

Targeting Mitochondria- and Reactive Oxygen Species-Driven Pathogenesis in Diabetic Nephropathy

Runa Lindblom^{1,2,3,4}, Gavin Higgins^{1,2,4,5},
Melinda Coughlan^{1,2,3,6,7}, and Judy B. de Haan^{2,7,8}

¹ Glycation, Nutrition and Metabolism Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia. ² Diabetic Complications Division, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia. ³ Department of Medicine, Central Clinical School, Monash University, Melbourne, Victoria, Australia. ⁴ Equal contribution by first authors. ⁵ Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia. ⁶ Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Victoria, Australia. ⁷ Equal contribution by senior authors. ⁸ Oxidative Stress Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia. Address correspondence to: Judy B. de Haan, Head, Oxidative Stress Laboratory, Diabetic Complications Division, Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, Victoria 3004, Australia, e-mail: judy.dehaan@bakeridi.edu.au

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■ Abstract

Diabetic kidney disease is one of the major microvascular complications of both type 1 and type 2 diabetes mellitus. Approximately 30% of patients with diabetes experience renal complications. Current clinical therapies can only mitigate the symptoms, and delay the progression to end-stage renal disease, but do not prevent or reverse it. Oxidative stress is an important player in the pathogenesis of diabetic nephropathy. The activity of reactive oxygen and nitrogen species (ROS/NS), which are by-products of the diabetic milieu, has been found to correlate with pathological changes observed in the diabetic kidney. However, many clinical studies have failed to establish antioxidant therapy to be renoprotective. The discovery that increased ROS/NS activity is linked to mitochondrial dysfunction, endoplasmic reticulum stress, inflammation, cellular senescence, and cell death calls for a refined approach to antioxidant therapy. It is becoming clear that mitochondria play a key role in the generation of ROS/NS and their consequences on the cellular

pathways involved in apoptotic cell death in the diabetic kidney. Oxidative stress has also been associated with necrosis via induction of mitochondrial permeability transition. This review highlights the importance of mitochondria in regulating redox balance, modulating cellular responses to oxidative stress, and influencing cell death pathways in diabetic kidney disease. ROS/NS-mediated cellular dysfunction corresponds with progressive disease in the diabetic kidney, and consequently represents an important clinical target. Based on this consideration, this review also examines current therapeutic interventions to prevent ROS/NS-derived injury in the diabetic kidney. These interventions, mainly aimed at reducing or preventing mitochondrial-generated oxidative stress, improving mitochondrial antioxidant defense, and maintaining mitochondrial integrity, may deliver alternative approaches to halt or prevent diabetic kidney disease.

Keywords: diabetic nephropathy · reactive oxygen species · apoptosis · necrosis · mitochondrial dysfunction

1. Introduction

Diabetes is recognized as the leading cause of end-stage renal disease (ESRD) in developed countries [1]. Statistics show a steady increase in the global prevalence of type 2 diabetes, with the worldwide obesity epidemic recognized as

a major risk factor [2]. With an estimated 7.7% of the world population aged between 20-79 predicted to be diabetic by 2030, microvascular complications such as diabetic nephropathy are expected to contribute to a significant percentage of the worldwide morbidity rate [3]. Furthermore, chronic kidney disease (CKD), which affects ap-

proximately 5-30% of people with type 2 diabetes, is a significant risk factor for the development of cardiovascular disease (CVD) [4, 5]. Indeed, diabetic patients with microalbuminuria are 2-4 times more likely to experience cardiovascular impairments than non-diabetic patients [6], whilst those diabetic patients with overt kidney disease show a 4-8-fold increased risk of cardiovascular disease [7]. Despite the administration of glucose-, lipid-, and blood pressure-lowering drugs, nearly half of all diabetic patients continue on to develop renal and cardiovascular complications. Thus, the search for effective therapies is still a major goal in the treatment of diabetic patients.

Oxidative stress and inflammation, induced by chronic elevations in blood glucose in diabetic patients, are increasingly being recognized as risk factors [8] and significant mechanistic contributors [9-11] inseparably linked with the development of diabetic complications including CKD and ultimately ESRD. There is evidence that reactive oxygen and nitrogen species (ROS/NS) play key roles in the pathogenesis of diabetic nephropathy. This has initiated increased efforts to reduce or prevent the cumulative damage of ROS/NS-induced injury. However, clinical antioxidant trials with primary endpoints of CVD reduction have shown little success, which emphasizes the need for more knowledge about the major sites of ROS/NS production and the processes they are involved in [12-14].

Increased attention is currently paid to the significance of mitochondrial ROS/NS generated in response to elevated glucose level as the major player in the process of cell death. This impacts not only the ability of a cell to function optimally, but ultimately and collectively it dictates how an organ like the diabetic kidney responds to its challenging high-glucose environment. This review will focus on the latest evidence, both clinically and pre-clinically, to evaluate the contribution of ROS/NS to acute kidney failure in diabetes. Therapies that specifically target mitochondrial ROS to prevent cell death will be discussed and newer intervention options around this paradigm will be presented.

2. Contribution of ROS/NS production to diabetic nephropathy

Oxidative stress by definition is due to an overproduction of ROS/NS and/or a deficiency in enzymatic and non-enzymatic antioxidant defense, such that the balance is tipped in favor of ROS/NS accumulation and cell damage. Evidence from pre-clinical studies supports the role of ROS/NS in-

Abbreviations:

8-OHdG – 8-hydroxy-2'-deoxyguanosine
8-oxodG – 8-oxo-7,8-dihydro-2'-deoxyguanosine
ADP – adenosine diphosphate
AGE – advanced glycation end-product
AIF – apoptosis-inducing factor
aPC – activated protein C
ATM – ataxia telangiectasia mutated
ATP – adenosine triphosphate
ATR – ataxia telangiectasia and Rad3-related
BER – base excision repair
BMF – Bcl-2 modifying factor
CKD – chronic kidney disease
CVD – cardiovascular disease
DIABLO – direct IAP-binding protein with low pI
EMT – endothelial-mesenchymal transition
EndoG – endonuclease G
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
ESRD – end-stage renal disease
ETC – electron transport chain
FADD – FAS-associated protein with death domain
GPx1 – glutathione peroxidase 1
H₂O₂ – hydrogen peroxide
ICR – Institute of Cancer Research
IDH2 – isocitrate dehydrogenase 2
IMS – intermembrane space
MAPK – mitogen-activated protein kinase
MCP-1 – monocyte chemotactic protein 1
MIF – macrophage migration inhibitory factor
MIOX – myo-inositol oxygenase
MOMP – mitochondrial outer membrane permeability
mPTP – mitochondrial permeability transition pore
mtDNA – mitochondrial DNA
MTH1 – mut T homolog 1
MUTYH – mut Y homolog
NADPH – nicotinamide adenine dinucleotide phosphate hydrogen
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
*NO₂ – nitrogen dioxide
NOX – NADPH-oxidase
NRK – normal rat kidney
O₂^{•-} – superoxide
OGG1 – oxoguanine glycosylase
OH[•] – hydroxyl radical
ONOO – peroxynitrite
OPG – osteoprotegerin
OXPHOS – oxidative phosphorylation
PARP – poly (ADP-ribose) polymerase
PKC – protein kinase C
RAGE – receptor for AGEs
RAS – renin-angiotensin system
ROS/NS – reactive oxygen and nitrogen species
Smac – second mitochondrial-derived activator of caspases
Smad2 – mothers against decapentaplegic homolog 2
SOD2 – superoxide dismutase 2
STZ – streptozotocin
TCA – tricarboxylic acid
TGF – transforming growth factor
TRAIL – tumor necrosis factor-related apoptosis-inducing ligand
VCAM-1 – vascular cell adhesion molecule 1
ZDF – Zucker diabetic fatty

involvement in the pathogenesis of diabetic CKD and ESRD. It has been shown that elevated levels of markers for ROS/NS damage accumulate in diabetic kidneys [15-18] and, under high-glucose conditions mimicking diabetes, in various kidney-derived cultured cells [19, 20]. Data from our laboratory have clearly demonstrated increased oxidative stress and advanced kidney disease in type 1 diabetic mice lacking the major cytosolic and mitochondrial antioxidant enzyme glutathione peroxidase 1 (GPx1) [21]. In particular, our study demonstrated a strong link between increased oxidative stress, enhanced inflammatory response (monocyte chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1)), and increased pro-fibrotic mediators (transforming growth factor (TGF) β , collagens I and III) in the diabetic kidney cortex. Indeed, the ROS/NS, peroxynitrite, has been shown to play a key role in the pathogenesis of the diabetic glomerular lesion [22]. Other pre-clinical studies with genetic alterations of the major ROS/NS producing enzymes, the NADPH-oxidases (NOX), in particular NOX4, lend further support to the theory of oxidant-mediated diabetic kidney injury [23]. Ablation of NOX4 in a mouse model of type 1 diabetes caused marked protection from both structural and functional kidney damage [9]. Thus, targeting NOX4 with pharmacological inhibitors may offer a viable therapeutic strategy to reduce diabetic kidney injury.

Clinically, type 2 diabetes patients demonstrate elevations of excreted urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, which was positively correlated with the extent of tubulointerstitial kidney injury in these patients [24]. Furthermore, a complementary marker of DNA damage, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), strongly predicted the progression of the disease in type 2 diabetics over a 5-year follow-up period [25].

At the molecular level, ROS/NS, produced as a consequence of elevated glucose levels, affect cell signaling pathways associated with metabolism, cell proliferation, and cell death [26]. Of particular relevance to diabetic kidney disease, a number of studies have shown the involvement of ROS in glucose-mediated activation of the protein kinase C (PKC) pathway in mesangial cells, leading to increased TGF- β levels [27, 28], an important mediator of kidney fibrosis [29, 30]. ROS have also been shown to activate NF- κ B signaling in mesangial cells, causing a pro-inflammatory response [31].

Evidence from antioxidant studies has further supported a role for ROS/NS-mediated modulations of cellular signaling. Indeed, our own studies

with the antioxidant and GPx1 mimetic ebselen have shown reductions in the activation of p38 MAP kinases (MAPK) and JNK in normal rat kidney (NRK) cells [21], suggesting the involvement of ROS/NS in these pathways linked to diabetic kidney disease. Others have used disparate antioxidants, and found a reduction in TGF- β -stimulated ROS and endothelial-mesenchymal transition (EMT), as well as lower levels of phosphorylated Smad2, p38 MAPK, and extracellular signal-regulated kinase (ERK) in renal tubular epithelial cells [32]. In conclusion, there is sufficient preclinical evidence supporting the role of glucose-stimulated ROS/NS in disrupting the relevant signal transduction cascades and transcription factors involved in cell function and metabolism, leading to glomerular mesangial expansion and tubulointerstitial fibrosis.

3. Mitochondrial ROS/NS in the pathogenesis of diabetic nephropathy

The importance of intact, efficiently functioning mitochondria is imperative to the prevention of kidney damage and renal failure [33, 34]. Mitochondria are involved in many biological processes, such as energy production, calcium homeostasis, and the regulation of cell death pathways, including apoptosis, programmed cell death, and unregulated necrosis [35]. Mitochondria play paradoxical roles in fueling cellular homeostasis through ATP produced by oxidative phosphorylation (OXPHOS), and in initiating cell death via the release of intermembrane space cell death proteins, such as cytochrome *c*, Smac/DIABLO, apoptosis-inducing factor (AIF), and endonuclease G (EndoG). These actions can be linked to an imbalance in redox regulation within the mitochondrial matrix, an association that plays a significant role in diabetic nephropathy. This line of evidence will be further discussed in the next sections.

OXPHOS involves the transfer of electrons across complexes I-IV of the electron transport chain (ETC) to generate a membrane potential ($\Delta\psi_m$) that is essential to establish the proton motive force driving ATP production. While OXPHOS is critical to meet the energy requirements of all eukaryotic cells, this is most evident in proximal and distal tubule cells that depend on ATP to drive Na^+/K^+ antiporters and Ca^{2+} transporters, respectively [36-38]. However, the byproduct of OXPHOS is the production of superoxide ($\text{O}_2^{\cdot-}$) that occurs as a result of electrons escaping from complex I and III and reacting with molecular oxygen. In intact

mitochondria, complex I and the Q_1 site of complex III release O_2^{\bullet} toward the mitochondrial matrix, whilst the Q_0 site of complex III releases O_2^{\bullet} toward the intermembrane space. [39, 40]. Impairment of complex I can result in an increase in the formation of reactive species and the development of renal damage similar to that seen in diabetic nephropathy, as demonstrated in *Ndufs6* knockout mice [41]. Moreover, O_2^{\bullet} can be converted into other ROS/NS, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{\bullet}), and can react with nitric oxide to form peroxynitrite ($ONOO^{\bullet}$) and nitrogen dioxide (NO_2^{\bullet}) [33]. For proximal tubules, this is thought to be exacerbated under hyperglycemic conditions, where an increase in glucose reabsorption may result in increased glycolysis and OXPHOS, resulting in increased oxidative stress [34]. In addition, methylglyoxal, an α -dicarbonyl byproduct of glycolysis, can induce the formation of advanced glycation end-products (AGEs) that exacerbate ROS production [42].

AGEs accumulate during diabetes. They are associated with an increase in oxidative stress through signaling via the receptor for AGEs (RAGE) [33, 43]. Our group has demonstrated that RAGE-induced cytosolic ROS/NS promotes mitochondrial O_2^{\bullet} production via activation of complex I of the mitochondrial ETC, triggering mitochondrial permeability transition pore (mPTP) formation and cell death, which can be attenuated in diabetic *RAGE*^{-/-} mice [44]. Formation of AGEs through methylglyoxal accumulation results in modification of subunits, core protein 1 and cytochrome *c*₁, of complex III causing O_2^{\bullet} production, as shown in the cortex of diabetic rat kidneys [45]. Overexpression of glyoxalase I, the enzyme responsible for detoxifying methylglyoxal, has been shown to reduce the expression of complex I, II, and III, while reducing ROS production and apoptosis under hyperglycemic conditions in mouse mesangial cells [46]. In conclusion, existing evidence argues in favor of a significant role for ROS/NS produced as byproducts of the ETC in diabetic nephropathy. Indeed, Brownlee and coworkers proposed the unified hypothesis of diabetic complications where ETC-generated ROS/NS induce diabetic complications [47-49]. Current thinking has expanded on these ideas to speculate that the actions of ETC-generated ROS/NS most likely involve additional pathways and ROS/NS-producing enzymes. These aspects are further discussed below.

The regulation of redox homeostasis within the diabetic milieu has been the subject of two recent studies on diabetic nephropathy. The tubular-

specific enzyme myo-inositol oxygenase (MIOX) was identified as a regulator of redox imbalance in the mitochondria under hyperglycemic conditions [50]. These studies showed an increase in MIOX in the renal cortex of streptozotocin (STZ)-induced diabetic ICR (Institute of Cancer Research) outbred male mice. Moreover, an increase in ROS and the incidence of apoptosis associated with increased MIOX expression was observed in the kidney proximal tubule cell line, LLC-PK1 [50]. Likewise, the redox enzyme p66^{Shc}, which localizes within mitochondria, has been implicated in the regulation of ROS/NS production and apoptosis during exposure to increased levels of glucose in diabetic nephropathy [51]. This enzyme has been shown to be epigenetically suppressed by the coagulation protease, activated protein C (aPC), in podocytes in diabetic nephropathy [51].

A significant focus of recent research has been on the NADPH oxidases within the renal cortex in the diabetic milieu, particularly NOX4, as discussed above. Block *et al.* demonstrated that NOX4 is upregulated under hyperglycemic conditions within the mitochondrial compartment of the cell [52]. NOX4 has been localized within renal cortex membrane fractions [52]. Since NOX isoenzymes reside within cells and span membranes of cells [53], it is likely that NOX4 spans the inner membrane of mitochondria, and that O_2^{\bullet} or H_2O_2 are released into the intermembrane space (IMS) of mitochondria. Initially, it had been proposed that NOX4 produces H_2O_2 only [54], but it is also capable of producing O_2^{\bullet} , as shown by Block *et al.* [52].

NOX4 has also been localized in the mitochondrial fraction of kidney glomerular mesangial cells that are known to be primary targets for glucose-mediated oxidative injury [23]. While $NADP^+$ / $NADPH$ can traverse the outer mitochondrial membrane, it is impermeable to the inner membrane. NOX4 requires $NADPH$ to become active. The fact that functional O_2^{\bullet} -producing NOX4 has been identified in diabetic mesangial cells suggests that the carboxyl-terminus of NOX4 is positioned in contact with the intermembrane space [52]. Furthermore, NOX4 may indirectly influence the citric acid cycle, which is located within the mitochondrial matrix, through its influence on isocitrate metabolism. This includes the conversion of isocitrate into α -ketoglutarate by isocitrate dehydrogenase 2 (IDH2), which is dependent on $NADP^+$ [55]. Recently, the use of metabolomics has revealed that NOX4 plays a key role in regulating the tricarboxylic acid (TCA) urinary metabolite fumarate in diabetic Akita mice by exerting its ef-

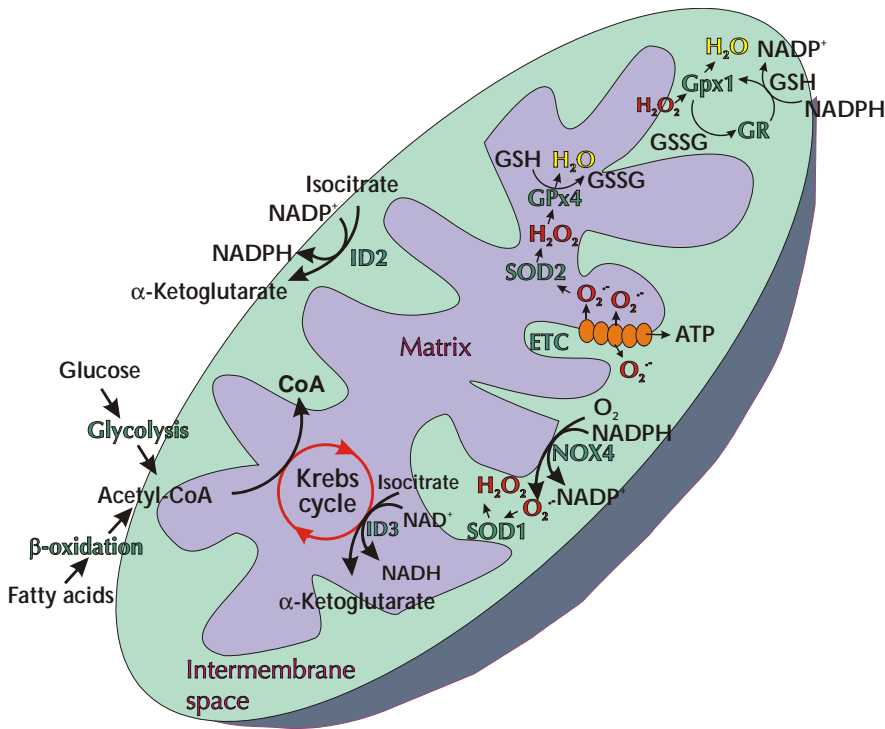


Figure 1. Redox signaling within mitochondria. ROS in the form of superoxide ($O_2^{\cdot -}$) can be produced via the electron transport chain (ETC) or NOX4 within the inner mitochondrial membrane (IMM). $O_2^{\cdot -}$ is released from complexes I and III of the ETC into the matrix or intermembrane space. $O_2^{\cdot -}$ released from NOX4 or complex III enters the intermembrane space where it is dismutated by SOD1 into hydrogen peroxide (H_2O_2). $O_2^{\cdot -}$ within the matrix is converted into H_2O_2 by SOD2. In turn, H_2O_2 is detoxified into H_2O via GPx1 in the intermembrane space or via GPx4 within the matrix. This delicate balance of ROS within the mitochondria is dependent upon the Krebs (citric acid) cycle, the conversion of $NAD(P)^+$ to $NAD(P)H$, and *vice versa*. Under diabetic conditions this redox signaling is disrupted, resulting in increased oxidative stress.

fect on the mitochondrial enzyme fumarate hydratase. Importantly, interventions with a NOX1/NOX4 inhibitor restored fumarate hydratase and fumarate levels and reduced albuminuria, glomerular hypertrophy, and mesangial matrix accumulation in these diabetic mice [56]. Nonetheless, it is evident that $O_2^{\cdot -}$ produced by mitochondrial NOX4 cannot have a direct link to deficiencies in the ETC or mitochondrial DNA (mtDNA) integrity that are housed within the inner mitochondrial membrane or matrix respectively, unless converted to membrane permeable molecules such as H_2O_2 or $ONOO^-$, which we have demonstrated to be increased in the diabetic kidney [57].

If NOX4 has any deleterious influence within the mitochondria then it is likely to occur within the IMS. It is also likely that glutathione reductase competes with NOX4 for NADPH to maintain the balance of glutathione used for Gpx1 and Gpx4 within the mitochondria to detoxify H_2O_2 by conversion into H_2O . Indeed, the NOX inhibitor apocynin has been shown to restore the glutathione/glutathione disulfide (GSH/GSSG) ratio in Zucker diabetic fatty (ZDF) rats [58]. Within the IMS, $O_2^{\cdot -}$ produced by NOX4 must be detoxified by SOD1. Otherwise, it exerts damage to the inner and outer mitochondrial membrane [59]. It will be a matter of future research to clarify whether diabetes-induced NOX4 upregulation is sufficient to rupture the outer membrane, initiate the mPTP, or induce mitochondrial outer membrane permeabilization (MOMP), and thereby trigger the redistribution of the IMS cell death proteins. Indeed, it has been demonstrated that NOX4 upregulation medi-

ates TGF- β -induced apoptosis in podocytes, although it was not investigated whether IMS cell death proteins were engaged in this process [60].

Investigations have also focused on deficiencies in the antioxidant network within the mitochondria, including coenzyme Q, SOD2, and GPx4 [34]. The consequence of an imbalance in these ROS generators and antioxidants within the diabetic renal cortex is an elevation in oxidative stress within the mitochondria, which can trigger the intrinsic cell death cascade [61]. **Figure 1** summarizes the key players involved in redox signaling in the mitochondria. Under diabetic conditions, this redox signaling is disrupted, resulting in increased oxidative stress.

Finally, it should be mentioned that results from studies on the occurrence of mitochondrial ROS/NS in diabetes and the amounts produced are inconsistent to some extent. While it is now generally accepted that mitochondria-derived ROS/NS are increased in diabetes, some studies have reported an overall decrease [62, 63]. The discrepancy may result from the cellular heterogeneity and differing metabolic responses of the cells in the kidney [64]. Further research is necessary to clarify this issue.

4. Consequences of oxidative stress in the diabetic kidney

4.1 Nuclear DNA damage

Oxidative stress is known to contribute to a range of harmful intracellular events, including DNA damage within the nucleus. This damage may ultimately cause dysfunction, cell death, and long-term risk of oncogenesis. Generally, oxidative DNA damage, in the form of oxidized bases, occurs as frequently as one million times per day in the human body as a consequence of normal metabolism and other environmental factors [65, 66]. Cells rely on high energy DNA repair mechanisms to clear DNA damage. This includes the recruitment of the base excision repair (BER) protein complex, amongst others, to repair this type of damage [67]. The formation of general DNA repair complexes (e.g. ataxia telangiectasia mutated (ATM), ataxia and rad-related kinase (ATR)) involves the activation of proteins that target gene expression and protein production to respond rapidly to detected damage. Two of the more significant transcription factors are p53 and NF- κ B, both of which regulate gene expression.

In cases where the DNA damage level is high, the recruitment of large volumes of repair complexes can result in rapid depletion of available ATP due to elevated energy demand, e.g. by cleavage of the damage-sensing protein poly (ADP-ribose) polymerase (PARP) [68, 69]. This creates the potential for unregulated cell death (i.e. necrosis) as there is insufficient energy for cells to undergo ATP-dependent programmed cell death (apoptosis) [70]. This "switch" in DNA damage-related cell death PARP-1 activation has been observed in canine renal cells [71, 72]. However, if the DNA damage by ROS is sub-lethal, cells can sufficiently repair the damage and even adapt to chronic change [73].

Mitochondria are involved in sensing nuclear DNA damage. They are able to influence cellular

responses, including the release of apoptosis-inducing factor (AIF), a protein involved in DNA fragmentation and chromatin condensation during apoptosis [74]. In the pathogenesis of diabetic nephropathy, oxidative stress-related damage to mitochondria contributes to apoptosis and loss of podocytes, which are key events in the progression of the disease [75].

The ability for cells to adapt to increased levels of ROS/NS is a critical component in homeostatic protection. Due to the frequency of oxidative DNA damage across the body, cells have robust mechanisms to identify and repair this damage to prevent cumulative genotoxicity or mutations. BER is one of the mechanisms employed by mammalian cells to repair DNA damage caused by oxidation. [76, 77]. In normal cells, the BER pathway is regulated by 3 main proteins: OGG1, MTH1, and MUTYH that constitute a part of the BER repair complex [78, 79]. When an oxidized deoxyguanosine base (8-oxo-dG) is repaired by BER, the dissected base (8-OHdG) is discharged from the cell and filtered through the kidney [80].

Recent evaluations of DNA breaks induced by oxidative stress showed that they are increased in patients with type 1 and type 2 diabetes, although most studies involved analyses of peripheral lymphocytes [81-83]. The same phenomenon has also been observed in gestational diabetes [84, 85]. Of particular interest is the association with renal complications. The direct effect of high glucose on ROS/NS production and subsequent DNA damage has been assessed in rat models of type 1 diabetes [86]. These studies support the hypothesis that oxidative stress-induced damage occurs in the kidneys of diabetic patients. Clinical investigation into the effects of oxidative stress-induced DNA damage on renal cells is limited, in part because of the need for renal biopsies. However, it is possible to determine indirectly the level of oxidative damage in a patient by analyzing the urinary excretion of oxidized DNA bases in the form of 8-OHdG. This validated method has revealed increased excretion rates in patients with diabetes, corresponding to an increase in generalized oxidative stress-induced DNA damage throughout the body [80].

Interestingly, the occurrence of 8-oxo-dG base damage has been found to coincide with diabetic complications and may be a predictive biomarker for detecting the severity of diabetic complications, including retinopathy and nephropathy [80, 87, 88]. Although a higher excretion rate is correlated with severity of diabetic nephropathy, this critical biomarker can give an idea of systemic DNA damage only (i.e. relative damage in the entire body),

but not the specific incidence in an individual organ. Another limiting factor for the use of 8-OHdG as biomarker for renal damage is that the excretion of 8-OHdG is affected by other compounding factors, including exercise and environmental toxins [89-91]. This limits the clinical implications of these studies as an individual's baseline excretion rate can vary significantly.

4.2 Mitochondrial DNA damage

In addition to the effects of nucleic DNA damage, mtDNA in the diabetic kidney is also susceptible to damage by ROS/NS [18]. The close proximity of mtDNA to the ETC within the mitochondrial matrix makes this type of DNA highly susceptible to damage by ROS/NS produced during OXPHOS. This mechanism is generally considered to account for the increased observation of oxidized mtDNA compared with nuclear DNA damage [92]. Although mtDNA damage is ultimately repaired after a short insult, longer insults lead to persistent damage that could subsequently result in apoptosis [93]. MtDNA damage is also repaired through the BER pathway by the excision of 8-OHdG, although there seems to be some slight differences between the recruited proteins in each organelle. For example, nucleic DNA is repaired primarily by DNA ligase I, whilst mtDNA damage is repaired by DNA ligase III [94].

The role of oxidative stress in DNA damage and mitochondrial dysfunction has already been established in other pathologies such as in neurodegeneration, heart failure, and diabetic retinopathy [95-97]. In a recent clinical study, a link was found between diabetes, damaged mtDNA, and functionally altered mtDNA. Indeed, changes in mtDNA preceded bioenergetic dysfunction, leading the authors to propose that systemic mitochondrial dysfunction initiated by glucose-induced mtDNA damage may be involved in the development of diabetic nephropathy [98].

4.3 Compromised DNA repair mechanisms

Susceptibility to oxidative stress-induced DNA damage has been shown to correlate with certain gene polymorphisms in the BER pathway in type 2 diabetes complications such as distal symmetric polyneuropathy [99, 100]. One study examined changes in the gene expression of the BER complex protein MUTYH, an important protein in the response to cellular stress, following treatment with TGF- β 1. The investigators found that expression of the protein in renal proximal tubule cells

differs from that in interstitial cells [101]. Furthermore, oxidative DNA damage, as measured by 8-oxoG, was positively associated with renal fibrosis, suggesting that MUTYH mediates tubulointerstitial damage [101]. Other studies have indicated that suppression of MUTYH may actually be protective of renal disease [102, 103]. Importantly, polymorphisms found in these DNA repair pathway genes have also been shown to increase the risk of ESRD progression in a cohort of mixed-cause patients undergoing hemodialysis [102]. It would be interesting to examine this effect in the context of diabetic nephropathy, and earlier in the progression of the disease, to determine whether this correlates with patient risk of progression to ESRD.

Besides BER, additional repair pathways such as homologous recombination and mismatch repair respond to critical DNA damage [104]. When DNA repair fails to proceed adequately, there is a danger of cell death by necrosis due to the high energy requirements of the DNA repair processes [70]. Thus, DNA repair proteins may be inactivated where possible to downregulate their functional activity for the salvation of apoptotic pathways [104]. However, loss of important repair processes, whether mediated by oxidative stress itself or by other factors, is thought to compromise the integrity of mitochondrial and nuclear DNA. Further investigation is required to find out how the repair mechanisms are compromised in diabetic nephropathy.

5. Modes of cell death during diabetic renal disease

Cell death is a natural consequence of oxidative stress when damage exceeds the ability of the cell to respond and repair the insult. The two main types of cell death observed in diabetic nephropathy are apoptosis and necrosis. Apoptosis is a controlled process that is highly dependent on the continued production of ATP to fuel this high-energy process [69]; it is characterized by cell shrinkage, chromatin condensation, and DNA fragmentation [61]. Apoptosis occurs through two types of pathways. One is the death receptor pathway (extrinsic apoptotic pathway) that includes FAS/FAS-ligand; the second is the mitochondrial pathway (intrinsic apoptotic pathway) that requires regulatory proteins such as the Bcl2-related family [105]. Necrosis, by comparison, is considered uncontrolled and catastrophic. In necrotic cell death, ATP production is insufficient to

meet the energy demands of the cell, leading to rupture of cellular contents [106]. This generates a high-level inflammatory response, which is not observed in apoptosis. Each type of cell death has physiological consequences that correspond to the clinical presentation of a disease. Finally, ER stress [107] and cellular senescence within key cell types of the kidney [108] are two additional mechanisms that are now regarded as contributing to cell death processes in diabetic nephropathy.

5.1 Induction of apoptosis

In diabetic nephropathy, pathogenic lesions are characterized by initial hypertrophy followed by a gradual loss of renal mass, sclerosis, and fibrosis. Apoptosis is known to contribute to the later process in both humans and animals [109-111]; it includes the upregulation of cell surface markers which encourages phagocytosis (removal) by resident and circulating immune cells. These surface markers include CD36, CD95 (FAS-ligand), CD74 (MIF-R), OPG, and TRAIL, which are increased in diabetic kidneys [112-115]. FAS-ligand binds to FAS. It has been shown that plasma FAS levels are elevated in diabetes, and that the rate of apoptosis begins early in the development of proteinuria in renal cells [116, 117]. One study in diabetic female rats found that an increase in FAS/FAS-ligand could be attenuated by blockade of the renin-angiotensin system (RAS) [118]. The inner cell membrane portion of the FAS-ligand includes a FAS-associated protein with death domain (FADD). It has been shown that FADD and its dimeric counterpart FADD-DD play an important role in mediating cell death in renal tubule cells in the context of acute renal injury, and that they can mediate early diabetic renal injury [119, 120]. However, it is clear that additional pathways are involved since increased FAS-ligand levels do not directly correlate with apoptosis [116, 117].

Another event that may occur in diabetic nephropathy includes changes in internal pro-apoptotic proteins such as BASP1, Bcl-2 family proteins, and p53 [109]. The Bcl-2 family of proteins are highly conserved in diabetic nephropathy, and are involved in the regulation of both pro-survival and pro-apoptotic signaling. The numerous family members interact downstream of p53 and NF- κ B to regulate cell survival. The anti-apoptotic protein Bcl-2 has been shown to play an important role in diabetic nephropathy [121] as it antagonizes the pro-apoptotic Bax and Bak proteins that together control mitochondrial cell death. An increase in the pro-apoptotic Bax relative to the anti-apoptotic

Bcl-2 establishes a more pro-apoptotic phenotype; this ratio is a commonly used technique to establish the susceptibility of cells to apoptotic cell death. This change in ratio has been demonstrated in a murine model of type 2 diabetic nephropathy [122]. The increase in the observed pro-apoptotic Bax/Bcl-2 ratio could be reversed through blockade of the RAS pathway in mice overexpressing angiotensinogen in renal proximal tubular cells [123].

In human diabetic nephropathy, the data are limited regarding changes in Bcl-2 and Bax expression. However, it has been identified as a contributing factor in other forms of renal disease [124, 125]. Additionally, other Bcl-2 family members may also be linked with diabetic nephropathy. For example, overexpression of Bcl-2 modifying factor (BMF) is involved in apoptosis in both animal and human studies [126]. BMF is a BH3 only protein (a sub-class of smaller proteins within the Bcl-2 family) that indirectly activates Bax and Bak by inhibiting the related proteins Bid, Noxa, and Bik to promote cell death [127]. Other pathways implicated in diabetic nephropathy include Akt and p38 MAPK [128].

In the diabetic kidney, it is generally considered that ROS/NS-induced apoptosis is associated with specific changes observed in the disease, in particular tubulointerstitial changes [129]. For example, damage by high-glucose-induced ROS/NS has been associated with PKC δ that regulates the cellular response protein p66Shc [130]. P66Shc mediates high-glucose and ANG II responses to oxidative stress and renal proximal tubule cell (PTC) injury via mitochondrial dysfunction and apoptosis pathways [131]. A recent review on renal tubular apoptosis in diabetic nephropathy reported that high-glucose is one of the major contributors to free radical- and ROS-induced cell death in these cells [61]. Further stressors relating to PTC injury include uric acid transport which is also associated with oxidative stress injury and apoptosis [132].

Prolonged oxidative stress is known to cause adaptive changes in cells. Cells that fail to adapt to the changes die by one of the many cell death pathways available to detect and respond to harmful cellular stress conditions. One pathway by which ROS/NS contributes to cell death is the classical apoptotic cascade engaging the Bcl-2 family proteins [133]. This pathway includes the activation of the Bax protein which translocates to the mitochondria of damaged cells and elicits changes in mitochondrial outer membrane permeability (MOMP) by the formation of the mitochondrial outer membrane pore. The downstream effects in-

clude the release of cytochrome *c* and subsequent caspase-3 cleavage.

Bax is activated by a range of transient protein interactions initiated by ROS/NS damage. However, *in silico* modeling also proposes a direct activation of Bax via oxidative interactions [134]. Bax is well known as part of the mitochondrial (intrinsic) pathway of apoptosis, although its full repertoire of cellular interactions is not yet understood. It has a clear role in p53-activated apoptosis, but evidence suggests the involvement of Bax in other forms of cell death as well [135, 136]. In apoptotic death, Bax undergoes a conformational change following activation which allows it to associate with mitochondrial membranes and to take part in the formation of the MOMP. In addition to Bax, the related protein Bak is also capable of forming these pores. Bak is a membrane-bound protein, whereas Bax is cytosolic in its inactive state. Studies show that Bax and Bak can oligomerize during MOMP formation [137]. While the precise morphology of this pore is still a matter of debate, it is hypothesized that there are two distinct pore types [138]. Bax expression has been found to be increased in a diabetic STZ rat model and in dbdb mice [122, 139]. However, expression in human renal cells is relatively unexplored in diabetic nephropathy.

Although necrotic cell death has also been observed in diabetic nephropathy [140], the full implications of its contribution to the disease are not yet understood.

5.2 Endoplasmic reticulum stress

Mitochondrial function is closely linked to endoplasmic reticulum (ER) function. ER stress is another important aspect of cellular pathology. It occurs simultaneously with oxidative stress in the context of the diabetic milieu and progression of diabetic complications [107, 141, 142]. In normal conditions, the ER is responsible for regulating protein folding. In situations of high demand or protein damage (as seen in oxidative stress), the unfolded protein response (UPR) is activated to restore ER homeostasis. This is mediated by the proteins XBP1 and IRE1, and allows for transient protection against damage [143, 144]. When the unfolded protein response is overwhelmed ER stress occurs. This is a cytotoxic process involving cellular dysfunction and the activation of proapoptotic factors such as IRE1 [144].

Cells with a high protein production rate are at an increased risk of cellular dysfunction by ER stress. Pancreatic beta-cells are an important ex-

ample due to the continual production of proinsulin. In type 2 diabetes, this is particularly relevant as the development of insulin resistance and maximum insulin production places the pancreatic cells at an increased risk of ER stress [145, 146]. Additionally, proteinuria and hyperglycemia can directly induce ER stress in renal PTCs *in vitro* [147]. In fact, the upregulation of genes associated with the UPR positively associate with increased severity of diabetic nephropathy, which is regarded as a protective change [147]. ER stress has been shown to mediate renal pathology in diabetic nephropathy and to correspond with disease severity [148, 149]. Examples include albuminuria, which has been shown to cause ER stress by the induction of caspase-12 expression [150]. Furthermore, accumulation of protein in the proximal tubules is known to follow aldosterone administration in rat models (physiological elevated equivalent) and leads to PTC damage if not cleared by autophagy [151].

The ER is primarily responsible for regulating Ca^{2+} . Oxidative stress has been found to alter Ca^{2+} homeostasis [152]. This alteration includes a release of Ca^{2+} from the ER into the cytosol, which in turn affects mitochondria and mitochondrial function [153]. In fact, calcium leakage has been shown to directly cause elevated ROS/NS production in mitochondria via interactions with OXPHOS [154]. Other proteins have been implicated in the reduction of elevated ROS/NS production via oxidative phosphorylation mechanisms in diabetes [155]. However, most of this research has focused on neurodegenerative or skeletal muscle models, not diabetic nephropathy.

In many disease processes, cell death by ER stress occurs via the mitochondrial apoptosis pathway [156]. In type 2 diabetes, ER stress appears to be upregulated and linked with an increase in both apoptosis and necrosis correlating with changes in inflammatory cytokine expression [140]. The translocation of Bax and Bak to the ER membrane may occur during ER stress-mediated apoptosis [157]. Furthermore, caspase-12 cleavage occurs downstream, indicating a pathway of cell death that is potentially independent of the mitochondria in human fibroblast cells [158]. In comparison, the upregulation and accumulation of another pro-apoptotic Bcl-2 family protein, BIM, at the ER membrane is associated with mitochondrial death pathways following caspase-12 activation [159, 160].

Bax/Bak oligomerization at the ER membrane followed by caspase-12 activation has also been demonstrated in mouse models [161]. However,

murine caspase-12 is a homologue of human caspase-4. This variant has also been associated with cell death following ER stress [162]. Additionally, caspase-4 has been observed to mediate PTC death in some types of nephropathy [163], but is yet to be confirmed in diabetic kidney disease. Although human caspase-12 has been analyzed in many studies, its relevance to the general population has been questioned as the full homologue of the gene is only expressed in 2.8% of humans [164].

Additional caspases may be activated downstream of ER stress, including caspase-7 [158] and caspase-8 [165, 166]. It seems that the distribution of Bax to different organelles relates to the type of cell death induced [167]. The structure of the reported ER membrane pore is not yet known, but early results point to changes in membrane permeability [157].

Autophagy is another cell death pathway that has been observed when key components of the mitochondrial apoptotic pathway (i.e. Bax/Bak, caspase-9) are disrupted [165]. Although this aspect is of importance in the field of cancer research and drug resistance, in the context of diabetic nephropathy, it is interesting to consider the implications of altered mitochondrial function in this pathway, particularly as the link between mitochondria and ER relays important signal transfer during cell death [153]. Furthermore, Bcl-2 family proteins, Bax and Bak, have also been linked to this interaction by regulating Ca^{2+} ER homeostasis and efflux to the cytosol [168]. Bcl-2 family proteins play a major role in mediating the response of the ER and mitochondria to oxidative damage. The role of Bax in the progression from ER stress to apoptosis is an interesting area for exploration in diabetic nephropathy, considering the role of Bax in the mitochondria-dependent cell death pathways. Mediation of ER stress could potentially provide a therapeutic target for the prevention of renal disease.

5.3 Cellular senescence

Another potential outcome of oxidative stress is cellular senescence. This is likely to be due to a range of factors, including the inherent growth-inhibition response to DNA mutagenic stimuli. Senescence is a useful state induced by cells to protect from mutagenesis without depleting cell mass by apoptosis. Cells may persist indefinitely in cell cycle arrest. This forms an important physiological function in terminally differentiated tissues such as neurons and adult stem cells, which retain the ability to exit cell cycle arrest.

Senescence of cells can be triggered by oxidative stress. This has been observed in many human cell lines with fibroblasts being particularly susceptible [169-171]. Oxidative stress-induced senescence has also been demonstrated in normal renal cells, and identified as a contributing factor to diabetic nephropathy in animal models and type 2 diabetic patients [169, 172, 173]. The senescent response to oxidative stress can include an increase in mitochondrial mass and mtDNA copy number [174]. Although increased circulatory mtDNA has been detected in patients with type 2 diabetes [98, 175], it may be possible that senescent cells are more susceptible to the effects of ROS and NOS, instead of inducing a protective state. For example, renal podocytes exist as terminally differentiated cells functionally equivalent to senescent cells, but highly susceptible to oxidative stress. There is evidence to suggest that dedifferentiation of these cells occur in diabetes, indicating exit from cell cycle arrest [108] and resulting in podocyte loss. Podocyte loss, as measured by urinary excretion, is associated with early glomerular pathology in diabetic patients [176].

6. Mitochondrial permeability transition as a pathway to renal injury

Since the kidney relies on oxidative phosphorylation (OXPHOS) to provide the bulk requirements of ATP for tubular reabsorption [177], it is not surprising that mitochondrial homeostasis is essential for an optimally functioning kidney. Indeed, in experimental diabetic nephropathy or renal proximal tubule cells exposed to high glucose, mitochondrial ATP content [178] and production [179, 180] are depleted. Mitochondria are able to compensate for decreased cellular ATP production by fusion. This mechanism is an effective adaptive response, as previously observed in other organs [181]. Notably, changes in mitochondrial morphology within renal proximal tubules in both human [182] and animal models of early diabetic nephropathy [183] have long been identified as part of the disease, with mitochondrial enlargement and swelling. Diabetes-induced increases in mitochondria-derived ROS/NS have been shown to provoke the dynamic changes in mitochondrial shape [184]. Another stimulator of change in mitochondrial shape is the induction of mitochondrial permeability transition. This mechanism is independent of the mitochondrial dynamics machinery and oxidative stress, and has been historically detected by electron microscopy imaging showing mitochondrial swelling.

Mitochondrial permeability transition (mPT) is an abrupt increase in inner mitochondrial membrane (IMM) permeability that allows the passage of solutes with molecular masses of less than 1,500 Da. This event is caused by unlocking the mitochondrial permeability transition pore (mPTP), an evolutionary, highly conserved channel [185]. The unlocked pore allows for immediate dissipation of the mitochondrial transmembrane potential and influx of solutes, causing expansion of the matrix and mitochondrial swelling. The loss of the inner mitochondrial membrane potential and the inability to maintain a pH gradient due to proton influx also disrupts mitochondrial ATP synthesis, leading to energy depletion; persistent opening can induce necrotic cell death [186]. Sufficient swelling of the mitochondrial matrix may result in rupture of the outer mitochondrial membrane. This causes cytochrome *c* release with subsequent caspase activation, and results in cellular apoptosis [187]. Opening of the mPTP prevents mitochondria from generating ATP by OXPHOS, and allows reversal of the FoF1 ATP synthase, causing hydrolysis of the ATP produced by glycolysis or any remaining functional mitochondria [178].

The mPTP is composed of multiple macromolecular components which are not yet fully characterized [178, 180, 181]. It was initially thought that mPTP was comprised of the adenine nucleotide translocase (ANT), voltage-dependent anion channel (VDAC), and non-pore forming regulatory component cyclophilin D (CypD), the mitochondrial isoform of the peptidylprolyl cis-trans isomerase cyclophilin chaperone family [188, 189]. The mPTP was thought to be generated as one contiguous pore spanning the intermembrane space [190]. However, gene ablation studies suggested that VDAC and ANT play only a limited role in mPT [191-193]. Also, a significant role for CypD in mPT was established through the development of CypD-deficient mice (*Ppif*^{-/-}) whose mitochondria do not undergo cyclosporin A (CsA)-sensitive mPT [194]. CypD is considered to regulate mPT by facilitating a calcium-triggered conformational change in the mPTP, converting it into an open state [195]. CypD-deficient mice (*Ppif*^{-/-}) are protected from ischemia/reperfusion injury of the heart and focal cerebral ischemia, implicating a role for CypD-dependent mPT opening and subsequent necrotic cell death in end-organ injury [194, 196]. Furthermore, mouse hepatocytes from CypD-deficient mice showed resistance to necrotic cell death induced by ROS and calcium overload [197]. In an updated model of the mPTP, dimers of the F1Fo

ATP synthase form the mPTP within the IMM, with CypD binding to the lateral stalk of the FoF1 ATP synthase [198]. Further studies have identified the c-subunit ring of the Fo (i.e. ab_2c_{10-14} , *Escherichia coli* nomenclature) of the FoF1 ATP synthase to form the inner pore of the mPTP [199]. However, this model has not yet been validated.

Mitochondrial permeability transition is induced by mitochondrial sequestration of high levels of calcium, and is sensitized by factors such as decreased ATP levels, inorganic phosphate, and alkaline pH [178]. Hydrogen peroxide and other ROS may lead to induction of mPT [200]. mPT can also induce changes to the mitochondrial respiratory chain, including inhibition of complex I [201] and loss of cytochrome *c* [202]. Both of these conditions contribute to a reduced and decelerated flow of electrons through the respiratory chain, favoring the one-electron reduction of molecular oxygen. Finally, these events result in the generation of superoxide radicals [203, 204]. Loss of cytochrome *c* from the mitochondrial IMS reduces ATP synthesis, and further increases electron leak and ROS generation, setting up a feed-forward cycle of ROS-induced ROS generation [205]. A recent study has found that Bax mediates mPTP opening, which may cause ATP depletion and cellular necrosis [206].

Recently, a role for mPT has been implicated in renal injury in the kidney. Swelling of mitochondria within renal proximal tubules of the kidney has been demonstrated in early experimental diabetic nephropathy [183] and in kidneys from diabetic patients [182]. *Ppif*^{-/-} mice are protected from acute ischemic renal injury [207]. Moreover, in a short-term model of streptozotocin (STZ)-induced diabetes, rats treated with cyclosporin A (CsA), an inhibitor of mPT, were protected against glomerular hypertrophy and extracellular matrix (ECM) accumulation [208]. A previous study has suggested that renal mitochondria from Goto-Kakizaki rats, a model of type 2 diabetes, had enhanced mPT. However, mPT was only measured indirectly by extramitochondrial calcium flux and its amelioration by CsA *in vitro* [209]. Recent studies from our laboratory have suggested that mPT is a candidate for mediating ROS-induced renal damage [179]. However, interruption in physiological mPT and the downstream consequences, including cell death, have not been extensively studied in models of diabetic nephropathy. It is possible that enhanced susceptibility to the mPT contributes to the development of diabetic nephropathy.

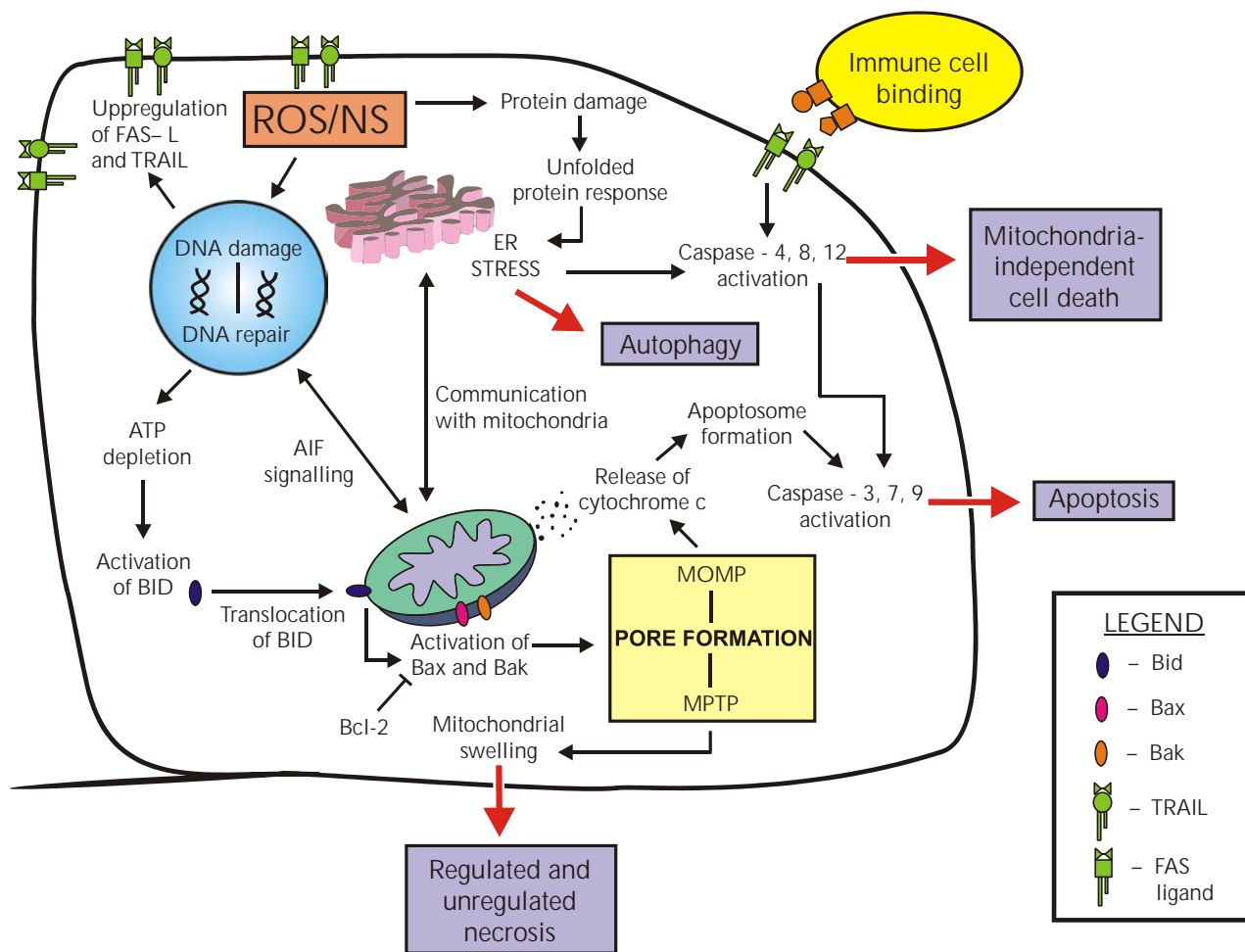


Figure 2. Lethal consequences of ROS/NS. The presence of high levels of ROS/NS can lead to downstream activation of cell death pathways. The type of cell death that occurs depends on the parts of the cell that are damaged. For example, oxidized protein damage can induce ER stress and mediate mitochondrial-independent cell death. However, crosstalk with the mitochondria can induce apoptosis via release of cytochrome c if suitable stimuli are present. DNA damage by ROS/NS can induce a range of signaling pathways, including the upregulation of gene expression of the cell surface markers FAS-L and TRAIL. These markers encourage cytotoxic immune cells to bind and activate the extrinsic apoptosis pathway. Furthermore, communication with mitochondria can lead to apoptotic or necrotic cell death if ATP is depleted. Activation of Bax and Bak can cause mitochondrial pore formation and opening, resulting in either mitochondrial swelling and necrosis or apoptosis via cytochrome c release. This process can be blocked by the anti-apoptotic protein BCL-2. The persistence of ROS/NS in diabetes may result in loss of cell mass and subsequent decrease in renal function.

In summary, diabetes-induced oxidative stress is able to stimulate numerous cellular pathways that eventually lead to cell death. Different types of cell death may occur depending on the parts of the cell that are damaged, as illustrated in **Figure 2**. Apoptosis, mostly induced by MOMP formation, mitochondrial shrinkage, and cytochrome c release, activates caspases via intrinsic or extrinsic pathways. Necrosis induced by MPTP formation results in mitochondrial swelling and lysis. As

highlighted in this review, evidence for these major cell death pathways can be found in the diabetic kidney.

7. Targeting mitochondrial ROS in diabetic nephropathy

Despite increasing evidence supporting the role of mitochondrial oxidative stress in experimental diabetic nephropathy, the evidence from clinical

trials using antioxidants has not confirmed a beneficial effect. For example in the Heart Outcomes Prevention Evaluation (HOPE) trial, vitamin E treatment for 4.5 years failed to confer benefits in cardiovascular outcomes or nephropathy [13]. Lack of specificity and failure to target the correct sites of ROS production are two reasons often mentioned for the potential failure of vitamin E in the clinic. Therefore, the idea of specifically targeting ROS within ROS-producing organelles such as the mitochondria has been investigated. This strategy aims to increase specificity and to avoid targeting important physiological ROS outside the mitochondria because such ROS may be required for cell signaling. Mitochondria-targeting antioxidants have therefore been suggested as a potential therapeutic strategy [210] for the prevention of cisplatin-induced nephropathy [211], acute kidney injury [212], CKD, and diabetic nephropathy. This strategy may also hold promise for the treatment of cardiovascular diseases [213].

The most effective antioxidants would be those that can cross the outer and inner mitochondrial membrane. Therefore, antioxidants were conjugated with a positively charged triphenylphosphonium cation (TPP⁺) to yield compounds such as quinone analogues, mitoquinone (Mito Q), SkQ1 and SkQR1, as well as mitovitamin E, mitophenyltertbutylamine, and the SOD mimetic Mito-CP [212]. These antioxidants accumulate within the mitochondrial matrix at concentrations several-fold higher than within the cytosolic compartment due to the high negative membrane potential of the IMM. A study by Chacko *et al.* in type 1 diabetic Ins2-Akita mice showed that oral administration of MitoQ for 12 weeks prevented diabetic kidney damage [214]. Interstitial fibrosis and glomerular damage were significantly reduced in the treated animals. MitoQ also reduced the expression of the pro-fibrotic transcription factors phospho-Smad2 and 3, and prevented the nuclear accumulation of β -catenin, a member of the Wnt pathway that has been implicated in pathological processes such as fibrosis. Similarly, Mito-CP, the SOD mimetic composed of a lipophilic cationic nitroxide conjugated to TPP⁺, prevented cisplatin-induced renal injury by effectively limiting oxidative and nitrosative stress, preventing mitochondrial structural damage and tubular injury, attenuating renal inflammation, preventing renal dysfunction, and reducing renal cell death in 6-8 week-old male C57Bl/6J mice [211].

Other strategies to increase the antioxidant defense in the mitochondria include overexpression

of hemoxygenase-1 (HO-1), an antioxidant enzyme that is not normally present in mitochondria [215]. To achieve this, a plasmid containing a mitochondrial permeability sequence was fused to the HO-1 gene sequence and transfected into HEK293 renal epithelial cells. As a result, Mito-HO-1-expressing cells were protected from hypoxia-dependent cell death and loss of mitochondrial membrane potential. However, the treatment was afflicted with long-term limitations due to the negative impact on heme-containing mitochondrial proteins.

Ebselen is a synthetic selenocysteine-containing mimetic of the cytosolic and mitochondrial enzyme glutathione peroxidase-1 (GPx1) [216]. In our type 1 diabetic ApoE knockout mouse model, ebselen had renoprotective effects, with reductions in renal inflammation and fibrosis [21, 217]. Ebselen has been shown to cross into the mitochondrial matrix without the need for a leader sequence. It is activated by the intramitochondrial glutathione and thioredoxin systems, making this an extremely attractive therapy for diabetic nephropathy [218]. In summary, these results support the hypothesis that mitochondrially targeted therapies may be beneficial in the treatment of diabetic nephropathy.

An alternative approach has been the development of mitochondrially targeted Szeto-Schiller (SS) tetrapeptides that bind to cardiolipin on the IMM. The SS tetrapeptide/cardiolipin complex protects cardiolipin from peroxidative damage by cytochrome *c*, thus protecting mitochondrial crista and preserving mitochondrial structure and function. Use of the SS-31 peptide, which is known to scavenge ROS and inhibit mPTP opening, protects mitochondrial structure and function. In a rat model of ischemic kidney injury, it accelerated recovery of ATP, reduced apoptosis and necrosis of tubular cells, and abrogated tubular dysfunction [219]. Furthermore, SS-31 peptide decreased ischemia/reperfusion-mediated oxidative stress and the inflammatory response in tubular cells. The SS peptide known as Bendavia (Stealth Peptides, Newton Centre, Massachusetts) has advanced into the clinic as a phase 2a randomized controlled trial (EMBRACE STEMI) to evaluate its safety, tolerability, and efficacy on reperfusion injury in patients after myocardial infarction [220]. A second clinical trial will evaluate the efficacy of Bendavia on renal parameters (estimated glomerular filtration rate (eGFR), renal volume and perfusion, inflammation and urinary osmolarity). This is a promising strategy for the restoration of mito-

chondrial injury, but needs first to be tested pre-clinically in models of diabetic nephropathy.

The strategy of specifically targeting the mPTP has seen the development of mPTP-opening inhibitors such as cyclosporin A (CsA) or sangliferin A [221]. CsA is a well-known immunosuppressant and inhibitor of mPTP; it acts via inhibition of peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of cyclophilin D [222, 223]. Clinically, CsA has already shown benefits in reducing human cardiac ischemia/reperfusion injury [224]. However, CsA is nephrotoxic [225], and interacts with the calcineurin pathway, which is activated in diabetic nephropathy [208]. These adverse properties limit its application in the treatment of diabetic nephropathy. Analogues of CsA have been generated, including Debio-025 (Alisporivir). Debio-025 is non-immunosuppressive [226], and unlike CsA, it does not display affinity for calcineurin [227], but it selectively inhibits cyclophilin D, prevents cell death [228, 229], and restores mitochondrial function [230]. To date, Debio-025 has not been tested in experimental models of diabetes.

MitoTempo is a mitochondrially-targeted SOD1. In response to excessive ROS production and calcium overload, it prevents mPTP opening in cultured proximal tubular epithelial cells [231]. Moreover, a recent study targeting glycogen synthase kinase (GSK) 3 β through the use of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), a highly selective small-molecule inhibitor of GSK3 β , has shown promise in protecting podocytes against mPTP opening, ROS production, and apoptosis induced by adriamycin [232].

Other treatments have improved diabetic nephropathy end-points by increasing antioxidant capacity through upregulation of antioxidant response genes using Nrf2 activators such as bardoxolone methyl [233, 234], curcumin [235], sulforaphane [236], cinnamic aldehyde [236], resveratrol [237], and ebselen [21]. Recent studies have shown that metformin, an oral hypoglycemic drug, possesses antioxidant properties, and is effective in lowering end-points of diabetic nephropathy in a rat model [238]. Other studies have investigated the targeting of mitophagy pathways in the hope of restricting the accumulation of fragmented mitochondria in the renal cortex, a process shown to be linked with the development of diabetic nephropathy [34]. Furthermore, some treatments, not necessarily targeted at mitochondria, have included mangiferin, a natural C-glucosyl xanthone and polyhydroxy polyphenol compound that has shown protection against diabetic nephropathy in STZ-induced diabetic Wistar rats via different mecha-

nisms, including inhibition of the PKC, MAPKs (p38, JNK and ERK1/2), NF- κ B, and TGF- β 1 pathways [239].

Finally, the specific targeting of the main enzymatic source of ROS/NS in the kidney, NOX4, with specific NOX1/NOX4 inhibitors has shown tremendous potential for the treatment of diabetic nephropathy [240], as discussed earlier in section 3. It is unknown whether these inhibitors enter the mitochondria *per se*, but recent evidence suggests that they mediate their inhibitory effect on NOX4, and thus they impact diabetic nephropathy via pathways that include enzymes located within the mitochondria [56]. The existence of disparate NOX inhibitors such as the pan-NOX inhibitors, VAS2870 and VAS3947 [241], await further pre-clinical analysis with respect to their effectiveness in preventing or reversing diabetic nephropathy. The more specific NOX1/NOX4 inhibitor, GKT137831, which is developed by Genkyotex, has completed phase 1 clinical trials with encouraging results regarding safety and tolerability. A phase 2 clinical study is now planned in diabetic nephropathy patients [242].

8. Concluding remarks and future perspective

Diabetic nephropathy is a leading cause of ESRD. Despite significant advances in the understanding of the cellular mechanisms that are responsible for its initiation and progression, it remains therapeutically elusive. It is clear that blood glucose and blood pressure control are insufficient to prevent its progression, and that new, more effective treatment options are urgently needed. The therapeutic targeting of diabetic kidney mitochondria is a relatively new area that has gained attention due to the involvement of mitochondria in cell death pathways such as apoptosis and necrosis implicated in key cell types such as podocytes.

The involvement of oxidative and nitrosative stress in mediating many of the observed changes has focused attention on antioxidants that specifically target mitochondria. Several approaches have been attempted, as outlined in this review, with a more nuanced approach required to restore redox homeostasis rather than depletion of important oxidants required for signaling. The interwoven involvement of ER stress with oxidative stress means that new targets may be found by closer examination of ER stress-driven pathways.

Preventing the consequences of increased ROS/NS on nuclear and mitochondrial DNA damage via enhanced surveillance by DNA repair

mechanisms may offer an alternative solution. Preventing mPT pore formation is another new area with the potential to regulate renal injury. It is only by careful analysis of the cellular mechanisms governing diabetic renal injury that novel targets will be recognized and verified preclinically prior to translation into the clinic. These ap-

proaches promise to pave the way for new, more effective treatment options for diabetic nephropathy.

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