Oxidative Stress as a Major Culprit in Kidney Disease in Diabetes

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It is postulated that localized tissue oxidative stress is a key component in the development of diabetic nephropathy. There remains controversy, however, as to whether this is an early link between hyperglycemia and renal disease or develops as a consequence of other primary pathogenic mechanisms. In the kidney, a number of pathways that generate reactive oxygen species (ROS) such as glycolysis, specific defects in the polyol pathway, uncoupling of nitric oxide synthase, xanthine oxidase, NAD(P)H oxidase, and advanced glycation have been identified as potentially major contributors to the pathogenesis of diabetic kidney disease. In addition, a unifying hypothesis has been proposed whereby mitochondrial production of ROS in response to chronic hyperglycemia may be the key initiator for each of these pathogenic pathways. This postulate emphasizes the importance of mitochondrial dysfunction in the progression and development of diabetes complications including nephropathy. A mystery remains, however, as to why antioxidants per se have demonstrated minimal renoprotection in humans despite positive preclinical research findings. It is likely that the utility of current study approaches, such as vitamin use, may not be the ideal antioxidant strategy in human diabetic nephropathy. There is now an increasing body of data to suggest that strategies involving a more targeted antioxidant approach, using agents that penetrate specific cellular compartments, may be the elusive additive therapy required to further optimize renoprotection in diabetes. *Diabetes* 57:1446–1454, 2008

Renal disease in diabetic patients is characterized by functional as well as structural abnormalities (1). Within the glomeruli there is thickening of basement membranes, mesangial expansion, hypertrophy, and glomerular epithelial cell (podocyte) loss. In conjunction, the disease progresses in the tubulointerstitial compartment causing expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis, and arteriosclerosis. To date, the most effective treatments for progressive diabetic nephropathy appear to be antihypertensive agents, particularly those that target the renin-angiotensin system (RAS), such as ACE inhibitors, angiotensin receptor-1 antagonists, or their combination (2). Although these treatments retard the relentless progression to end-stage renal disease that occurs in diabetic patients susceptible to nephropathy, these agents do not prevent this disorder.

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AGE, advanced glycation end product; FFA, free fatty acid; G6PDH, glucose-6-phosphate dehydrogenase; NOS, nitric oxide synthase; RAGE, receptor for AGEs; RAS, renin-angiotensin system, ROS, reactive oxygen species; SOD, superoxide dismutase.

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THE CONCEPT OF OXIDATIVE STRESS

Oxidative stress (or oxidant-derived tissue injury) occurs when production of oxidants or reactive oxygen species (ROS) exceeds local antioxidant capacity. When this occurs, oxidation of important macromolecules including proteins, lipids, carbohydrates, and DNA ensues. Although animal studies have demonstrated potent inhibition of oxidative stress with certain antioxidants (3) with associated end-organ protection under experimental diabetic conditions, human studies with various antioxidants including α-tocopherol (4) have been generally disappointing. Thus, the general view has been that conventional antioxidant therapy is not likely to have particular benefit as part of the strategy to reduce diabetes complications including nephropathy. In this Perspective on the News, we focus on the diverse sources of ROS generation in a diabetic milieu and postulate that more targeted, rationally designed antioxidant approaches may ultimately be worth considering as part of the therapeutic strategy to optimize renoprotection in diabetes. Each of these suggested points of targeted intervention are highlighted throughout this review and presented within Fig. 2.

There are a number of enzymatic and nonenzymatic sources of ROS in the diabetic kidney, including autooxidation of glucose, transition metal–catalyzed Fenton reactions, advanced glycation, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase (NOS) and NAD(P)H oxidase. ROS include free radicals such as superoxide ($^{\bullet}O_2^-$), hydroxyl ($^{\bullet}OH$), and peroxyl ($^{\bullet}RO_2$) and nonradical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). It is important to note that there are also reactive nitrogen species produced from similar pathways, which include the radicals nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2^-$), as well as the nonradical peroxynitrite ($ONOO^-$), nitrous oxide (HNO_2), and alkyl peroxynitrates (RONOO). Of these, $^{\bullet}O_2^-$, $^{\bullet}NO$, H_2O_2, and $ONOO^-$ have been the most widely investigated in the diabetic kidney; therefore, this review will focus on the sources of these ROS (Fig. 1).

GLUCOSE AS OUR PRIMARY FUEL SOURCE

Arguably, the most important factor in the excessive intracellular generation of ROS by hyperglycemia is the ability of individual cell types to process glucose. It is critical that cells are able to decrease the transport of glucose across the plasma membrane into the cytosol when exposed to hyperglycemia in order to maintain

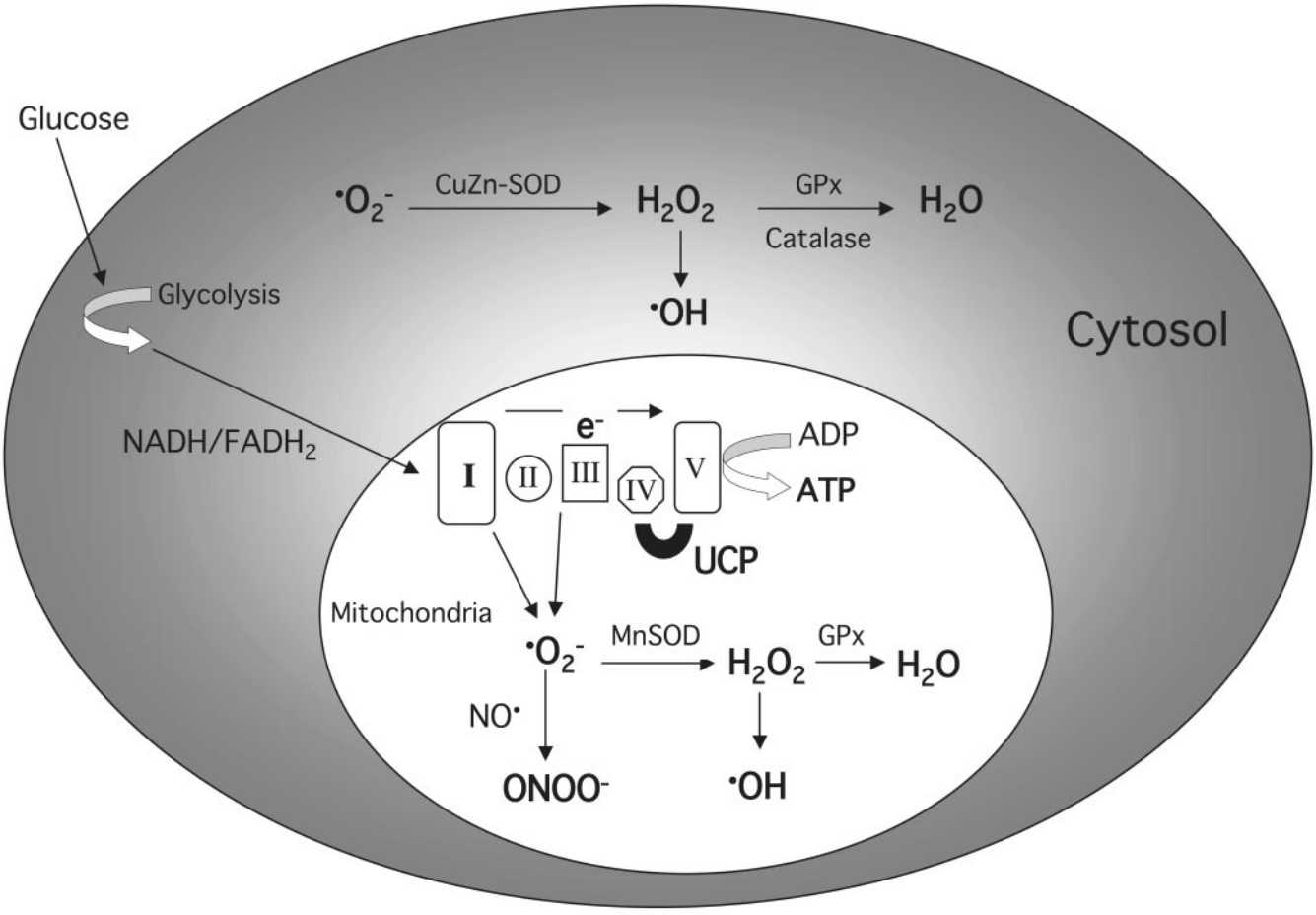

FIG. 1. Major ROS generated within renal cells in diabetic milieu and the classic antioxidant pathways for their detoxification. I, NADH dehydrogenase (complex I); II, succinate reductase (complex II); III, ubiquinol-cytochrome C reductase (complex III); IV, cytochrome oxidase (complex IV); V, ATP synthase; ADP/ATP, adenosine bi(tri)phosphate; $FADH_2$, flavin adenine dinucleotide; GPx, glutathione peroxidase; NADH, nicotinamide adenine dinucleotide (reduced form); UCP, uncoupling protein; $^{\cdot}O_2^-$ superoxide radical; $^{\cdot}OH$, hydroxyl radical; H_2O_2, hydrogen peroxide; $^{\cdot}NO$, nitric oxide; $ONOO^-$, peroxynitrite radical.

intracellular glucose homeostasis. However, of direct relevance to diabetes complications, certain cell populations including the retinal capillary endothelial cells, mesangial cells in the renal glomeruli, and neuronal and Schwann cells in peripheral nerves are unable to decrease glucose transport rates adequately to prevent excessive changes in intracellular glucose concentrations (5). Indeed, enhanced glucose uptake has been identified in many of the cell populations within the diabetic kidney, including glomerular epithelial cells (6), mesangial cells, and proximal tubular epithelial cells. Thus, these specific cell populations may be particularly susceptible to the changing milieu of diabetes, since they are unable to prevent intracellular hyperglycemia in the setting of elevations in systemic glucose concentrations. Although intensive glycemic control is the most desirable method to prevent progressive diabetic renal disease (7), another early intervention that may limit cellular ROS generation in the diabetic kidney may be to enhance the ability of these specific susceptible cell populations to decrease glucose uptake in hyperglycemic environments (Fig. 2, *1*). Relevant to this approach, a number of studies have shown therapeutic benefit in experimental diabetic nephropathy with interventions to prevent membrane localization of glucose transporters, in particular GLUT1 (5,8).

MITOCHONDRIAL SOURCES OF ROS

Oxidative phosphorylation. The ultimate fate of most glucose once it enters the cell is as a fuel for the mitochondrial respiratory chain via oxidative phosphorylation. Once inside the cell, glucose is rapidly converted to pyruvate and eventually NADH (reduced form) in addition to reduced $FADH_2$ by the glycolytic pathway. NADH and $FADH_2$ are then transported into the mitochondria via either the malate-aspartate or the glycerol phosphate shuttle systems. NADH is the main electron donor to the mitochondrial respiratory chain, and it is hypothesized that hyperglycemia increases the NADH/NAD^+ ratio in complication-prone cell populations (9). Therefore, therapies that would partially decrease the excess chronic glycolysis present in these cells may be of therapeutic benefit in diabetes complications (10) by decreasing the fuel availability to the mitochondrial electron transport chain, as is explored further within this article (Fig. 2, *2*).

Mitochondria also utilize free fatty acids (FFAs) as fuel for oxidation reactions. β-Oxidation and oxidation of FFAs in the tricarboxylic acid cycle generate the same electron donors for oxidative phosphorylation (NADH and $FADH_2$); therefore, excess FFAs can replicate hyperglycemia-induced mitochondrial defects. It is likely that in the context of established renal disease, control of dietary fat

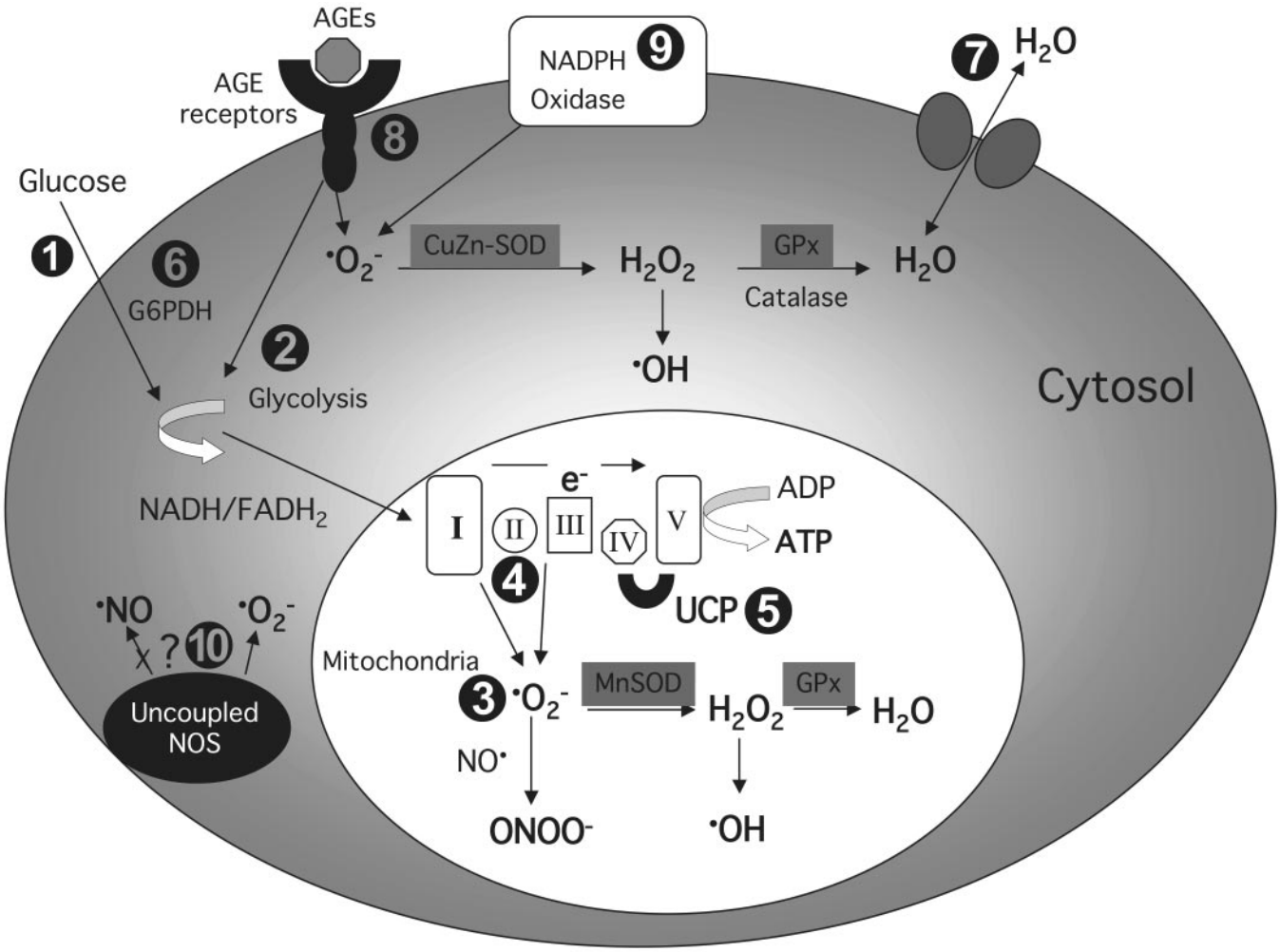

FIG. 2. Potential cellular sites for specific therapeutic intervention to decrease ROS formation.

intake, and circulating LDL cholesterol and triglycerides with HMG-CoA reductase inhibitors should further protect the mitochondria from FFA-induced oxidative damage.

The generation of ROS, specifically $^{\cdot}O_2^-$, by damaged or dysfunctional mitochondria, has been postulated as the primary initiating event in the development of diabetes complications (11). Therefore, decreasing mitochondrial ROS generation has increasingly been considered a relevant aim in ameliorating the burden of diabetic renal disease (Fig. 2, *3*). During oxidative phosphorylation, in which over 90% of oxygen in humans is metabolized, electrons from glucose and other fuels are transferred to molecular oxygen, involving complex reactions utilizing complexes I–IV and finally ATP synthase. Protons are pumped across the mitochondrial membrane creating a voltage gradient, which is collapsed to generate ATP. This series of reactions is tightly regulated. Nevertheless, it is estimated that up to 1% of oxygen is only partially reduced to $^{\cdot}O_2^-$, instead of fully to water, under physiological conditions (Fig. 1). The two major sites of electron leakage are at NADH dehydrogenase (complex I) and at the interface between Coenzyme Q and complex III (12). Therefore, in diabetes, where there is an excess of fuels supplied as a result of chronic hyperglycemia feeding into the respiratory chain, it has been hypothesized, based primarily on in vitro studies (11), that excess production of $^{\cdot}O_2^-$ is via the premature collapse of the mitochondrial membrane potential, which, rather than driving ATP production, leaks electrons to oxygen to form $^{\cdot}O_2^-$. While these findings are exciting, these predominantly tissue culture studies (13) remain to be fully substantiated in vivo, particularly with respect to nephropathy.

Specifically, dysfunction of the mitochondrial respiratory chain (Fig. 2, *4*) has been postulated to contribute to various disease pathologies, and patients with genetic defects that decrease the activity of complex I have vastly elevated rates of mitochondrial $^{\cdot}O_2^-$ production (14). Additional evidence for mitochondrial oxidative phosphorylation as a candidate in the pathogenesis of diabetes complications comes from the disease Friedreich's ataxia, a genetic disorder due to frataxin mutations causing excessive mitochondrial ROS generation in association with downregulation of mitochondrial complex I (15). Indeed, in addition to the well-characterized cardiac dysfunction seen in this disorder, some individuals with Friedreich's ataxia develop renal disease. A role for mitochondria in the development of diabetic kidney disease is further strengthened by the recent observation that up to 50% of children with mitochondrial diseases have renal impairment (16). Furthermore, some of these subjects with mitochondrial respiratory chain defects have demonstrated renal disease as their primary pathology, including a newly described mitochondriopathy involving a deficiency in coenzyme Q10, which also has primary renal

involvement (17). This suggests that investigation into specific mitochondrial defects and their contribution to diabetic kidney disease are warranted and should be highlighted as a research priority.

Intramitochondrial $^{\bullet}O_2^-$ production initiates a range of damaging reactions through the production of H_2O_2, ferrous iron, $^{\bullet}OH$, and $ONOO^-$, which can then damage lipids, proteins, and nucleic acids. A number of functional enzymes within the mitochondria are particularly susceptible to ROS-mediated damage, leading to altered ATP synthesis, cellular calcium dysregulation, and induction of mitochondrial permeability transition, all of which predispose the cell to necrosis or apoptosis.

Idebenone is a new generation mitochondrial antioxidant that has a high uptake into organs such as the kidney, where one-third of its intracellular content is localized within the mitochondria. Studies in humans with Friedreich's ataxia suggest that this antioxidant is a safe and highly efficient way to protect mitochondrial function from oxidative damage (18). Interestingly, unlike traditional antioxidants such as α-tocopherol, idebenone has been shown to reduce cardiomyopathy in these subjects (18). It remains to be determined whether such an agent may have renoprotective effects in a setting such as diabetes. Mito Q is another new generation antioxidant with selective uptake into mitochondria due to its covalent attachment of its antioxidant moiety to the lipophilic triphenylphosphonium cation, which is being tested in patients with Alzheimer's disease (http://www.antipodeanpharma.com). This molecule accumulates 5- to 10-fold in mitochondria, but changes in the membrane potential can increase its uptake by between 100- to 500-fold (19). The efficacy of these relatively selective mitochondrial antioxidants in diabetic nephropathy remains to be determined; however, their targeted specificity for mitochondria suggests that intensive preclinical and subsequent clinical investigation is warranted for these agents.

Uncoupling of the respiratory chain—dissipating energy as heat. In nature, the collapse of the mitochondrial membrane potential can occur via uncoupling of the respiratory chain where electrons are utilized for heat rather than for ATP synthesis. Indeed, chronic uncoupling decreases ATP synthesis and increases the leakage of electrons to oxygen to form $^{\bullet}O_2^-$. There are three major isoforms of uncoupling proteins, UCP-1 to -3, that bind to the respiratory chain at the location of ATP synthase. Studies in diabetic neural tissues and retinal endothelial cells have suggested that chronic overexpression of uncoupling proteins is responsible for the "back up" of electrons in the respiratory chain and their leakage to $^{\bullet}O_2^-$, although this phenomenon is unsubstantiated in vivo in renal tissues (20). Therefore, therapeutic agents that decrease the levels of these proteins, thereby lowering mitochondrial superoxide generation, may lead to a novel treatment strategy for renal disease (Fig. 2, *5*). Interestingly, this has been used successfully in other experimental models of disease including β-cell death (21). Paradoxically, however, low levels of artificial uncouplers may be useful in disorders such as obesity. Thus, the challenge is to create an agent that sufficiently attenuates mitochondrial ROS production without significantly compromising ATP generation.

CYTOSOLIC SOURCES OF ROS

Glycolysis. Once transported inside the cell, glucose is converted via glycolysis to glucose-6-phosphate, which is then sequentially processed to pyruvate. Cellular glycolysis can promote the production of excess ROS. On one hand, in diabetes complications it is intuitive that restricting cellular glucose uptake in order to maintain intracellular glucose homeostasis is critical in susceptible cell populations to minimize cellular damage and ROS generation. On the other hand, there is evidence suggesting that restriction of cellular glucose uptake causes production of small quantities of cellular ROS, which ultimately improve cell survival (22). This finding is further supported by data in C. elegans, which demonstrate that antioxidant therapies impair cellular survival by restoring glucose uptake (22). Theoretically, it is thought that exposure to low-grade stress and associated elevations in ROS primes cells against pathological injury during extreme changes in cellular glycolysis. Indeed, in support of this, either caloric restriction (23) or intermittent feeding patterns are renoprotective in rodent models of diabetic nephropathy (24). Since a major issue in human health appears to be long-term compliance to such a dietary-oriented regimen, caloric restriction mimetics are currently being tested in ageing (25), which itself is associated with declining renal function. However, the ultimate effects of these mimetics currently remain unknown. Nevertheless, disruption of glycolysis (Fig. 2, *2*), perhaps most interestingly by both enhancement or suppression, can ultimately facilitate the excessive generation of ROS by a number of pathways as outlined below.

Glucose-6-phosphate dehydrogenase. The rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PDH) is involved in the pentose phosphate pathway. The pentose phosphate pathway is ultimately responsible for ribose synthesis, which is the main source of NAD(P)H, glutathione reductase, and aldose reductase. A number of studies have identified that altered activity of G6PDH results in cellular oxidative stress (26). Indeed, deficiencies in the activity of G6PDH are common human enzymopathies, resulting in increased ROS generation and decreases in antioxidants such as glutathione (27). Interestingly, the activity of G6PDH is increased in kidneys from rodents with experimental diabetic nephropathy. Clearly, further investigation of the potential of this pathway as a source of ROS in diabetic nephropathy is warranted (Fig. 2, *6*).

Flux through the sorbitol pathway. Increased flux through the sorbitol/polyol pathway was documented more than 40 years ago in the hyperglycemic setting. The cytosolic enzyme aldose reductase converts high intracellular glucose concentrations to sorbitol using NAD(P)H derived from the pentose phosphate pathway as a cofactor. It is likely that during hyperglycemia, consumption of NAD(P)H by this reaction inhibits replenishment of reduced glutathione, which is required to maintain glutathione peroxidase activity. This would ultimately decrease cellular antioxidant activity. Subsequently, sorbitol is oxidized to fructose via sorbitol dehydrogenase, with NAD^+ reduced to NADH, providing increased substrate to complex I of the mitochondrial respiratory chain. Since the mitochondrial respiratory chain is thought to be a major source of excess ROS in diabetes, provision of additional electrons for transfer to oxygen-forming superoxide would augment mitochondrial ROS production. In addition, since sorbitol does not cross cell membranes, its intracellular

accumulation results in osmotic stress. Osmotic stress per se increases cellular cytosolic generation of H_2O_2. Indeed, administration of osmotic diuretics protects proximal tubular cells from ROS-mediated apoptosis (28) (Fig. 2, *7*).

Although inhibition of sorbitol accumulation with aldose reductase blockade has been shown to delay, prevent, and, at early stages, to reverse experimental diabetic neuropathy, trials have in general been disappointing despite decades of clinical investigation. Currently, the clinical utility of aldose reductase inhibitors in diabetic renal disease remains to be determined.

Advanced glycation. Nonenzymatic glycation of free amino groups on proteins and amino acids begins with covalent attachment of sugar moieties at a rate determined by a number of factors including intracellular glucose concentrations, pH, and time in a biochemical reaction termed the "Maillard reaction." Physiologically, this is thought to be an evolutionary pathway for labeling of senescent cellular proteins for their recognition and ultimate turnover. In both major forms of diabetes, persistent hyperglycemia and oxidative stress act to hasten the formation of advanced glycation end products (AGEs) (29). This causes long-lived proteins to become more heavily modified, in addition to rendering shorter-lived molecules as targets for advanced glycation. Furthermore, elevated intracellular glucose degradation products such as glyoxal resulting from glycolysis and the tricarboxylic acid cycle initiate the glycation of intracellular proteins far more rapidly than glucose itself. These AGEs can be generated from intracellular auto-oxidation of glucose to glyoxal, decomposition of early glycation (Amadori) products to 3-deoxyglucosone, and fragmentation of metabolites of the pentose phosphate pathway such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to the reactive carbonyl methylglyoxal (30). Excess ROS are generated during the formation of AGEs, causing a self-perpetuating cycle of ROS/AGE formation in diseases such as diabetes. The proposed sources of ROS in the Maillard reaction are many, including the autoxidation of glucose (Wolff pathway), Schiff bases (Namiki pathway), and Amadori adducts (Hodge pathway), as well as AGE proteins themselves (29).

Since the ultimate fate of most AGEs within the body is renal clearance, they can also interact with a number of renal cellular binding sites that mediate many of their biological effects. Arguably, the most important of these binding sites is the receptor for AGEs (RAGE), a member of the immunoglobulin superfamily (31). RAGE is a multiligand pattern recognition receptor involved in the amplification of immune and inflammatory responses primarily via activation of nuclear factor-κB and production of interleukin-1β and tumor necrosis factor-α (32). Previously, cytosolic generation of ROS has been demonstrated in vitro through activation of the RAGE receptor in both proximal tubular and mesangial cells, most likely through NAD(P)H oxidase (33). This further supports the interaction of AGEs with full-length cellular RAGE and subsequent cytosolic ROS generation as a major player in the development of nephropathy. This contribution of AGE-RAGE interactions to ROS generation in the pathogenesis of diabetic nephropathy has also been suggested in complementary in vivo studies (34).

There are a number of antioxidant systems in place to limit tissue damage initiated by the Maillard reaction including detoxification systems such as the glyoxalase pathway, aldose reductases, aldehyde dehydrogenases, and the chelation of metal ions (35,36). However, the ultimate development of tissue injury depends on the balance between the rate of formation of AGE-modified proteins and protection by these various systems in addition to renal clearance. Of particular interest, AGE modifications occur on antioxidant enzymes such as CuZnSOD, complex I, and MnSOD in diabetic nephropathy (37), and this would alter the activity of these enzymes, ultimately further contributing to excess cellular ROS accumulation.

In models of experimental diabetic nephropathy, there are clear benefits associated with a variety of AGE inhibitors that act in disparate ways in the context of improvements in cellular ROS generation (Fig. 2, *8*). In experimental diabetic nephropathy, alagebrium chloride decreases renal mitochondrial ROS generation, which is not seen with RAS blockade (38). Indeed, the utility of alagebrium chloride is currently being investigated in type 1 diabetic patients with microalbuminuria treated with concomitant ACE inhibition (PHASE IIb, http://www.alteon.com). In addition, benfotiamine has also shown efficacy in the treatment of patients with painful diabetic neuropathy, but to date it has not been studied in clinical diabetic nephropathy (39). Although blockade of RAGE signal transduction (33,40) is also a useful strategy to improve diabetic renal disease, concomitant amelioration of renal ROS generation with this approach has not been documented to date. Furthermore, the clinical utility of such agents targeting AGEs and/or RAGE in preventing or retarding diabetic nephropathy is yet to be determined but is an area of active preclinical and clinical investigation.

NAD(P)H oxidase. NAD(P)H oxidase is a cytosolic enzyme complex initially discovered in neutrophils, where it plays a vital role in nonspecific host-pathogen defense by producing millimolar quantities of $^{\bullet}O_2^-$ by electron transport. The enzyme complex is composed of five subunits comprising a membrane-associated $p22^{phox}$ and a $gp91^{phox}$ subunit and at least four cytosolic subunits: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and GTPase *rac*1 or *rac*2. In addition, $gp91^{phox}$ has other renal homologues, namely, nox-3 and nox-4, which have been identified in fetal kidney and renal cortical tissues, respectively (41). In addition to residing in phagocytic cells, NAD(P)H oxidase is present in nonphagocytic renal cell types such as mesangial and proximal tubular cells, vascular smooth muscle cells, endothelial cells, and fibroblasts (42). In these cell types, however, $^{\bullet}O_2^-$ production is proportionally lower than in activated neutrophils. Therefore, the intrinsic function of NAD(P)H oxidase in nonphagocytic cells is clearly different from that seen in phagocytic and other white cell populations. ROS are generated in these nonphagocytic cells, in this context, in the intracellular compartment, most likely in order to act as second messengers. Indeed, binding of several cytokines and hormones such as tumor necrosis factor-α, platelet derived growth factor, and angiotensin II to their cognate receptors rapidly activates NAD(P)H oxidase followed by intracellular $^{\bullet}O_2^-$ and H_2O_2 generation. This is evident from studies using pharmacological inhibition of NAD(P)H oxidase, mice with deletions of the various NAD(P)H oxidase subunits, or treatment with anti-sense oligonucleotides.

In addition to providing second messengers for nonpathogenic redox signaling pathways, nonphagocytic NAD(P)H oxidase can also generate excessive ROS production, contributing to cellular oxidative stress. This has been shown in renal pathological states such as diabetic nephropathy (43), hypertension, inflammation, and isch-

emia-reperfusion injury. Within the kidney, various subunits of NAD(P)H oxidase are increased in experimental diabetic nephropathy (44). Furthermore, pharmacological inhibition of NAD(P)H oxidase with apocynin prevents upregulation of $p47^{phox}$ and $gp91^{phox}$ overexpression and retards the mesangial matrix expansion seen in experimental diabetic nephropathy (43,45). In addition, a more specific therapeutic approach using anti-sense oligonucleotides to Nox-4, the renal $gp91^{phox}$ homologue, inhibited NAD(P)H-dependent ROS generation in renal cortical and glomerular homogenates, resulting in attenuation of renal hypertrophy (46). These data highlight the importance of NAD(P)H oxidase as a potential pathogenic mediator of hyperglycemia-induced ROS production (Fig. 2, *9*).

Xanthine oxidase. Xanthine oxidase is the enzyme that catalyzes the oxidation of hypoxanthine to uric acid using molecular oxygen as the electron acceptor, liberating a number of ROS including $^{\bullet}O_2^-$, $^{\bullet}OH$, and H_2O_2. Under normal physiological conditions, levels of xanthine oxidase activity are unmeasurable in most cell types, although sensitive electron spin technologies have confirmed xanthine oxidase as an important source of vascular superoxide generation in experimental models of type 1 diabetes (47). Despite this, there is no direct evidence of abnormalities in this pathway within renal tissues in experimental or human diabetes, and thus the contribution of this enzyme to the pathogenesis of diabetic nephropathy remains to be determined.

Uncoupling of NOS. There are three major isoforms of NOS, inducible (iNOS), neuronal (nNOS), and endothelial (eNOS). Each of these isoforms requires five cofactors/prosthetics such as flavinmononucleotide (FMN), bihydrobiopterin (BH_4), calmodulin, and flavin adenine dinucleotide (FAD) to produce $^{\bullet}NO$. In diabetes, uncoupling of NOS due to restricted substrate (L-arginine) availability or the absence of cofactors, is thought to generate $^{\bullet}O_2^-$ in preference to $^{\bullet}NO$. Indeed, one study in experimental diabetic nephropathy has suggested that uncoupling of NOS and NADPH oxidase provides two major sources of glomerular superoxide (48). In that study, restoration of physiological levels of BH_4 attenuated ROS production and improved renal function.

However, the status of $^{\bullet}NO$ and its role in diabetic nephropathy remains controversial. Based on current findings, it is reasonable to suggest that early nephropathy in diabetes is associated with increased intrarenal $^{\bullet}NO$ production (49) mediated primarily by constitutively released $^{\bullet}NO$ (eNOS and nNOS) (48). Indeed, enhanced $^{\bullet}NO$ production may contribute to the hyperfiltration and other hemodynamic changes that characterize early diabetic nephropathy. This is supported by studies in early diabetic nephropathy where L-NAME reversed hemodynamic changes and renal damage (50).

On the other hand, the majority of the studies in advanced diabetic renal disease indicate that severe proteinuria, declining renal function, and hypertension are associated with a state of progressive $^{\bullet}NO$ deficiency (51). Advanced renal changes attributed to $^{\bullet}NO$ are thought to be mediated through multiple mechanisms such as glucose and AGE quenching and inhibition and/or posttranslational modification of NOS, which changes the activity of both endothelial and inducible isoforms. Indeed, several authors have reported no effect (52) or aggravation of renal damage by chronic NO inhibition in models of type 1 and type 2 diabetic nephropathy, respectively (53).

Therefore, owing to the complex temporal changes in $^{\bullet}NO$ production during the evolution of diabetic nephropathy, there is ongoing controversy as to the clinical applicability of approaches that inhibit NOS activity.

ANTIOXIDANTS

In response to excess ROS production during respiration and metabolism, mammals have evolved numerous antioxidant systems including free radical scavengers and enzymes (Fig. 1). The first and perhaps most important of these antioxidant enzymes is superoxide dismutase (SOD), which exists in three major cellular forms: copper zinc (CuZnSOD, SOD1), manganese (MnSOD, SOD2), and extracellular (SOD3). These enzymes are responsible for the detoxification of superoxide radicals to hydrogen peroxide and water in different cellular compartments. Glutathione peroxidase (GPx) and catalase are other antioxidant enzymes that catalyze the conversion of hydrogen peroxide to water. Although it is appreciated that there are numerous other antioxidants present within cells, such as glutathione and numerous vitamins, these are not discussed here in the context of diabetic nephropathy. Furthermore, as highlighted above, a number of these antioxidants have proven to play a minimal if any role in the treatment of diabetic nephropathy in humans.

Decreases in expression, and in some instances the activity of each of these antioxidant enzymes, has been previously reported in diabetic microvascular disease (54). Indeed, the overexpression of CuZnSOD protects against end organ damage in models of type 2 diabetic nephropathy (55). Other studies in mice with genetic deletions of various antioxidant enzymes have also provided insight into the specific relative contributions of MnSOD (56) to the development of diabetes complications. MnSOD mimetics such as MnTBAP have also shown efficacy in preventing ROS-induced injury in vitro (11), although the utility in vivo of such drugs may be limited (57). Further strengthening a potential role for the antioxidant MnSOD, specific polymorphisms of the MnSOD gene are associated with the development of diabetic nephropathy (58).

Interestingly, GPx-1–deficient mice have no increased risk for microvascular disease, in particular diabetic nephropathy (59), most likely because of redundancy with respect to other renal GPx isoforms, in particular the GPx-3 isoform.

Overexpression of catalase in experimental models of type 2 diabetic nephropathy appears to be protective (60). In contrast to MnSOD, however, studies in humans have indicated no relationship between catalase gene polymorphisms that interfere with its cellular expression and the incidence of nephropathy in type 2 diabetic patients (Fig. 2) (61).

LINKING ROS GENERATION TO THE PROMINENT PATHOGENIC PATHWAYS IN DIABETIC NEPHROPATHY

It is increasingly evident that changes in cellular function resulting in oxidative stress play a key role in the development and progression of diabetic nephropathy (Fig. 3). Major early points for therapeutic intervention to reduce ROS generation would include first decreasing the cellular uptake of glucose and second retarding the feeding of glucose derived metabolites into cellular respiration. It is, however, increasingly considered that maintenance of oxidative phosphorylation and normalization of mitochondrial function are key strategies to reduce the progression of diabetic nephropathy. Further to this, a "unifying hy-

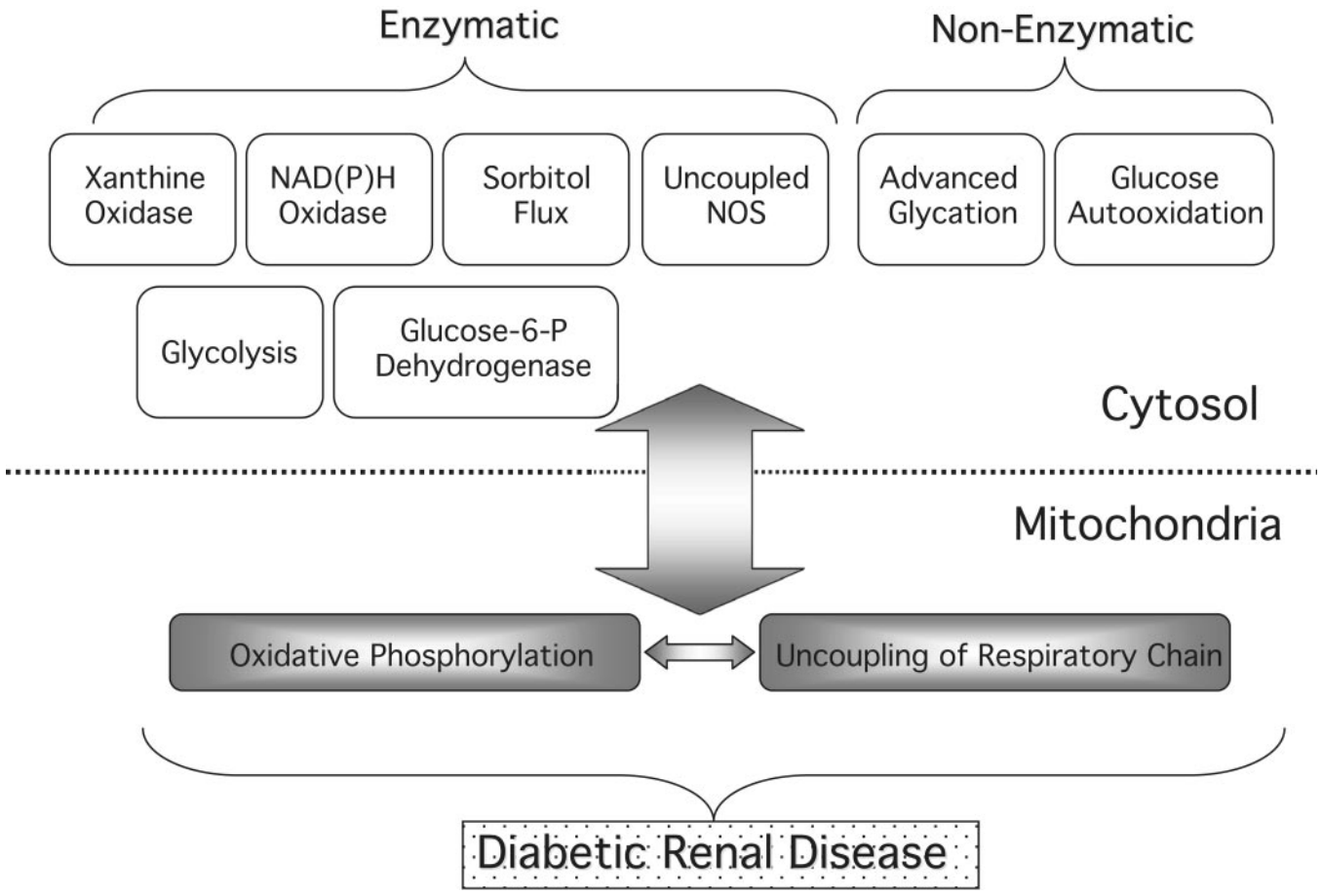

FIG. 3. Cytosolic and mitochondrial sources of ROS implicated in the pathogenesis of diabetic nephropathy.

pothesis" (11) suggests that the initiator of hyperglycemia-induced damage in the diabetic kidney is excess generation of mitochondrial $^{\bullet}O_2^-$, which then leads to activation of four major biochemical pathways, including increased AGE formation, activation of protein kinase C isoforms, and increased flux through the polyol and hexosamine pathways (11). In addition, each of these pathways can contribute to perpetuation and in some cases initiate cellular ROS generation. Inhibition of other cellular pathways including NADPH oxidase or reversing the uncoupling of eNOS may also warrant further investigation to assess their relative importance in progressive renal disease, in particular, their role in human disease.

Current treatments and ROS production. As mentioned earlier, current strategies to treat, prevent, or reverse diabetic nephropathy rely on widespread use of agents that interrupt the RAS. Indeed, angiotensin II itself can produce ROS primarily via NADPH oxidase (42), and it is likely that strategies that interrupt the RAS significantly decrease ROS generation. However, one cannot exclude that RAS blockade may not fully suppress ROS generation, particularly from other sources such as mitochondria (38), and indeed this could explain the persistent progression, albeit at a slower rate, seen in subjects with diabetic nephropathy concomitantly treated with agents that interrupt the RAS. Therefore, it is worth considering as an important strategy identification of new therapeutic targets that could lead to new treatments that confer synergistic effects with those seen with RAS blockade.

Nevertheless, although a single cellular source of ROS as the initiator of diabetic nephropathy is an attractive prospect and potentially simplifies therapeutic targets, it is unlikely that this fully explains what occurs in the kidney, which has marked heterogeneity in cell populations. Therefore in vitro studies in individual renal cell types are essential; however, one must be cautious in their interpretation. Indeed, these must be performed in association with appropriate in vivo models of diabetic nephropathy, although these also have limitations. It is clear from the data presented within this article that more than one source of ROS in diabetic nephropathy may be pathogenic. Ultimately, the goal of designing new generation antioxidant therapies is to identify agents that are potentially effective and penetrative of certain cell compartments. Thus, these would provide superior renoprotection upon their combination with RAS blockade and then following extensive testing may be translated into clinical research and practice.

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