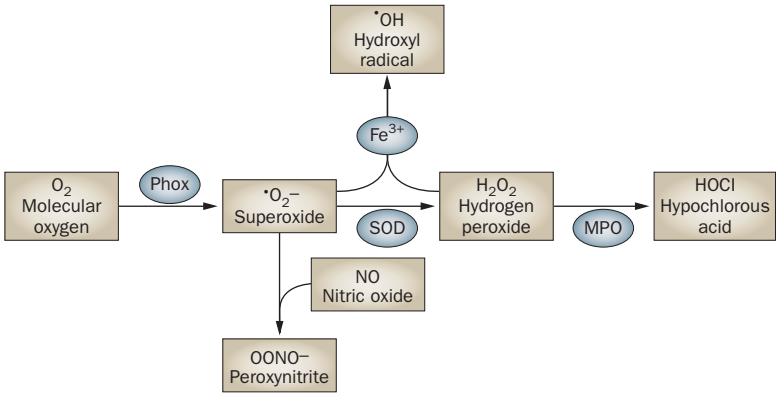

Figure 3 | Reactive oxygen and reactive nitrogen species. Some of the prominent ROS include free radicals such as superoxide ($^{\bullet}O_2^-$) and hydroxyl ($^{\bullet}OH$) and nonradical molecules such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). These oxidative molecules with an unpaired electron in the outer orbit are highly reactive and bring about adverse changes, when in contact with other cell structures and molecules. In addition to ROS, another group of oxidant species, the reactive nitrogen species (RNS), are major contributors to oxidative stress. The most important RNS is peroxynitrite ($OONO^-$), which is formed as a result of the reaction of superoxide with nitric oxide.[69] Adapted with permission from Macmillan Publishers © Lambeth, J. D. *Nat. Rev. Immunol.* **4**, 181–189 (2004).

Oxidative phosphorylation of glucose

Glucose is a major fuel for the mitochondrial respiratory process, where it undergoes oxidative phosphorylation and conversion to pyruvate. This process generates large amounts of NAD^+ and flavin adenine dinucleotide (FAD). Both NADH/NAD^+ and $FADH_2$/FAD are electron transport systems with very similar roles. NADH is the main electron donor to the mitochondrial respiratory chain and is produced at several steps during glucose and free fatty acid metabolism. More than 90% of oxygen is metabolized during the process of oxidative phosphorylation, with mobilization of electrons from glucose and other fuel substrates, to produce ATP.[1,14] Small amounts of ROS are produced during conditions of normal glucose supply. However, in patients with diabetes mellitus, the uninhibited supply of glucose can overburden the mitochondrial electron transport chain, which causes early leakage of electrons and excess ROS production.[12]

Polyol pathway

Unused glucose in the cytosol is diverted to the polyol pathway, where it is reduced to sorbitol by aldose reductase, utilizing NADPH from the pentose phosphate pathway as a cofactor. Reduction of NADPH produces NADH, which is taken up by the mitochondrial respiratory chain reactions, resulting in enhanced production of ROS in the form of superoxide.[13] Chronic hyperglycemia increases ROS generation by multiple mechanisms: it causes excessive consumption of NADPH in the polyol pathway, which inhibits replenishment of reduced glutathione—the vital substrate for glutathione-peroxidase-mediated cellular antioxidant activity—and increased superoxide production by transfer of NADH to the respiratory chain.[14]

NADPH oxidase pathway

The NADPH oxidase pathway constitutes the most important source of ROS in individuals with diabetes mellitus.[15] NADPH oxidase is a cytosolic enzyme complex located in the lysosomal membrane of phagocytic cells, such as neutrophils, and in the plasma membrane of various renal cell types, including mesangial and proximal tubular cells, vascular smooth muscle cells, endothelial cells and fibroblasts.[16] The enzyme catalyzes the transfer of electrons from NADPH to molecular oxygen to generate ROS (superoxide ions and hydrogen peroxide) as part of the defense against pathogens.[17] In the renal milieu, the primary role of the NADPH complex is as a signaling molecule; however, enhanced protein kinase C (PKC)-mediated activation of NADPH oxidase, for example, by angiotensin 2 or transforming growth factor β (TGF-β), leads to excessive generation of free radicals, which are detrimental to renal cells. Angiotensin 2 is one of the most potent inducers of NADPH oxidase and markedly contributes to ROS generation in early diabetic nephropathy.[18]

The NADPH oxidase complex comprises several isoforms, now designated the Nox family, that have important physiological roles in several organ systems, such as intestines, kidney, thyroid, testis and lymphoid organs.[19] The Nox4 isoform, a 578-amino acid protein and a major source of ROS in the renal milieu, is currently under intensive investigation with respect to its role in oxidative stress and potentiation of diabetic nephropathy.[20,21] Nox4 has been isolated in the cytosol as well as in the mitochondria of mesangial cells[21] and plays a pivotal part in promoting hyperglycemia-induced oxidative stress through excessive ROS generation, resulting in activation of Akt (also known as protein kinase B) and extracellular signal-regulated kinases 1 and 2. The modulation of these profibrotic pathways in chronic hyperglycemia is potentiated through Nox4-mediated activation of angiotensin 2 expression, which promotes renal hypertrophy and fibronectin expression in early diabetic nephropathy.[20]

Advanced glycation

AGEs are a diverse group of molecules generated as a result of nonenzymatic covalent bonding of glucose residues with free amino groups of proteins, lipids and nucleic acids. This biochemical reaction is termed the 'Maillard reaction'.[22] Chronic hyperglycemia results in excess ROS generation from various substrates of the Maillard reaction, such as auto-oxidation of glucose (Wolff pathway), Schiff bases (Namiki pathway), Amadori adducts (Hodge pathway) and AGE proteins themselves.[23]

Increased production of AGEs has been implicated in the pathogenesis of glomerulopathy[22] and tubulopathy[24] in diabetic nephropathy. AGEs are freely filtered by the glomerulus and are metabolized by the proximal tubule through the receptor megalin (also known as LDL receptor-related protein 2).[25] Binding of AGEs to their receptor RAGE on the tubular surface has been implicated in the pathogenesis of tubular cell injury, potentially via NADPH oxidase.[24] Binding to RAGE activates NFκB, which in turn induces production of various inflammatory cytokines and generation of mitochondrial ROS, thereby further aggravating oxidative stress in the kidney.

Excess mitochondrial production of ROS, as a result of damaged mitochondria caused by chronic hyperglycemia, has been proposed to be the primary event in the pathogenesis of tissue damage in diabetes mellitus.[12] In the normoglycemic cytosolic milieu, surplus ROS generated by activation of RAGE are neutralized by cellular antioxidant mechanisms. However, in chronic hyperglycemia, RAGE activation results in opening of the mitochondrial transition pore, which, in turn, causes enhanced production of superoxide ions through electron leakage.[26] The mitochondrial damage may become progressive in chronic hyperglycemia and can fuel the cytosolic production of ROS. This notion supports a role for AGE–RAGE interaction and resultant production of excessive ROS in the pathogenesis of early diabetic nephropathy. In addition, mitochondrial ROS production has been implicated in the activation of cyclooxygenase 2 and prostaglandin E_2 in mesangial cells.[27]

Minor sources of ROS

In addition to the above-mentioned pathways, several other pathways can, if defective, potentially contribute to increased production of ROS in diabetes mellitus. These pathways include primary inherited mitochondrial dysfunction, mitochondrial uncoupling of the respiratory chain, cytokine and growth factor signal transduction and amplification, the glutathione pathway, the xanthine oxidase pathway, uncoupling of nitric oxide synthase (NOS), altered activity of antioxidant and catalytic iron and sequestration of nitric oxide to perioxynitrite.[1,14]

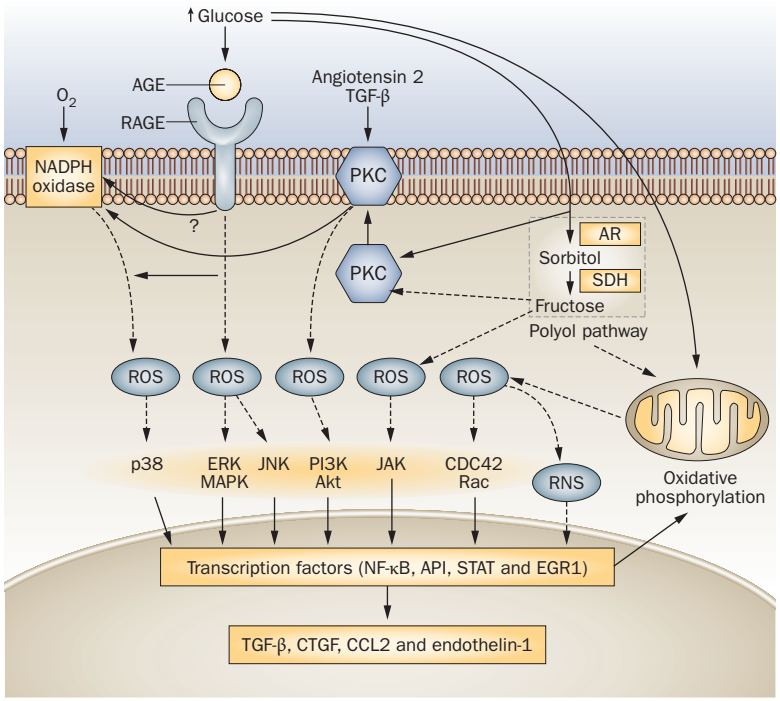

Figure 4 | Glucotoxicity pathways in diabetic nephropathy. Organ damage can be triggered by both extracellular and intracellular hyperglycemia. Increased extracellular glucose leads to nonenzymatic glycosylation of proteins and subsequent formation of advanced glycation end products (AGE) that interact with the receptor for AGE (RAGE) on the plasma membrane and promote the production of reactive oxygen species (ROS). Increased intracellular glucose drives mitochondrial activity, increases the activity of protein kinase C (PKC) and NADPH oxidase and promotes flux through the polyol pathway, all of which effect cellular metabolism and phenotype. Excessive ROS production in the vasculature drives changes in cell phenotype that are mediated by a range of signaling pathways and transcription factors. Kidney cells also undergo cell-specific and organ-specific phenotypic changes as a result of hyperglycemia-mediated ROS production. Abbreviations: AP1, activator protein 1; AR, aldose reductase; CCL2, CC-chemokine ligand 2 (also known as MCP1); CDC42, cell division cycle 42; EGR1, early growth response protein 1; ERK, extracellular signal-regulated kinase; JAK, Janus-activated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; RNS, reactive nitrogen species; SDH, sorbitol dehydrogenase; STAT, signal transducer and activator of transcription. Adapted with permission from Macmillan Publishers © Calcutt, N. A. *et al. Nat. Rev. Drug Discov.* **8**, 417–430 (2009).

ROS and nitric oxide metabolism

Patients with early diabetic nephropathy have mild albuminuria of mixed glomerular and tubular origin in the pre-microalbuminuric stage,[28] which is suggestive of generalized derangements in these systems. One of the earliest signs of endothelial cell dysfunction in the pre-microalbuminuric stage is enhanced sensitivity to renal vasoconstrictors and reduced nitric oxide-dependent vasodilation (Figure 1).[29]

The impaired ability of endothelial cells to modulate the vascular tone is a result of low bioavailability of nitric oxide in the vascular lumen.[30] In its physiological state, the endothelium has a constitutive supply of nitric oxide via endothelial NOS (eNOS). However, under certain pathological states such as inflammation, production of nitric oxide can occur via an inducible NOS isoform (iNOS), which results in increased release of nitric oxide into the subendothelial space rather than the vascular lumen and thus impairs modulation of the vascular tone.[31]

Furthermore, during normal cellular metabolism, superoxide reacts with nitric oxide, to generate the highly reactive nitrogen intermediate peroxynitrite. When endothelial cell dysfunction occurs, enhanced production of superoxide causes excessive consumption of nitric oxide, thus reducing its bioavailability.[32] Low levels of nitric oxide in endothelial cells may potentially result in ineffective suppression of ROS and could indirectly lead to enhanced vasoconstriction. These alterations of nitric oxide metabolism promote oxidative stress, especially in the renal milieu (glomerular and tubulointerstitial cells).[33]

Oxidative stress in the renal milieu

The kidney may be particularly vulnerable to early structural and functional damage caused by hyperglycemia-induced

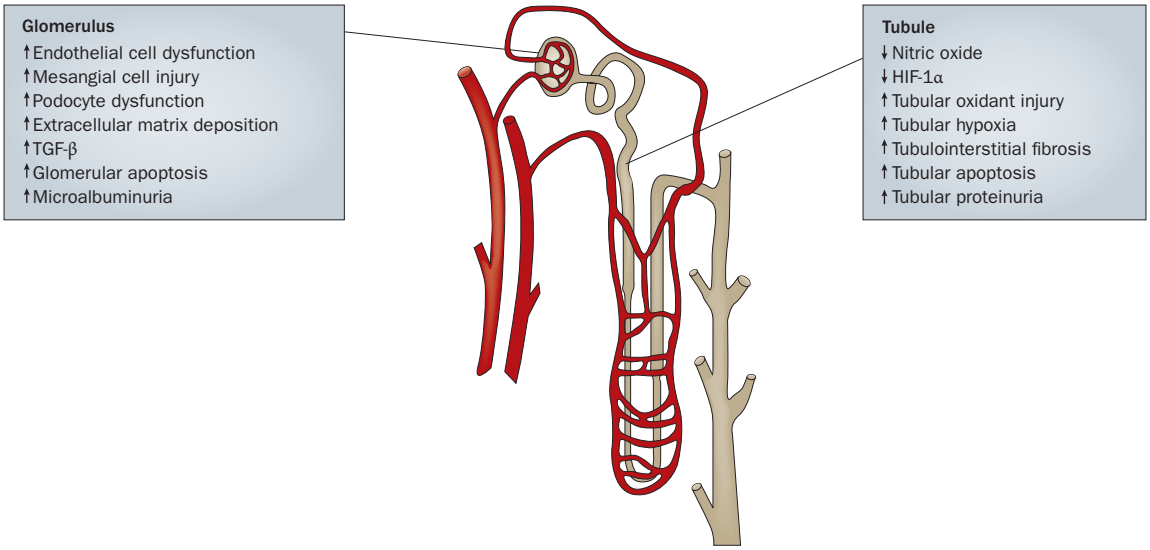

Figure 5 | Effects of oxidative stress in early diabetic nephropathy. Abbreviations: HIF-1α, hypoxia-inducible factor 1α; TGF-β, transforming growth factor β.

oxidative stress. Several factors might contribute directly or indirectly to this susceptibility (Figure 5). The glomerular mesangial cells and tubular cells do not require insulin for glucose uptake and consequently have no control over glucose movement across the cells.[34] Chronic hyperglycemia in the renal milieu stimulates the production of AGEs, the polyol pathway and activation of PKC, all of which lead to increased ROS formation and oxidative stress.[35]

Renal cells (glomerulus, tubules and fibroblasts) express NADPH oxidase and contribute to ROS formation in small amounts in the healthy state. In chronic hyperglycemia, dysfunctional renal cells can increase ROS generation, which may enhance renal tissue injury.[36] In addition to structural and functional derangements, presence of excessive ROS in the renal milieu can promote dysregulation of renal medullary blood flow leading to renovascular hypertension.[37]

Oxidative stress in the glomerulus

The glomerular endothelial cells are particularly vulnerable to chronic hyperglycemia owing to their strategic location as a biological barrier between the blood and the mesangium.[37] Chronic hyperglycemia and elevated levels of free fatty acids in diabetes mellitus are potent stimulators of NADPH oxidase in these cells.[38] In addition, ROS production is further exacerbated by hyperglycemia through an increase in intrarenal concentration of angiotensin 2.[18]

Increased production of ROS in the glomerular microcirculation reduces the bioavailability of nitric oxide, resulting in dysregulation of mesangial contraction and arteriolar tone and persistent oxidative stress, giving rise to endothelial dysfunction, leukocyte adherence and glomerular cell apoptosis.[39] Chronic hyperglycemia essentially facilitates a state of chronic oxidative stress in the glomerular milieu, the long-term consequence of which is progressive loss of glomerular cells.

The earliest morphological change in diabetic nephropathy is a thickening of the glomerular basement membrane as a result of mesangial matrix deposition and hypertrophy of mesangial cells.[40] With disease progression enhanced deposition of the extracellular matrix occurs, owing to increased production and/or decreased degradation of its components, type IV collagen, laminin and fibronectin. In addition, a widening of the foot processes of podocytes—these visceral epithelial cells wrap around the capillaries of the glomerulus and leave slits between them to filter the blood—and loss of glomerular nephrin, a zipper-like protein that forms the slit diaphragm, has been described.[41]

Increased oxidative stress in conjunction with raised inflammatory markers in chronic hyperglycemia activates several signaling pathways (Figure 4), such as the PKC,[42] mitogen-activated protein kinase[43] and JAK–STAT (Janus kinase–signal transducer and activator of transcription) signaling pathways.[44] This process is associated with activation of redox-sensitive transcription factors that include NFκB, AP1 (Fos and Jun proteins), STAT and early growth response protein 1.[45,46] These factors enhance the transactivation of genes coding for cytokines such as TGF-β[47] and connective tissue growth factor,[48] which upregulate the expression of extracellular matrix proteins.[45]

In addition, new evidence suggests that glomerular podocytes have a key role in early proteinuria, and reduced podocyte number has been reported in patients with diabetic nephropathy.[49] Chronic hyperglycemia-induced ROS production has been implicated in early podocyte damage and apoptosis.[50] Experimental studies suggest that amelioration of oxidative stress by antioxidant therapies may potentially prevent podocyte damage in early diabetic nephropathy.[49,51]

Oxidative stress in tubules

Enhanced oxidative stress contributes to early tubular injury and apoptosis and is mediated via signaling of

multiple caspases, such as caspase 3, 8 and 9.[52] In addition, enhanced oxidative stress in conjunction with increased angiotensin 2 levels activates TGF-β, which is a key regulator of extracellular matrix remodeling in the mesangium, tubulointerstitium and tubular epithelial–mesenchymal transition.[53] Enhanced and sustained activation of TGF-β by increased ROS production in diabetes mellitus may result in excessive extracellular matrix remodeling in the mesangium and promotion of fibrotic processes in the tubulointerstitium.[54]

Physiologically, given the limited blood supply and disproportionate energy requirements, tubular and interstitial cells in the juxtamedullary region and outer medulla are, even under normal conditions, in a state of relative hypoxia, which may worsen in the hyperglycemic milieu.[55] In addition, chronic hyperglycemia may give rise to alterations in the regulation of intrarenal blood flow, resulting in reduced oxygen delivery and low oxygen tension in this region.[33] High concentrations of nitric oxide are constantly maintained in the renal medulla to effectively restrain mitochondrial respiration in a dose-dependent manner, especially during hypoxic states.[56]

Hypoxia-inducible factor (HIF-1α) is a major mediator of cell adaptation to hypoxia. HIF-1α manifests its effect by promoting vasculogenesis, improving oxygen availability and modulating cellular metabolism and protects against fibrotic processes in the kidney by modulation of fibrosis-promoting genes.[57] Hyperglycemia impairs the stabilization of HIF-1α against protease degradation in a dose-dependent fashion, thus reducing its availability and promoting interstitial fibrosis.[58] ROS is an important modulator of HIF-1α and regulates its degradation in hypoxic conditions.[59] Low levels of ROS have an important role in oxygen sensing and influence the stability of HIF-1α. Sustained stimulation of HIF-1α by enhanced ROS generation in the context of reduced nitric oxide availability may adversely affect HIF-1α stability, leading to tubulointerstitial fibrosis via induction of inflammatory responses.[60]

Measurement of oxidative stress

Evidently, oxidative stress, either from increased production of ROS and/or reduced bioavailability of nitric oxide, has a pivotal role in endothelial cell dysfunction and, hence, early diabetic nephropathy. Unfortunately, no reliable and reproducible method to measure oxidative stress or endothelial cell dysfunction is available to date. The presence of these two factors is usually inferred through surrogate markers, such as flow-mediated vasodilation after induction of transient ischemia[61] or evaluation of changes in vascular resistance in small or large arteries in response to a physiological stimulus.[62] These techniques basically assess the capacity of endothelial cells to release nitric oxide in response to various stimuli.

In addition to these approaches, a number of methods that allow determination of plasma levels of various ROS are available. Nevertheless, estimation of ROS levels in plasma is difficult because of the highly reactive nature of these molecules. Several studies have measured the total antioxidant buffering capacity of plasma or, alternatively, specific markers of free radical-mediated damage such as F2-isoprostane or oxidized LDL.[63] However, these research tools are yet to be validated in large populations and their application in the routine clinical setting is far off.

Antioxidative stress therapies

Antioxidants

In the physiological state, a balance between promoters and inhibitors of oxidant injury is maintained through various intrinsic renal cytoprotective antioxidant factors and enzymatic responses. Some of the major antioxidant molecules are superoxide dismutase (SOD), glutathione peroxidase, catalase, heme oxygenase and biliverdin reductase.[64]

SOD is the first-line physiological defense against oxidative stress and reacts with superoxide to generate hydrogen peroxide (Figure 3), which, in turn, is degraded by catalase[65] and glutathione peroxidase.[66] In animal models of diabetic nephropathy, addition of the exogenous SOD mimetic tempol has shown encouraging results, with suppression of albuminuria, TGF-β levels, collagen synthesis and oxidative stress.[66,67] In addition, tempol has been demonstrated to diffuse through the cell membrane and, hence, can react with both intracellular and extracellular ROS.[66]

Glutathione peroxidase uses glutathione to reduce hydrogen peroxide to water and lipid peroxides, along with acting as a peroxynitrite reductase.[68] Chronic treatment of Zucker diabetic rats with ebselen, a glutathione mimic, has demonstrated potent antiperoxynitrite and antioxidant activity that substantially ameliorate tubulointerstitial pathology, inflammation and markers of nitrosative and oxidative stress in early diabetic nephropathy.[69]

The antioxidant enzyme catalase has generated much interest after the demonstration that catalase overexpression in the proximal tubules of transgenic diabetic mice attenuates interstitial fibrosis and tubular apoptosis.[70] This effect was attributed to increased degradation of hydrogen peroxide, which decreases oxidative stress and thus reduces stimulation of angiotensin 2-mediated activation of TGF-β.[65,70]

Heme oxygenase converts heme, a pro-oxidant, into iron, carbon monoxide and biliverdin.[71] Biliverdin generated in this reaction is then transformed into a potent antioxidant, bilirubin, by biliverdin reductase.[71]

Current therapies

Given the difficulties in measurement of markers of oxidative stress and endothelial cell dysfunction, target-specific antioxidant therapies are still elusive. As the driver of oxidative stress is hyperglycemia, strict glycemic control remains the cornerstone of antioxidant therapy in diabetes mellitus. In addition to strict glycemic control, many of the current standard therapeutic approaches may also help to ameliorate oxidative stress, directly, indirectly or as pleiotropic effects. These therapies include angiotensin-converting enzyme (ACE) inhibitors,[72] angiotensin-receptor blockers[73] and aldosterone blockers (spironolactone),[74] which, along with control of systemic and intrarenal blood pressure, activate eNOS, increase bioavailability of nitric oxide, inhibit synthesis

of angiotensin 2 and TGF-β and thus help to ameliorate or prevent tubulointerstitial fibrosis in diabetic nephropathy. Similarly, pioglitazone, an antihyperglycemic agent, has been shown to markedly reduce glomerulosclerosis, glomerular hypertrophy, mesangial expansion, tubulointerstitial fibrosis and urinary albumin excretion in diabetic nephropathy.[75,76] These effects may decrease oxidative stress through a reduction of hyperglycemia and insulin resistance.[76]

Lipid-lowering agents such as statins, which inhibit HMG-CoA reductase, have been demonstrated to activate eNOS, maintain glomerular filtration rate and renal cortical blood flow and ameliorate glomerular lesions.[77,78] Benfotiamine, a drug used in the treatment of diabetic neuropathy, has been demonstrated to reduce ROS formation and thus oxidative stress by activation of eNOS and may decrease hyperfiltration and proteinuria in patients with diabetic nephropathy.[79]

Potential therapies

The ideal antioxidant therapy would be target-specific, have minimal adverse effects and influence all pathways of ROS generation and, more selectively, mitochondrial ROS generation. Several potentially beneficial therapies, although still in the experimental phase, are being studied.

Inhibition of ROS generated by AGE

In view of the vast generation of ROS as a result of excessive advanced glycation in diabetes mellitus, a variety of pharmacological approaches are being evaluated in experimental and early clinical studies that inhibit AGE formation. Alagebrium, a new prototypic compound with appreciable biological activity, has been shown to reduce vascular stiffness and renal AGEs in animal and human models.[80] Other investigational therapies of anti-AGE formation include aminoguanidine[81] and pyridoxamine,[82] which exhibit their effect by trapping reactive carbonyl intermediates on the basis of their nucleophilic potential. Hydrallazine[83] and ascorbic acid[84] are other agents with anti-AGE effects, although evidence of any therapeutic benefit is limited.

Some encouraging results on the potentially beneficial role of ACE inhibitors and angiotensin-receptor blockers in reducing ROS and AGE formation have been obtained from small studies.[85] In addition, in view of the beneficial effects of tempol and ebselen in animal models, further evaluation of these agents is warranted to examine their potential as antioxidant replenishment agents in humans with diabetic nephropathy.

Inhibition of PKC and TGF-β

Chronic hyperglycemia results in overexpression of PKC and TGF-β, which causes increased generation of ROS. Ruboxistaurin, an inhibitor of PKC, has shown benefit in some small clinical studies.[86,87] Pirfenidone, a TGF-β inhibitor, has been demonstrated to ameliorate oxidative stress and reverse renal fibrosis in rodent models of diabetes.[88,89] Studies on both these agents are preliminary and further evaluation is needed.

Other approaches

Other potential agents with antioxidant properties are currently under investigation. L-propionylcarnitine,[90] an intracellular superoxide scavenger, has been shown to ameliorate DNA damage and improve mitochondrial function experimentally. Pentoxifylline is an established inhibitor of phosphodiesterases and platelet aggregation and has potent antioxidant properties when administered alone[91] or in combination with ACE inhibitors in small studies of patients with diabetic nephropathy.[92]

Bioflavonoids[93] have antioxidant, free-radical-scavenging and DNA cleavage properties. Lipoic acid acts as a mitochondrial antioxidant and was shown to ameliorate endothelial cell dysfunction.[94,95] Vitamin C and E exert their antioxidant properties by scavenging free radicals, decreasing nitric oxide inactivation and increasing eNOS activity. These agents have been examined in experimental[96] and small clinical studies,[95,97] with some preliminary indications of benefit. A small clinical study in patients with chronic kidney disease (including those with diabetes mellitus) demonstrated beneficial effects of Allopurinol, with a substantial reduction in inflammation and progression of chronic kidney disease.[98] These positive changes indirectly suggest an improvement in overall oxidative stress, although this measure was not an endpoint of the study.[98]

Conclusions

Oxidative stress, though under-recognized, is ubiquitous in patients with diabetes mellitus. It occurs early and plays an important part in the pathogenesis of endothelial cell dysfunction and microalbuminuria. Chronic hyperglycemia is the single most important factor in the generation of early and sustained oxidative stress. Other complications of chronic hyperglycemia that promote oxidative stress include enhanced production of AGEs, decreased nitric oxide production, increased cytokine activation and levels of inflammatory markers. Enhanced ROS formation and reduced bioavailability of nitric oxide is the hallmark of oxidative stress in diabetes mellitus.

Oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities, even before the onset of microalbuminuria. These changes include extracellular deposition of matrix in the mesangium, promotion of a hypoxic environment by early microvascular damage, induction of cellular oxidant injury and apoptosis and, finally, promotion of tubulointerstitial fibrosis by activation of TGF-β, which stimulates several pathways of fibrosis.

In view of the potential hazards of sustained oxidative stress, an effective, target-specific antioxidant agent to treat patients with diabetic nephropathy would be highly desirable. Currently, apart from the primary prevention of chronic hyperglycemia, standard medications, such as antilipid and antihypertensive agents, have demonstrated potent pleiotropic antioxidant effects, in addition to their primary effects. However, these agents are yet to be examined for their clinical benefits as stand-alone antioxidants. A surge in the search for an appropriate antioxidant has

lead to the determination of several promising new agents currently under development. However, until definitive antioxidants are available, regular monitoring of patients with diabetes mellitus with emphasis on clinical parameters (tight glycemic, lipid and blood pressure control) and lifestyle measures (regular exercise, healthy diet and cessation of smoking) remains the cornerstone of therapy.

Review criteria

A search for original articles published between 1980 and 2010 was performed in PubMed, Google and Scopus. The search terms used were "diabetes mellitus", "diabetic nephropathy", "endothelial cell dysfunction", "oxidative stress", "reactive oxygen species", "nitric oxide", "advanced glycation end products" and "anti-oxidant therapy".

1. Hakim, F. A. & Pflueger, A. Role of oxidative stress in diabetic kidney disease. *Med. Sci. Monit.* **16**, RA37–RA48 (2010).
2. Foley, R. N. *et al.* Cardiac disease in diabetic end-stage renal disease. *Diabetologia* **40**, 1307–1312 (1997).
3. Mogensen, C. E., Christensen, C. K. & Vittinghus, E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. *Diabetes* **32** (Suppl. 2), 64–78 (1983).
4. Schalkwijk, C. G. & Stehouwer, C. D. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clin. Sci. (Lond.)* **109**, 143–159 (2005).
5. Singh, D. K., Winocour, P. & Farrington, K. Endothelial cell dysfunction, medial arterial calcification and osteoprotegerin in diabetes. *Br. J. Diabetes Vasc.* **71**, 54–56 (2010).
6. Goligorsky, M. S., Chen, J. & Brodsky, S. Workshop: endothelial cell dysfunction leading to diabetic nephropathy: focus on nitric oxide. *Hypertension* **37**, 744–748 (2001).
7. Stehouwer, C. D. Endothelial dysfunction in diabetic nephropathy: state of the art and potential significance for non-diabetic renal disease. *Nephrol. Dial. Transplant.* **19**, 778–781 (2004).
8. Naidoo, N. P. The link between microalbuminuria, endothelial dysfunction and cardiovascular disease in diabetes. *Cardiovasc. J. S. Afr.* **13**, 194–199 (2002).
9. Yamagishi, S., Nakamura, K., Jinnouchi, Y., Takenaka, K. & Imaizumi, T. Molecular mechanisms for vascular injury in the metabolic syndrome. *Drugs Exp. Clin. Res.* **31**, 123–129 (2005).
10. Steinberg, H. O., Brechtel, G., Johnson, A., Fineberg, N. & Baron, A. D. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Invest.* **94**, 1172–1179 (1994).
11. Yaqoob, M. *et al.* Relationship between markers of endothelial dysfunction, oxidant injury and tubular damage in patients with insulin-dependent diabetes mellitus. *Clin. Sci. (Lond.)* **85**, 557–562 (1993).
12. Nishikawa, T. *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* **404**, 787–790 (2000).
13. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820 (2001).
14. Forbes, J. M., Coughlan, M. T. & Cooper, M. E. Oxidative stress as a major culprit in kidney disease in diabetes. *Diabetes* **57**, 1446–1454 (2008).
15. Cave, A. C. *et al.* NADPH oxidases in cardiovascular health and disease. *Antioxid. Redox Signal.* **8**, 691–728 (2006).
16. Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D. & Alexander, R. W. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.* **74**, 1141–1148 (1994).
17. Thomas, S. R., Witting, P. K. & Drummond, G. R. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid. Redox Signal.* **10**, 1713–1765 (2008).
18. Li, J. M. & Shah, A. M. ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy. *J. Am. Soc. Nephrol.* **14** (Suppl. 3), S221–S226 (2003).
19. Geiszt, M. & Leto, T. L. The Nox family of NAD(P)H oxidases: host defense and beyond. *J. Biol. Chem.* **279**, 51715–51718 (2004).
20. Gorin, Y. *et al.* Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. *J. Biol. Chem.* **280**, 39616–39626 (2005).
21. Block, K., Gorin, Y. & Abboud, H. E. Subcellular localization of Nox4 and regulation in diabetes. *Proc. Natl Acad. Sci. USA* **106**, 14385–14390 (2009).
22. Bucala, R. & Vlassara, H. Advanced glycosylation end products in diabetic renal and vascular disease. *Am. J. Kidney Dis.* **26**, 875–888 (1995).
23. Fu, M. X. *et al.* Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes* **43**, 676–683 (1994).
24. Simm, A. *et al.* Advanced glycation endproducts stimulate the MAP-kinase pathway in tubulus cell line LLC-PK1. *FEBS Lett.* **410**, 481–484 (1997).
25. Gugliucci, A. & Bendayan, M. Renal fate of circulating advanced glycated end products (AGE): evidence for reabsorption and catabolism of AGE-peptides by renal proximal tubular cells. *Diabetologia* **39**, 149–160 (1996).
26. Coughlan, M. T. *et al.* RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. *J. Am. Soc. Nephrol.* **20**, 742–752 (2009).
27. Kiritoshi, S. *et al.* Reactive oxygen species from mitochondria induce cyclooxygenase-2 gene expression in human mesangial cells: potential role in diabetic nephropathy. *Diabetes* **52**, 2570–2577 (2003).
28. Gibb, D. M. *et al.* Renal tubular proteinuria and microalbuminuria in diabetic patients. *Arch. Dis. Child.* **64**, 129–134 (1989).
29. Pflueger, A. C., Osswald, H. & Knox, F. G. Adenosine-induced renal vasoconstriction in diabetes mellitus rats: role of nitric oxide. *Am. J. Physiol.* **276**, F340–F346 (1999).
30. Balakumar, P., Chakkarwar, V. A., Krishnan, P. & Singh, M. Vascular endothelial dysfunction: a tug of war in diabetic nephropathy? *Biomed. Pharmacother.* **63**, 171–179 (2009).
31. MacNaul, K. L. & Hutchinson, N. I. Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions. *Biochem. Biophys. Res. Commun.* **196**, 1330–1334 (1993).
32. Buerk, D. G. Can we model nitric oxide biotransport? A survey of mathematical models for a simple diatomic molecule with surprisingly complex biological activities. *Annu. Rev. Biomed. Eng.* **3**, 109–143 (2001).
33. Palm, F. Intrarenal oxygen in diabetes and a possible link to diabetic nephropathy. *Clin. Exp. Pharmacol. Physiol.* **33**, 997–1001 (2006).
34. Heilig, C. W. *et al.* Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. *J. Clin. Invest.* **96**, 1802–1814 (1995).
35. Pacher, P., Obrosova, I. G., Mabley, J. G. & Szabó, C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr. Med. Chem.* **12**, 267–275 (2005).
36. Shiose, A. *et al.* A novel superoxide-producing NAD(P)H oxidase in kidney. *J. Biol. Chem.* **276**, 1417–1423 (2001).
37. Chabrashvili, T. *et al.* Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. **39**, 269–274 (2002).
38. Inoguchi, T. *et al.* High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* **49**, 1939–1945 (2000).
39. Pautz, A. *et al.* Cross-talk between nitric oxide and superoxide determines ceramide formation and apoptosis in glomerular cells. *Kidney Int.* **61**, 790–796 (2002).
40. Wolf, G., Reinking, R., Zahner, G., Stahl, R. A. & Shankland, S. J. Erk 1,2 phosphorylates p27(Kip1): Functional evidence for a role in high glucose-induced hypertrophy of mesangial cells. *Diabetologia* **46**, 1090–1099 (2003).
41. Kim, Y. *et al.* Differential expression of basement membrane collagen chains in diabetic nephropathy. *Am. J. Pathol.* **138**, 413–420 (1991).
42. Scivittaro, V., Ganz, M. B. & Weiss, M. F. AGEs induce oxidative stress and activate protein kinase C-beta(II) in neonatal mesangial cells. *Am. J. Physiol. Renal Physiol.* **278**, F676–F683 (2000).
43. Lander, H. M. *et al.* Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J. Biol. Chem.* **272**, 17810–17814 (1997).
44. Simon, A. R., Rai, U., Fanburg, B. L. & Cochran, B. H. Activation of the JAK-STAT pathway by reactive oxygen species. *Am. J. Physiol.* **275**, C1640–C1652 (1998).
45. Mason, R. M. & Wahab, N. A. Extracellular matrix metabolism in diabetic nephropathy. *J. Am. Soc. Nephrol.* **14**, 1358–1373 (2003).
46. Palmer, H. J. & Paulson, K. E. Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr. Rev.* **55**, 353–361 (1997).
47. Nath, K. A. *et al.* Redox regulation of renal DNA synthesis, transforming growth factor-beta1 and collagen gene expression. *Kidney Int.* **53**, 367–381 (1998).
48. Park, S. K. *et al.* Hydrogen peroxide is a novel inducer of connective tissue growth factor.

Biochem. Biophys. Res. Commun. **284**, 966–971 (2001).
49. Spurney, R. F. & Coffman, T. M. Stressed-out podocytes in diabetes? *J. Am. Soc. Nephrol.* **19**, 2035–2037 (2008).
50. Susztak, K., Raff, A. C., Schiffer, M. & Böttinger, E. P. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* **55**, 225–233 (2006).
51. Zheng, S. *et al.* Podocyte-specific overexpression of the antioxidant metallothionein reduces diabetic nephropathy. *J. Am. Soc. Nephrol.* **19**, 2077–2085 (2008).
52. Allen, D. A., Harwood, S., Varagunam, M., Raftery, M. J. & Yaqoob, M. M. High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *FASEB J.* **17**, 908–910 (2003).
53. Baricos, W. H., Cortez, S. L., Deboisblanc, M. & Xin, S. Transforming growth factor-beta is a potent inhibitor of extracellular matrix degradation by cultured human mesangial cells. *J. Am. Soc. Nephrol.* **10**, 790–795 (1999).
54. Lee, H. B., Yu, M. R., Yang, Y., Jiang, Z. & Ha, H. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. *J. Am. Soc. Nephrol.* **14** (Suppl. 3), S241–S245 (2003).
55. Singh, D. K., Winocour, P. & Farrington, K. Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy. *Nat. Clin. Pract. Nephrol.* **4**, 216–226 (2008).
56. Palm, F. *et al.* Reduced nitric oxide in diabetic kidneys due to increased hepatic arginine metabolism: implications for renomedullary oxygen availability. *Am. J. Physiol. Renal Physiol.* **294**, F30–F37 (2008).
57. Higgins, D. F. *et al.* Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am. J. Physiol. Renal Physiol.* **287**, F1223–F1232 (2004).
58. Catrina, S. B., Okamoto, K., Pereira, T., Brismar, K. & Poellinger, L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. *Diabetes* **53**, 3226–3232 (2004).
59. Callapina, M., Zhou, J., Schmid, T., Köhl, R. & Brüne, B. NO restores HIF-1alpha hydroxylation during hypoxia: role of reactive oxygen species. *Free Radic. Biol. Med.* **39**, 925–936 (2005).
60. Gupte, S. A. & Wolin, M. S. Oxidant and redox signaling in vascular oxygen sensing: implications for systemic and pulmonary hypertension. *Antioxid. Redox Signal.* **10**, 1137–1152 (2008).
61. Corretti, M. C. *et al.* Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J. Am. Coll. Cardiol.* **39**, 257–265 (2002).
62. Vincent, M. A., Barrett, E. J., Lindner, J. R., Clark, M. G. & Rattigan, S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am. J. Physiol. Endocrinol. Metab.* **285**, E123–E129 (2003).
63. Stephens, J. W., Khanolkar, M. P. & Bain, S. C. The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease. *Atherosclerosis* **202**, 321–329 (2009).
64. Wagener, F. A., Dekker, D., Berden, J. H., Scharstuhl, A. & van der Vlag, J. The role of reactive oxygen species in apoptosis of the diabetic kidney. *Apoptosis* **14**, 1451–1458 (2009).
65. Bagby, S. P. Diabetic nephropathy and proximal tubule ROS: challenging our glomerulocentricity. *Kidney Int.* **71**, 1199–1202 (2007).
66. Fujita, H. *et al.* Reduction of renal superoxide dismutase in progressive diabetic nephropathy. *J. Am. Soc. Nephrol.* **20**, 1303–1313 (2009).
67. Samuni, A. M., DeGraff, W., Krishna, M. C. & Mitchell, J. B. Cellular sites of H202-induced damage and their protection by nitroxides. *Biochim. Biophys. Acta* **1525**, 70–76 (2001).
68. Yamaguchi, T. *et al.* Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. *Stroke* **29**, 12–17 (1998).
69. Chander, P. N. *et al.* Nephropathy in Zucker diabetic fat rat is associated with oxidative and nitrosative stress: prevention by chronic therapy with a peroxynitrite scavenger ebselen. *J. Am. Soc. Nephrol.* **15**, 2391–2403 (2004).
70. Brezniceanu, M. L. *et al.* Attenuation of interstitial fibrosis and tubular apoptosis in db/db transgenic mice overexpressing catalase in renal proximal tubular cells. *Diabetes* **57**, 451–459 (2008).
71. Abraham, N. G. & Kappas, A. Heme oxygenase and the cardiovascular-renal system. *Free Radic. Biol. Med.* **39**, 1–25 (2005).
72. Kobayashi, N. *et al.* Critical role of bradykinin-eNOS and oxidative stress-LOX-1 pathway in cardiovascular remodeling under chronic angiotensin-converting enzyme inhibition. *Atherosclerosis* **187**, 92–100 (2006).
73. Ogawa, S. *et al.* Angiotensin II type 1 receptor blockers reduce urinary oxidative stress markers in hypertensive diabetic nephropathy. *Hypertension* **47**, 699–705 (2006).
74. Takebayashi, K., Matsumoto, S., Aso, Y. & Inukai, T. Aldosterone blockade attenuates urinary monocyte chemoattractant protein-1 and oxidative stress in patients with type 2 diabetes complicated by diabetic nephropathy. *J. Clin. Endocrinol. Metab.* **91**, 2214–2217 (2006).
75. Calkin, A. C. *et al.* PPAR-alpha and -gamma agonists attenuate diabetic kidney disease in the apolipoprotein E knockout mouse. *Nephrol. Dial. Transplant.* **21**, 2399–2405 (2006).
76. Okada, T. *et al.* Thiazolidinediones ameliorate diabetic nephropathy via cell cycle-dependent mechanisms. *Diabetes* **55**, 1666–1677 (2006).
77. Usui, H. *et al.* HMG-CoA reductase inhibitor ameliorates diabetic nephropathy by its pleiotropic effects in rats. *Nephrol. Dial. Transplant.* **18**, 265–272 (2003).
78. Endres, M. & Laufs, A. Effects of statins on endothelium and signaling mechanisms. *Stroke* **35** (Suppl. 1), 2708–2711 (2004).
79. Babaei-Jadidi, R., Karachalias, N., Ahmed, N., Battah, S. & Thornalley, P. J. Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. *Diabetes* **52**, 2110–2120 (2003).
80. Bakris, G. L. *et al.* Advanced glycation end-product cross-link breakers. A novel approach to cardiovascular pathologies related to the aging process. *Am. J. Hypertens.* **17**, 23S–30S (2004).
81. Bolton, W. K. *et al.* Randomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. *Am. J. Nephrol.* **24**, 32–40 (2004).
82. Williams, M. E. *et al.* Effects of pyridoxamine in combined phase 2 studies of patients with type 1 and type 2 diabetes and overt nephropathy. *Am. J. Nephrol.* **27**, 605–614 (2007).
83. Nangaku, M. *et al.* Anti-hypertensive agents inhibit *in vivo* the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. *J. Am. Soc. Nephrol.* **14**, 1212–1222 (2003).
84. Krone, C. A. & Ely, J. T. Ascorbic acid, glycation, glycohemoglobin and aging. *Med. Hypotheses* **62**, 275–279 (2004).
85. Miyata, T. *et al.* Angiotensin II receptor antagonists and angiotensin-converting enzyme inhibitors lower *in vitro* the formation of advanced glycation end products: biochemical mechanisms. *J. Am. Soc. Nephrol.* **13**, 2478–2487 (2002).
86. Gilbert, R. E. *et al.* Effect of ruboxistaurin on urinary transforming growth factor-beta in patients with diabetic nephropathy and type 2 diabetes. *Diabetes Care* **30**, 995–996 (2007).
87. Danis, R. P. & Sheetz, M. J. Ruboxistaurin: PKC-beta inhibition for complications of diabetes. *Expert Opin. Pharmacother.* **10**, 2913–2925 (2009).
88. RamachandraRao, S. P. *et al.* Pirfenidone is renoprotective in diabetic kidney disease. *J. Am. Soc. Nephrol.* **20**, 1765–1775 (2009).
89. Miric, G. *et al.* Reversal of cardiac and renal fibrosis by pirfenidone and spironolactone in streptozotocin-diabetic rats. *Br. J. Pharmacol.* **133**, 687–694 (2001).
90. Vanella, A. *et al.* L-propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector. *Cell Biol. Toxicol.* **16**, 99–104 (2000).
91. Navarro, J. F. *et al.* Urinary protein excretion and serum tumor necrosis factor in diabetic patients with advanced renal failure: effects of pentoxifylline administration. *Am. J. Kidney Dis.* **33**, 458–463 (1999).
92. Harmankaya, O., Seber, S. & Yilmaz, M. Combination of pentoxifylline with angiotensin converting enzyme inhibitors produces an additional reduction in microalbuminuria in hypertensive type 2 diabetic patients. *Ren. Fail.* **25**, 465–470 (2003).
93. Russo, A. *et al.* Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biol. Toxicol.* **16**, 91–98 (2000).
94. Heitzer, T. *et al.* Beneficial effects of alpha-lipoic acid and ascorbic acid on endothelium-dependent, nitric oxide-mediated vasodilation in diabetic patients: relation to parameters of oxidative stress. *Free Radic. Biol. Med.* **31**, 53–61 (2001).
95. Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A. & Salvetti, A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* **97**, 2222–2229 (1998).
96. Simsek, M., Naziroglu, M. & Erdinç, A. Moderate exercise with a dietary vitamin C and E combination protects against streptozotocin-induced oxidative damage to the kidney and lens in pregnant rats. *Exp. Clin. Endocrinol. Diabetes* **113**, 53–59 (2005).
97. Farvid, M. S., Jalali, M., Siassi, F. & Hosseini, M. Comparison of the effects of vitamins and/or mineral supplementation on glomerular and tubular dysfunction in type 2 diabetes. *Diabetes Care* **28**, 2458–2464 (2005).
98. Goicoechea, M. *et al.* Effect of allopurinol in chronic kidney disease progression and cardiovascular risk. *Clin. J. Am. Soc. Nephrol.* **5**, 1388–1393 (2010).
99. Yokoyama, M. & Hirata, K. Endothelial nitric oxide synthase uncoupling: Is it a physiological mechanism of endothelium-dependent relaxation in cerebral artery? *Cardiovasc. Res.* **73**, 8–9 (2007).
100. Versari, D., Daghini, E., Virdis, A., Chiadoni, L. & Taddei, S. Endothelium-dependent contractions and endothelial dysfunction in human hypertension. *Br. J. Pharmacol.* **157**, 527–536 (2009).

Author contributions
D. K. Singh researched the data for the article. All authors provided a substantial contribution to discussions of the content, contributed equally to writing the article and reviewed and/or edited the manuscript before submission.