Reactive Oxygen Species and Diabetic Nephropathy

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Abstract

Diabetic nephropathy remains a major microvascular complication of diabetes and the most common cause of end-stage renal disease requiring dialysis in the USA. Medical advances over the past century have substantially improved the management of diabetes mellitus and thereby increased patient survival. However, current standards of care reduce but do not eliminate the risk of diabetic nephropathy, and future studies are required to further understand the molecular mechanisms involved in the pathogenesis of diabetic nephropathy. There is an increasing body of evidence indicating that reactive oxygen species (ROS) may play a major role in the development of diabetic nephropathy. Oxidative stress is increased in diabetes, and the overproduction of ROS correlates with complications of diabetes, including diabetic nephropathy. Both NADPH oxidase and mitochondrial electron gradients seem to play critical roles in hyperglycemia-induced ROS generation. However, the key pathways by which hyperglycemia leads to enhanced ROS and structural changes associated with

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diabetic nephropathy are not well established. It is known that in addition to their ability to directly inflict macromolecular damage, ROS can function as signaling molecules resulting in transcriptional activation of profibrotic genes in the kidney. Here, we highlight the role of ROS in the development of the diabetic kidney disease. In particular, we will discuss recent advances in our understanding of the molecular mechanisms by which mitochondrial ROS might be implicated in the pathogenesis and progression of diabetic nephropathy.

Keywords
Diabetic nephropathy • Mitochondira • Mitochondrial dynamics • ROCK1 • ROS

Introduction

Diabetes is a worldwide pandemic affecting approximately 160 million individuals as of the year 2000 and is expected to rise to 366 million individuals by 2030, an estimated 50 % increase over 30 years in the number of individuals with diabetes (Wild et al. 2004). Diabetic nephropathy is a major microvascular complication of diabetes, affecting between 20 % and 40 % of diabetic patients (Hasslacher et al. 1989). Of note, in 1975, 3 years after the initiation of the end-stage renal disease program, patients with diabetes mellitus comprised only ~ 5 % of dialysis patients. However, in the intervening time, there has been an explosion in the incidence and prevalence of type 2 diabetes that has resulted in diabetes mellitus becoming the leading cause of end-stage renal disease (ESRD) in industrialized countries, including the USA (http://www.cdc.gov/diabetes /pubs /factsheet11.htm).

The mechanisms underlying the development and progression of diabetic nephropathy remain poorly understood; however, it is known that the level of hyperglycemia correlates with progression of diabetic nephropathy and retinopathy (Remuzzi and Ruggenenti 1993; Lewis et al. 1993), and improving glycemic control decreases the rate of progression of diabetic nephropathy and loss of kidney function (Lewis et al. 1993; 2003). The differential effect of chronic hyperglycemia on different tissues reflects the failure of cells to downregulate the uptake of glucose when extracellular glucose concentrations are elevated. Consistent with this notion, hyperglycemic damage is pronounced in cells and tissues which show no significant change in glucose transport rate, resulting in intracellular hyperglycemia and cell damage.

Although multiple recent published reviews have provided an excellent summary of the most popular pathways underlying hyperglycemia-induced diabetic cellular and kidney damage, the mechanisms leading to the development of diabetic nephropathy remain largely unknown. In general, it is believed that prolonged hyperglycemia leads to chronic metabolic and hemodynamic changes that modulate various intracellular signaling pathways, transcription factors, cytokines, chemokines, and growth factors (Soldatos and Cooper 2008; Remuzzi et al. 2002). These effects promote structural abnormalities in the kidney such as glomerular basement membrane thickening, podocyte injury, and mesangial matrix

expansion with the later development of irreversible glomerular sclerosis and tubulointerstitial fibrosis associated with declining GFR.

Experimental evidence suggests that the critical molecular pathways that may be involved in the development of diabetic nephropathy include increased oxidant stress, enhanced flux into the polyol and hexosamine pathways, activation of PKC and transforming growth factor (TGF)-β-SMAD-MAPK signaling pathways, and increased formation of advanced glycation end products (AGEs). In addition, high glucose can activate the proinflammatory transcription factor NF-κB, resulting in increased inflammatory gene expression in part through oxidant stress, AGEs, PKC, and MAPKs (Schmid et al. 2006; Lee et al. 2004). Finally, hemodynamic changes, in part through the probable activation of the renin-angiotensin system (RAS) and VEGF signaling axis, also play critical roles in the pathobiology of diabetic nephropathy (Anderson and Brenner 1988; Hostetter et al. 1982; Khamaisi et al. 2003; Cooper et al. 1999). Although all of these factors have been implicated in the pathogenesis of diabetic nephropathy, here we focus on some of the new concepts highlighting the role of ROS in the pathogenesis and progression of diabetic kidney disease. This chapter will discuss a summary of the latest published data on the molecular mechanisms associated with the role of ROS in pathological changes in the kidney during the development of diabetic nephropathy.

Generation of Oxidative Stress in Diabetic Nephropathy

ROS include a number of molecular species derived from oxygen that arise principally from superoxide ($O_2^{\bullet -}$) (Sena and Chandel 2012). ROS can be produced enzymatically or nonenzymatically. Among several enzymes which have been implicated in the generation of ROS, cytoplasmic NADPH oxidases located mainly on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells (Vignais 2002), and cytochrome P_{450}-dependent oxygenases are well-characterized sources of enzymatic ROS (Coon et al. 1992). In contrast, the nonenzymatic production of superoxide mainly involves mitochondrial electron transport chain, which contains several redox centers that may leak electrons to oxygen, constituting the primary source of superoxide in most tissues.

It is now clear that various types of cells including endothelial, vascular smooth muscle, mesangial, and tubular epithelial cells are capable of producing ROS under hyperglycemic condition (Remuzzi et al. 2002), and there is increasing evidence that the overproduction of ROS is one major factor in the development of diabetic complications, including diabetic nephropathy. But how enhanced ROS lead to structural changes associated with diabetic kidney disease is less understood. Physiological levels of ROS are important in diverse biological activities of the cell, and small fluctuations in the steady-state concentration of ROS play a role in signal transduction cascades. However, uncontrolled increases in the steady-state concentrations of these oxidants are regarded as toxic by-products of metabolism that cause damage to cellular components, including proteins, lipids, carbohydrates, and DNA. Furthermore, in addition to their ability to directly inflict macromolecular damage,

ROS can activate a number of cellular stress-sensitive pathways that cause cellular damage (Schmid et al. 2006). For instance, ROS mediate hyperglycemia-induced activation of signal transduction cascades and transcription factors leading to transcriptional activation of profibrotic genes (Lee et al. 2004). Protein kinase C (PKC), transforming growth factor-β1 (TGF-β1), and angiotensin II (Ang II) stimulated by hyperglycemia-induced ROS, in turn, generate and signal through ROS and thus ROS act as a signal amplifier in diabetes (Lee et al. 2004).

The glucose auto-oxidation, polyol pathway, AGE, mitochondrial electron transport chain (ETC), uncoupled eNOS, and NAD(P)H oxidases have been long considered as main sources of ROS generation in diabetes. Among all these potential sources of ROS generation in the diabetic kidney, we will, however, mainly focus on the roles of mitochondria and activated NADPH oxidase.

Mitochondrial ROS

Mitochondria are the main source of ROS within most mammalian cells, and it is generally believed that the majority of ROS in the mitochondria are by-products of mitochondrial respiration. The mitochondrial electron transport chain (ETC) contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of endogenous superoxide production (Andreyev et al. 2005; Turrens 2003a; Balaban et al. 2005). Indeed, two of the respiratory chain complexes (I and III) have been long recognized as important sources of superoxide production.

The mitochondria generate energy by oxidizing hydrogen derived from our dietary carbohydrates (TCA cycle) and fats (β-oxidation) with oxygen to generate heat and ATP. The production of ATP occurs in the mitochondrial inner membrane, in the ETC. Electron flow is carried out by four membrane-associated enzyme complexes (complexes I to IV), plus cytochrome *c* and the mobile carrier ubiquinone (QH2). In the mitochondrial matrix, two electrons donated from NADH to complex I (NADH dehydrogenase) or from succinate to complex II (succinate dehydrogenase, SDH) are passed sequentially to ubiquinone (coenzyme Q or CoQ) to give ubisemiquinone ($CoQH^{\bullet}$) and then ubiquinol ($CoQH_2$). Ubiquinol transfers its electrons to complex III (ubiquinol:cytochrome *c* oxidoreductase), which transfers them to cytochrome *c*. From cytochrome *c*, the electrons flow to complex IV (cytochrome c oxidase or COX) and finally to $^1/_2\ O_2$ to give H_2O. Each of these ETC complexes incorporates multiple electron carriers. Complexes I, II, and III encompass several iron-sulfur (Fe-S) centers, whereas complexes III and IV encompass the $b + c_1$ and $a + a_3$ cytochromes, respectively.

The energy released by the flow of electrons through the ETC is used to pump protons out of the mitochondrial inner membrane through complexes I, III, and IV. This creates an electrochemical gradient (−0.32 V to + 0.39 V) across the mitochondrial inner membrane, which is used for ATP synthesis by complex V (ATP synthase). As protons flow back into the matrix through a proton channel in complex V, ADP and P_i are bound, condensed, and released as ATP. Thus, the mitochondria generate most of the endogenous ROS as a by-product of OXPHOS.

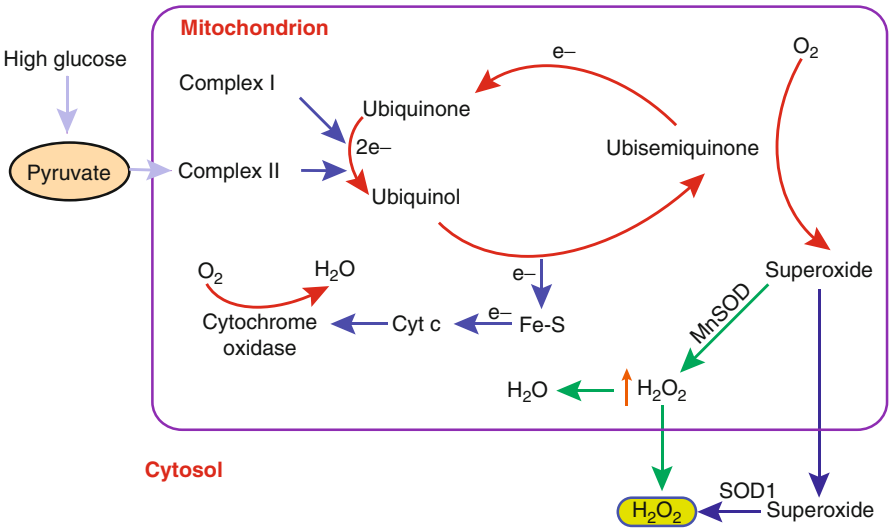

Fig. 117.1 Mitochondrial ETC and ROS production. The mitochondrial matrix contains the components of the TCA cycle and the β-oxidative pathway, which provide reduced NADH and $FADH_2$ to the ETC, leading to generation of a proton gradient across the inner mitochondrial membrane

ROS production is increased when excess electrons are provided to ETC. The excess electrons are transferred to oxygen, which is converted to superoxide and subsequently to hydrogen peroxide. The highest rate of ROS production occurs when the proton gradient is high and oxygen consumption (ATP demand) is low. Superoxide $O_2^{\bullet -}$ is converted to H_2O_2 by mitochondrial matrix enzyme Mn superoxide dismutase (MnSOD, *Sod2*) or by the Cu/ZnSOD (*Sod1*), which is located in both the mitochondrial intermembrane space and the cytosol.

Complex I and III of the ETC are important sources of ROS due to the formation of semistable radicals during electron transfer ($FMN^{\bullet}$ in complex I and $QH^{\bullet}$ in complex III) from which an electron may be transferred to molecular O_2, generating $O_2^{\bullet -}$. Approximately 0.1–0.2 % of the total mitochondrial oxygen consumption is due to $O_2^{\bullet -}$ production under normal physiological conditions (St-Pierre et al. 2002). Although it was initially assumed that the production of superoxide under normal conditions did not have any beneficial function, more recent studies have implicated ROS as an important cellular signaling molecule. For example, mitochondrial superoxide is a key component of angiogenesis and hypoxia-inducible factor (HIF) signaling cascades (Connor et al. 2005; Guzy et al. 2005).

Some of the potential mechanisms related to diabetes-induced mitochondrial ROS production are depicted in Fig. 117.1. Intracellular glucose oxidation begins with glycolysis in the cytoplasm, which generates NADH and pyruvate. Pyruvate can be transported into the mitochondria, where it is oxidized by the TCA cycle to produce four molecules of NADH and one molecule of $FADH_2$. In the diabetic milieu, there is an increased flow of the key substrates NADH and $FADH_2$ to the

respiratory chain, which overdrives the electron transport system in the mitochondria, resulting in increased superoxide anion production (Haidara et al. 2009; Palm et al. 2003; Nishikawa et al. 2000b).

Diabetes is associated with alterations in mitochondrial metabolism that result in both increased formation of ROS and failure of bioenergetics (Nishikawa et al. 2000a, b; Turrens 2003b). Mitochondrial dysfunction is a hallmark of diabetic nephropathy, and a central role for mitochondrial ROS in microvascular complications of diabetes has been proposed by several groups with multiple studies suggesting perturbations in mitochondria in both insulin-deficient and insulin-resistant states and in the related condition of obesity (Haidara et al. 2009; Palm et al. 2003; Brownlee 2001, 2003; Green et al. 2004; DeRubertis et al. 2004; Vincent et al. 2002; Kristal et al. 1997; Yamagishi et al. 2001; Giardino et al. 1996). It must be noted that the term "mitochondrial dysfunction" is poorly defined in the literature and evidence exists for a wide range of alterations in mitochondria, including changes in biogenesis, number, morphology, and dynamics, including fusion and fission.

Brownlee was the first to suggest that ROS produced by the mitochondrial ETC are the driving force in the pathogenesis of diabetic nephropathy. He proposed a "unifying mechanism" where several seemingly independent pathways, including protein kinase Cβ, aldose reductase, advanced glycation end products, and the hexosamine biosynthetic pathway, are activated by a single upstream event: mitochondrial overproduction of ROS (Nishikawa et al. 2000b; Brownlee 2001, 2003; Green et al. 2004; Vincent et al. 2002; Ishii et al. 1996; Inoguchi et al. 2003; Koya et al. 1997; Lee et al. 2003; Watts et al. 2002). Consistent with this hypothesis and with the critical role of ROS in microvascular complications of diabetes, normalization of the mitochondrial superoxide levels blocked three major pathways of hyperglycemia-induced injury (Palm et al. 2003; Brownlee 2001). The evidence for this model, however, comes mainly from experiments on cultured endothelial cells, where raising the glucose concentration from 5 to 30 mmol/l increased ROS production, as measured by the rate of oxidation of dichlorodihydrofluorescein (DCFH) to dichlorofluorescein (DCF) (Haidara et al. 2009; Palm et al. 2003; Green et al. 2004). DCF oxidation was blocked by inhibitors of mitochondrial pyruvate uptake and succinate dehydrogenase, but not by rotenone, a complex I inhibitor, suggesting that ROS generation at complex II may be important. One puzzling question in this observation is how the overexpression of mitochondrial manganese superoxide dismutase (MnSOD) prevented high glucose-induced DCFH oxidation (Green et al. 2004). DCFH is primarily sensitive to hydrogen peroxide, nitric oxide, or hydroxyl radicals, and it is not directly oxidized by superoxide. The overexpression of MnSOD should have converted the superoxide generated in the mitochondrial matrix to hydrogen peroxide (Fig. 117.1). Thus, MnSOD overexpression should have enhanced the DCF signal rather than abolishing it. A potential explanation for the effect of MnSOD overexpression is that MnSOD detoxifies superoxide into hydrogen peroxide within the mitochondrial matrix, preventing its escape into the cytosol. The hydrogen peroxide is then converted to water by glutathione peroxidase in the mitochondria. This finding

suggests that the nature of the ROS being measured in these experiments remains uncertain. DCF oxidation was also blocked by inhibitors of mitochondrial pyruvate uptake and of succinate dehydrogenase, but not by rotenone, suggesting that reverse electron transport was not involved.

A recent publication examined whether mitochondria-targeted antioxidant would prevent progression of diabetic nephropathy in the Ins2(+/)(AkitaJ) mouse model (Akita mice) of type 1 diabetes (Chacko et al. 2010). To test this hypothesis, the authors administered a mitochondria-targeted ubiquinone (MitoQ) over a 12-week period and assessed tubular and glomerular function. MitoQ treatment improved tubular and glomerular function in the Ins2(+/)$^{-}$(AkitaJ) mice. However, it did not have a significant effect on plasma creatinine levels, although it decreased urinary albumin levels to the same level as nondiabetic controls. Importantly, interstitial fibrosis and glomerular damage were significantly reduced in the treated animals. These results support the hypothesis that mitochondrial-targeted therapies may be beneficial in the treatment of diabetic nephropathy. Moreover, overexpression of catalytic antioxidants was shown to protect against diabetic injury. Craven et al. (2001a) demonstrated that diabetic mice transgenic for Cu/Zn SOD had significantly lower urinary albumin excretion, glomerular hypertrophy, and glomerular expression of TGF-β1 and collagen IV protein compared to non-transgenic mice. The same group also showed that overexpression of MnSOD suppresses increases in collagen accumulation induced by culture of mesangial cells in high glucose media (Craven et al. 2001b). Similarly, Du et al. showed that overexpression of MnSOD in bovine aortic endothelial cells prevented high glucose-induced activation of PKC, NK-κB, hexosamine, and advanced glycation end product (AGE) pathways (Du et al. 2003). Finally, Brezniceanu et al. demonstrated that renal catalase overexpression in *db*/*db* mice attenuated ROS generation, angiotensinogen, proapoptotic gene expression, and apoptosis in the kidneys of diabetic mice in vivo (Brezniceanu et al. 2007).

In a recent study, Wang et al. have convincingly shown that changes in the mitochondrial dynamics contribute to increased mitochondrial ROS and progression of diabetic nephropathy (Wang et al. 2012). Mitochondria are dynamic organelles, which are able to interchange their morphology between elongated interconnected mitochondrial networks and a fragmented disconnected arrangement. The dynamic nature of mitochondrial networks is due to two opposing processes, mitochondrial fission and fusion, that operate concurrently (Chan 2006). Mitochondrial fission and fusion are crucial for maintaining mitochondrial function and are thought to be important for rapid repair of damaged mitochondria and for intermixing of DNA and proteins between mitochondria (Fig. 117.2).

A growing number of studies have begun to investigate changes in mitochondrial morphology and dynamics as important parameters for many disease-related processes. Importantly, changes in mitochondrial morphology and increased mitochondrial fission have been recently implicated in the progression of Huntington's and Alzheimer's disease (Chen and Chan 2009). Our group has recently investigated the role of mitochondrial dynamics and specifically mitochondrial fission in the context of diabetic nephropathy (Wang et al. 2012). Condensed fragmented

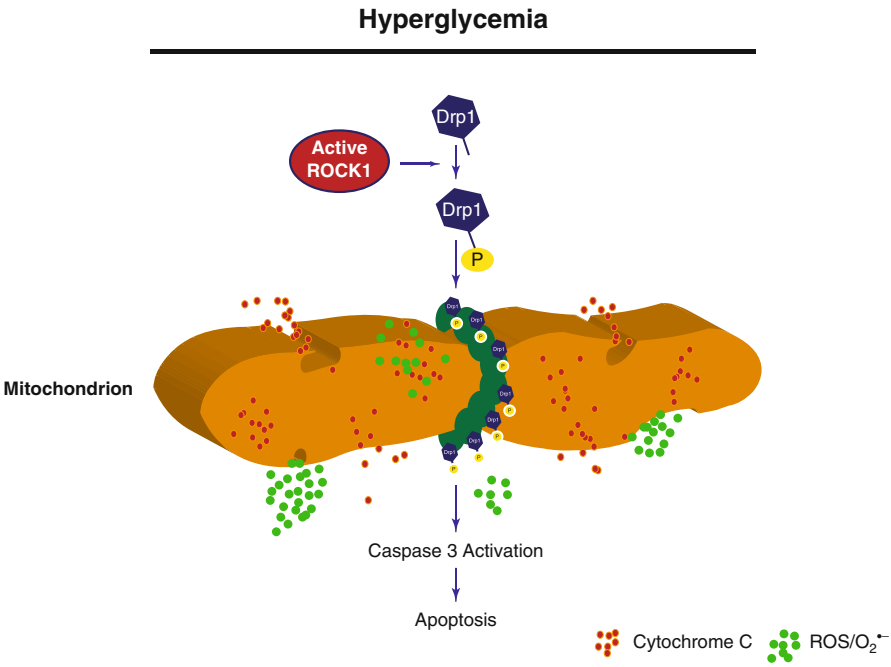

Fig. 117.2 High glucose treatment leads to mitochondrial fission. Mitochondrial fission is driven by Drp1, which resides primarily in the cytoplasm. Hyperglycemic conditions drive the activation of Rho kinase (ROCK1) which regulates a number of downstream substrates, including Drp1. Drp1 is recruited to mitochondria upon phosphorylation by activated ROCK1 at Ser613. Drp1 then forms multimeric spirals around mitochondria at fission sites, which promote the constriction of mitochondria followed by fission. Mitochondrial fragmentation is associated with release of mitochondrial cytochrome *C* which facilitates cleavage of caspase-3 and initiation of apoptosis

mitochondria were observed in the podocytes in kidneys from diabetic mice, which were associated with changes in phosphorylation status of the mitochondrial fission protein Drp1 (dyanmin-related protein-1).

So how does hyperglycemia trigger mitochondrial fission and fragmentation leading to increased ROS and apoptosis in podocytes? Drp1 (dyanmin-related protein-1) is one of the most relevant genes identified to date that directly mediate mitochondrial fission. Drp1 is a soluble dynamin-related GTPase which is localized predominantly in the cytosol and must be recruited to mitochondria for fission to occur (Chan 2006; Chen and Chan 2009; De Vos et al. 2005). Current evidence suggests that Drp1 promotes fission by tethering to mitochondria at specific positions known as constriction sites. Drp1 then forms multimeric spirals around mitochondria further constricting mitochondrial tubules leading to mitochondrial fission (Smirnova et al. 2001).

The study by Wang et al. demonstrated that Drp1 is phosphorylated by high glucose-induced Rho kinase (ROCK1) activation, where this modification

promotes the activity of Drp1, triggering its translocation from the cytosol to mitochondria, thus increasing fission. Whether inhibiting mitochondrial fission and Drp1 phosphorylation in the setting of DN would be beneficial is still unclear. However, consistent with these preclinical data, biopsies of skeletal muscle from subjects with type 2 diabetes reveal mitochondria of smaller size and number compared with control subjects (Kelley et al. 2002). Moreover, mitochondria of offspring of diabetic subjects are lower in density compared with those of controls (Morino et al. 2005).

Further evidence on the role of ROS in the development of diabetic nephropathy comes from studies on the potential role of uncoupling proteins on diabetic complications. Friederich et al. (2008) showed that diabetic rats express increased mitochondrial uncoupling protein-2 (UCP2) in proximal tubular cells associated with increased oxygen use and suggested that the increase in UCP2 was protective against oxidative stress. Manabe et al. (2008) reported that high glucose increased ROS fluorescence in human mesangial cells associated with potentially harmful cytokine expression, an effect that was blocked by astaxanthin, a carotenoid that accumulated in mitochondria. High glucose also reportedly increased H_2O_2 production by dichlorodihydrofluorescein fluorescence in human mesangial cells (Kiritoshi et al. 2003). This was suppressed by reduction in membrane potential by chemical inhibition or by UCP1 overexpression. Coughlan et al. (2007) demonstrated renal mitochondrial oxidative damage in streptozotocin-induced diabetic rats manifest as lucigenin luminescence in kidney slices, an effect that was reduced by alagebrium, a cross-link inhibitor of advanced glycosylation end product (AGE) accumulation. In another report, methylglyoxal formation (a precursor to AGEs) accompanied an increase in superoxide production by renal cortical mitochondria of 12-month STZ-diabetic rats (Rosca et al. 2005). Mitochondrial ROS also were implicated in renal pathology in the Goto-Kakizaki rat, a rodent model of type 2 diabetes (Rosen and Wiernsperger 2006). This study showed a reduction in tissue aconitase activity, a mitochondrial enzyme susceptible to inactivation by reactive oxygen, along with an increase in lipid peroxides.

In summary, the mitochondrial respiratory chain constitutes the main intracellular source of ROS in most tissues. Mitochondria, by virtue of numbers or functional properties or both, are critically involved in the pathophysiology of diabetes. The steady-state concentrations of ROS are maintained at nontoxic levels by a variety of antioxidant defenses and repair enzymes. This delicate balance between antioxidant defenses and ROS production may play a critical role in diabetic nephropathy in which the resulting oxidative insult could eventually cause kidney damage. New diabetes-treatment strategies are needed to address both mitochondrial function and ROS production. Pharmacologic interventions must focus on mechanisms regulating mitochondrial biogenesis, ROS, and respiration. Future examination of the members of the fission and fusion machinery may also enhance our understanding of the role of the mitochondrial dynamics in diabetic nephropathy. At the functional level, effective pharmacologic agents are needed that can be safely delivered to targeted sites within cells and within mitochondria.

NADPH Oxidase

NADPH oxidase is a multiprotein cytosolic enzyme complex initially identified in phagocytes, which generate ROS in response to bacterial infections. It catalyzes the transfer of electrons from NADPH to molecular oxygen via their catalytic subunits to generate superoxide. NADPH oxidase in phagocytic cells releases ROS as a defense against pathogens, whereas in endothelial cells (ECs), NADPH oxidase isoforms expressed in the endoplasmic reticulum (ER) and perinuclear membranes generate ROS as modulators of redox-sensitive signaling pathways.

How does NADPH oxidase generate ROS? NADPH oxidase is a heme-containing protein complex whose backbone is a Nox protein (also known as gp91phox). The Nox family is composed of 7 catalytic subunits termed Nox1-5 and Duox1 and Duox2 (for Dual Oxidase), regulatory subunits p22phox, p47phox, Noxo1, p67phox, Noxa1, p40phox, and the major binding partner Rac (Lambeth et al. 2000; Lambeth 2004). The enzyme is normally dormant in resting state but is rapidly activated upon appropriate stimulation in a process involving the translocation and association of cytoplasmic subunits. The activated cytoplasmic complex then associates with subunits in the membrane to form a functional enzyme with very specific regulatory mechanisms, tissue and subcellular patterns of expression, downstream targets, and functions.

The Nox isoforms are the catalytic subunits for ROS generation that are differentially expressed and regulated in various cell types but remain to be fully characterized. The reduced substrate NADPH binds to Nox isoforms on the cytoplasmic side of the membrane and releases two electrons, which are passed initially to FAD, then to the first and second heme groups, and finally accepted by two successive molecules of oxygen on the opposite side of the membrane, to produce two molecules of superoxide radical (Griendling and FitzGerald 2003a, b; Shiose et al. 2001; Jones et al. 1995; Radeke et al. 1991). In the kidney, all components of the NADPH oxidase complex, including p22phox, p47phox, and p67phox, as well as Nox isoforms 1, 2, and 4, are expressed, in a variety of cell types including fibroblasts, endothelial cells, vascular smooth muscle cells, mesangial cells, tubular cells, and podocytes.

An emerging body of evidence suggests that NADPH oxidase may play a pathogenic role in diabetic nephropathy. For instance, the mRNA expression of essential subunits of NADPH oxidase, NOX4 and $p22^{phox}$, in the kidneys of streptozotocin-induced diabetic rats were markedly increased as compared with control rats (Etoh et al. 2003). Immunohistochemical analysis showed that the expression of Nox4 and $p22^{phox}$ were increased in both distal tubular cells and glomeruli. Insulin treatment for 2 weeks completely restored the levels of these components in the diabetic kidney to control levels. Moreover, pharmacological inhibition of NADPH oxidase with apocynin prevented upregulation of p47phox and gp91phox overexpression and retarded the mesangial matrix expansion seen in experimental diabetic nephropathy (Asaba et al. 2005; Thallas-Bonke et al. 2008). And finally, using antisense oligonucleotides for Nox4, Gorin et al. reported

a significant improvement in renal hypertrophy and fibronectin accumulation in STZ rats (Gorin et al. 2005). These results suggest that the expression of NADPH oxidase subunits Nox4 and $p22^{phox}$ are upregulated in diabetic kidneys and that Nox4 may play a significant role in the pathogenesis of DN.

Several other reports have suggested that the expression of $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$ are upregulated in the aorta from animal models of diabetes (Kim et al. 2002; Hink et al. 2001) and in the saphenous vein and internal mammary artery from patients with diabetes and coronary artery disease (Guzik et al. 2002). Furthermore, NADPH oxidase-driven superoxide production was reported to be involved in vascular dysfunction in a type 2 diabetes animal model (Kim et al. 2002). In addition, at least one report showed that the activity of NADPH oxidase was increased in the retina of diabetic rats, suggesting that NADPH might be involved in the development of diabetic retinopathy (Ellis et al. 2000).

One major gap in our current understanding of the role of NADPH oxidase in diabetic nephropathy is to identify the mechanisms underlying activation of NADPH oxidase. In this regard, it has been shown that NADPH oxidase is triggered by AGE (Soro-Paavonen et al. 2008; Wautier et al. 2001). Importantly, incubation of human endothelial cells with AGE (carboxymethyl lysine-modified adducts) promotes intracellular generation of ROS, which is suppressed by DPI and an AGE inhibitor but not by L-NAME. Furthermore, a soluble form of receptor for advanced glycation end products (sRAGE) significantly inhibits expression of NADPH oxidase in diabetic mice (Soro-Paavonen et al. 2008; Wautier et al. 2001).

Finally, activation of NADPH oxidase is abolished in diabetic $PKC\beta^{-/-}$ mice, suggesting that NADPH oxidase is also activated via a PKC-dependent pathway (Ohshiro et al. 2006). Lack of PKCβ can protect against diabetes-induced renal dysfunction, fibrosis, and Nox-derived ROS production. Other PKC isoforms have also been implicated in NADPH oxidase activation in diabetes, e.g., PKCα is downstream of AGE-RAGE and mediates ROS generation by NADPH oxidase in the kidney of diabetic rats (Thallas-Bonke et al. 2008); PKCδ is responsible for high glucose-induced intracellular ROS production by NADPH oxidase in the adipocytes of diabetic mice (Taylor et al. 2005), while PKCζ is required for ROS generation from NADPH oxidase in mesangial cells treated with high glucose (Kwan et al. 2005). Angiotensin II is a potent stimulator of NADPH oxidase $O_2^{\bullet -}$ production in the vasculature. Accordingly, inhibitors of angiotensin II signaling slow the progression of diabetic complications such as nephropathy, retinopathy, and atherosclerosis, independent of their ability to lower blood pressure in both type 1 and type 2 diabetes (Wei et al. 2007).

Taken together, multiple studies have shown that activation of NADPH oxidase affects both cellular redox signaling and oxidative stress in diabetes. Recent advances in the identification of vascular NADPH oxidase subunits, their subcellular localization/regulation, and feedback inhibition of NADPH oxidase via the Nrf2/ARE pathway provide novel therapeutic targets to combat oxidative stress in diabetes. Therefore, strategies to restore basal NADPH oxidase activity offer a potential scope of treatment.

Conclusions

While progression to diabetic nephropathy cannot yet be prevented, multiple observations suggest that increased oxidative stress in the kidney may have a fundamental role in the development of microvascular complications of diabetes. However, these observations need to be evaluated cautiously since the evidence on the potential role of ROS in the development of diabetic kidney disease is mainly supported in experimental models. This is of particular interest since experimental rodent models of diabetes may not recapitulate many key aspects of phenotypes observed in patients with diabetic nephropathy. Indeed, conventional antioxidants such as vitamin E have shown little benefit on progression of diabetic kidney disease. Future studies are needed to translate into therapeutics the potential role of ROS in the pathogenesis and development of diabetic nephropathy in patients with this devastating disease.

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