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DIABETES AND APOPTOSIS

The role of reactive oxygen species in apoptosis of the diabetic kidney

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Abstract Increased levels of reactive oxygen species (ROS) by hyperglycemia can induce apoptosis of renal cells and diabetic nephropathy. The redox balance in the renal cell seems, therefore, of the utmost importance. ROS-mediated apoptosis may be further aggravated by an inadequate cytoprotective response against ROS. When there are insufficient cytoprotective and ROS scavenging molecules, ROS lead to considerable cellular damage and to a point of no return in apoptosis. Induction of cytoprotective proteins may prevent or attenuate apoptosis, renal cell injury, and finally diabetic nephropathy. Here, we discuss some mechanisms of apoptosis and several strategies that have been probed to ameliorate, or to prevent apoptosis in the diabetic kidney.

Keywords Diabetes · Apoptosis · Kidney · Diabetic nephropathy · Cytoprotective proteins · Anti-oxidants

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Abbreviations

AGE	Advanced glycation end products
Apaf-1	Apoptotic protease activating factor-1
Bcl2	B-cell lymphoma 2
CO	Carbon monoxide
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EPCR	Endothelial PC-receptor
FADD	Fas-associated death domain
GBM	Glomerular basement membrane
GSH	Glutathione
HO	Heme oxygenase
MAC	Mitochondrial apoptosis-induced channel
MAPK	Mitogen-activated protein kinases
MOMP	Mitochondrial outer membrane permeabilization
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
PAR-2	Protease-activated receptor-1
PARP	Poly (ADP-ribose) polymerase
PC	Protein C
PTPC	Permeability transition pore complex
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
Smac	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
STZ	Streptozotocin
TNFα	Tumor necrosis factor-α
TRADD	TNF-receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type MMTV integration site family

Introduction

Diabetes mellitus forms an enormous and still increasing health burden worldwide [1]. Mortality and morbidity are caused in particular by the occurrence of complications [2]. One of the microvascular complications is diabetic nephropathy in both T1DM and T2DM, which is the leading cause of adult renal failure at the moment [3]. A key role in the pathogenesis of diabetic nephropathy has been attributed to hyperglycemia as it leads to the formation of advanced glycation end products (AGEs) and the mitochondrial production of reactive oxygen species (ROS), and, subsequently, to cell death and kidney dysfunction [4]. In this review, we will focus on the pathogenic role of oxidative stress and apoptosis in diabetic nephropathy. Potential strategies to prevent or treat the renal damage inflicted by ROS-induced apoptosis are discussed.

Diabetic nephropathy

Diabetic nephropathy can be characterized by both glomerular and tubulointerstitial injury, each, however, to a hugely variable extent [5]. An important histological hallmark of diabetic nephropathy is proliferation of mesangial cells, and, as a result, the expansion of extracellular matrix (ECM) in the mesangium [6]. Thickening of the glomerular basement membrane (GBM) is another feature of diabetic nephropathy [7]. One of the clinical manifestations and main predictor of diabetic nephropathy is emerging microalbuminuria in the early phase of the disease that further develops to massive macroalbuminuria during overt diabetic nephropathy and end stage renal disease. Damage to the capillary filter, existing of podocytes, GBM and glomerular endothelium, may be responsible for the development of albuminuria. We have recently shown that heparanase-mediated loss of heparan sulfate in the GBM is a possible key mediator in the development of proteinuria and overt diabetic nephropathy. However, we could also observe changes in tubular heparan sulfate [8–12]. Notably, an aberrant expression of heparanase and heparan sulfate in certain cancer cells has been implicated to play a role in apoptosis [13, 14]. However, the possible role of renal heparanase expression in apoptosis during the development of diabetic nephropathy remains to be addressed. Historically, mesangial cells have been attributed a central role in glomerular-associated diabetic nephropathy. However, also other glomerular cell types, like endothelial cells, and podocytes have been implicated in the genesis of diabetic nephropathy, while inflammatory cells such as macrophages can further exacerbate the oxidative stress and inflammatory processes leading to apoptosis.

Alternatively, renal deterioration may also be related to chronic hypoxia, that can for example occur following vasoconstriction by decreased levels of nitric oxide (NO) or increased levels of angiotensin II [15]. The occurrence of interstitial fibrosis may additionally deteriorate the diffusion of oxygen to the renal cells. During hypoxia ROS formation can occur via increased electron flow at complex III of the mitochondrial electron transport chain and via NADPH oxidase [16, 17]. Experimental research has shown that hyperglycemia interferes with the beneficial VEGF response to hypoxia in proximal tubular cells [18]. As a result, these and numerous other factors may cause hypoxia, the formation of ROS, cellular injury and finally apoptosis of renal cells. In summary, both the glomerular and the tubular compartment may be involved in the initiation of diabetic nephropathy.

Redox balance in the kidney

Within renal cells a balance between pro- and anti-oxidants is kept under physiological conditions. Although ROS have important functions in normal cellular signaling and regulation, in excess, they may result in oxidative damage to proteins, lipids and DNA, ultimately leading to apoptosis and renal injury [19]. ROS can activate several pro-inflammatory transcription factors, resulting in the production of cytokines and chemokines and vascular adhesion molecules, and, subsequently, the influx of inflammatory cells into the kidney. This formation of renal inflammation initiated by ROS further prolongs and exacerbates ROS-mediated cell injury, apoptosis and kidney dysfunction. To keep the redox balance under control a complex network of pro- and anti-oxidant enzymes exists. However, it can be envisioned that in case the cytoprotective anti-oxidant factors, as exemplified in Fig. 1, get overwhelmed as may occur following hyperglycemia, the balance gets skewed towards oxidative stress, and may lead to apoptosis and kidney injury.

Mitochondria-derived ROS constitute the main source of intracellular ROS that are responsible for apoptosis and diabetic nephropathy [20, 21]. As described above, both the glomeruli and the tubulointerstitial compartments can be subjected to the hyperglycemia-induced increase in mitochondrial ROS production. During periods of hyperglycemia, renal cells fail to attenuate the entrance of glucose into the cell, resulting in such increased substrate levels for the mitochondrial electron transport chain that this leads to an overflow of the respiratory chain. Normally, a small amount of ROS are formed as a result of electron carriers reacting directly with oxygen, but during this overflow backed up electrons result in even more reactive electron carriers and thus enhanced production of ROS. It has been demonstrated that high glucose levels result in increased

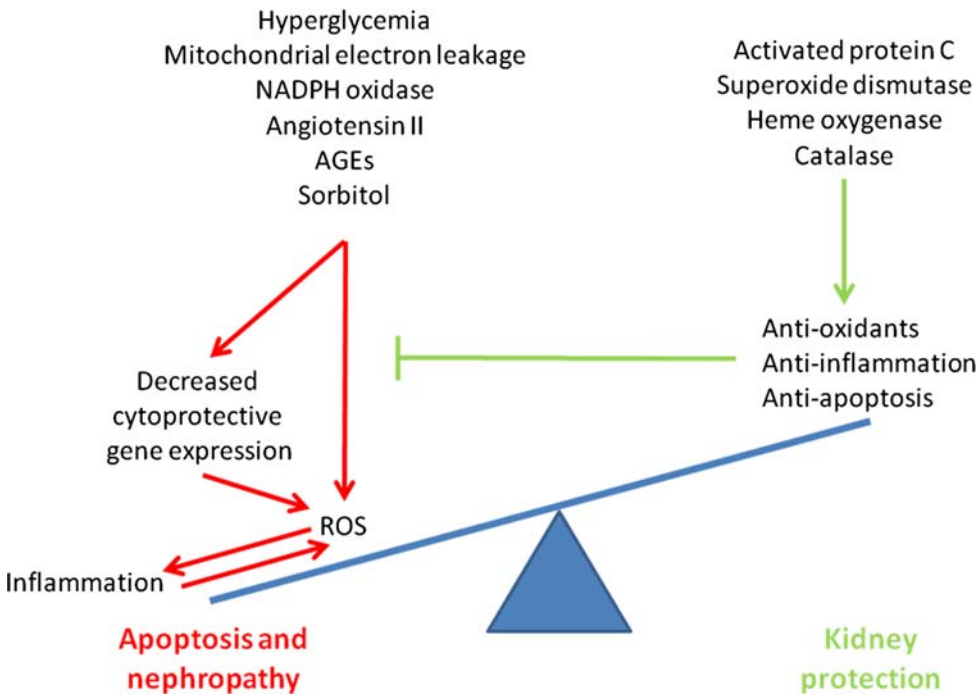

Fig. 1 Excess ROS leads to apoptosis and diabetic nephropathy. Model demonstrating the different causes of ROS formation that result in the induction of inflammation by pro-inflammatory genes, leukocyte infiltration, and aggravation of ROS formation. Ultimately, this may lead to apoptosis and kidney dysfunction. We postulate that in humans induction of cytoprotective proteins, such as HO-1, SOD or catalase, will restore this ROS-mediated skewing of the redox balance by generating products that protect against oxidative stress, and/or inflammation and apoptosis, or a combination

levels of ROS, AGEs and sorbitol, and higher activity of protein kinase C that have been demonstrated to contribute to cellular injury and apoptosis [22].

Other strong contributors to ROS formation in the diabetic kidney are the superoxide producing enzyme NADPH oxidase (NOX), and xanthine oxidase, which catalyzes oxidation of hypoxanthine (the occurrence of different NOX isoforms in different cells of the diabetic kidney is reviewed in [4]). Nitric oxide synthase produces the free radical nitric oxide, which, under normal conditions acts protective, but under oxidative circumstances, can be converted into other reactive nitrogen species (RNS), including peroxynitrite [23].

On the other hand, there are intrinsic renal cytoprotective anti-oxidant factors and protein responses that keep the ROS and RNS in balance. The cytoprotective anti-oxidant proteins include for example superoxide dismutases (SOD) that convert superoxide to hydrogen peroxide, which, in turn, is rendered inactive by catalase and glutathione peroxidase. The heme oxygenase (HO) system is another crucial anti-oxidant system in the kidney [24, 25]. HO degrades the pro-oxidant heme into iron, carbon monoxide and biliverdin [23]. Biliverdin is than directly converted into the strong anti-oxidant bilirubin by biliverdin reductase. HO-derived iron can, like heme, catalyze the Fenton reaction resulting in the production of reactive hydroxyl groups, but is scavenged by co-induced ferritin [23]. The strongly inducible HO-1 isoform has been shown to protect against oxidative stress, inflammation and apoptosis in the kidney [23, 26–28], and also the constitutively expressed HO-2 isoform is getting recognized for its protection against oxidative stress and inflammatory processes [29]. In addition to an increased formation of ROS, a decreased anti-oxidant capacity results in skewing of the redox balance. It is likely that in diabetes patients a combination of increased ROS formation and diminished anti-oxidant defense results in apoptosis of renal cells. This is exemplified by the findings of Quan et al., who demonstrated that hyperglycemia decreases HO-activity in the aortic endothelium and increases the production of ROS [30], suggesting that patients with hyperglycemia are deprived from the protective effects of the HO-effector molecules CO, bilirubin and ferritin. Moreover, hyperglycemia was shown to decrease the protective effect of the HO-system in the vascular system of animal models [30, 31], resulting in an impaired defense against oxidative stress and inflammation [30, 32]. In addition, it has been shown that HO-1 and the cytoprotective heat shock protein 72 expression is decreased in skeletal muscles of patients with type 2 diabetes (T2DM) [33]. In contrast, Hayashi demonstrated that during streptozotocin (STZ)-induced diabetes in rats, which is an established model for mainly T1DM, HO-1 levels were up-regulated in glomeruli, whereas the expression of other cytoprotective enzymes such as catalase, GSH peroxidase and Cu-, Zn- superoxide dismutase were unchanged compared to control rats [34, 35]. Several studies have shown a protective role of HO-1 against oxidants in the kidney, as reviewed in [36, 37]. In summary, when hyperglycemia leads to the sudden production of ROS, and the anti-oxidant capacity is not sufficient, induction of apoptosis leads to renal injury and diabetic nephropathy.

Mechanisms of apoptosis

The apoptotic process is regulated by certain proteins, the so-called cysteine-dependent specific aspartate proteases or caspases. Caspases have a role in the induction of apoptosis as well as in the execution phase of apoptosis, and play a central role in diabetic nephropathy. Caspase-dependent apoptosis can be induced by either the extrinsic or the intrinsic pathway.

The extrinsic pathway involves a death-inducing ligand such as for example Fas ligand (FasL) or TNFα interacting with their corresponding death receptor on the plasma membrane, for this example Fas receptor and TNF receptor, respectively. After binding of these ligands to the death receptors Fas-associated death domain (FADD) or TNF-receptor-associated death domain (TRADD), caspase-8 is recruited and activated to form a death-inducing signaling complex (DISC). DISC can propagate the death signal directly by activating the effector caspases-3, -6 and -7 [38, 39], or indirectly via cleavage of Bid, which upon translocation to the mitochondria leads to mitochondrial outer membrane permeabilization (MOMP) and subsequent release of pro-apoptotic proteins, such as cytochrome *c* [40]. Cytochrome *c* is part of the caspase-dependent intrinsic pathway discussed below and can interact with apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 to form an apoptosome. This complex is able to activate caspase 9, which in turn activates the effector caspases 3, 6 and 7, leading to apoptosis [41].

The second apoptotic pathway is the intrinsic pathway, in which the mitochondrion is the central regulator [42]. The point-of-no-return for the intrinsic pathway is MOMP, which is controlled by the family of Bcl-2 proteins [43]. Bcl-2 proteins are either anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-w) or pro-apoptotic (Bax, Bak, Bad, Bim) and the balance between these two groups ultimately determines cell survival or cell death. Especially the pro-apoptotic family member Bax seems important since it can oligomerize or form complexes with other proteins to form channels such as the permeability transition pore complex (PTPC) and mitochondrial apoptosis-induced channel (MAC) in the mitochondrial outer membrane, which can facilitate the release of pro-apoptotic molecules such as cytochrome *c* [44–46].

Diabetes and apoptosis in the kidney

As outlined above, both acute and chronic hyperglycemia leads to oxidative stress and this is the major trigger for tubular and glomerular cells to go into apoptosis as determined in animal models and in vitro cell culture systems [20, 22, 47]. High glucose levels cause ROS-dependent apoptosis of mesangial cells via Bax-mediated mitochondrial permeability and subsequent cytochrome *c* release [48]. In murine and human renal mesangial cells high glucose levels caused an increased Bax/Bcl-2 ratio, associated with cytochrome *c* release from mitochondria and subsequent the pro-apoptotic caspase-3 activation [49]. Enhanced levels of glucose generated NADPH oxidase-mediated and mitochondrial-mediated ROS formation that activated pro-apoptotic p38 MAPK and caspase-3 activation in podocytes in vitro, whereas inhibition of NADPH oxidase-induced ROS formation prevented podocyte apoptosis in vivo [50]. Inhibition of p38 MAPK inhibited the diabetes-mediated decrease in the anti-apoptotic Bcl-2 expression and activation of caspase-3 in mesangial cells [51].

In renal tubular epithelial cells high glucose-mediated oxidative stress induced an increased Bax protein expression, which was accompanied by a reduced Bcl-2 expression [52]. These data are in line with earlier findings that during diabetes gene expression of pro-apoptotic Bax was increased, whereas anti-apoptotic Bcl-2 and Bcl-X_L expression was down-regulated [53]. Different caspases, in particular caspase-3 and -9 play a crucial role in high glucose-induced apoptosis of proximal tubular epithelial cells [54].

In a gene profiling study of the tubulointerstitium from T1DM and T2DM nephropathy biopsy specimens it was shown that two death receptors (osteoprotegerin and Fas), and the death ligand TRAIL were strongly upregulated [55]. Glomerular and proximal tubular expression of TRAIL was confirmed by immunohistochemistry and was shown to be higher in diabetic kidneys than controls.

It was further found that protein C (PC) modulates mitochondrial apoptosis in diabetic nephropathy [56]. In glomerular endothelial cells, a complex of thrombomodulin and thrombin activates PC, that subsequently activates the protease-activated receptor-1 (PAR-1) in the proximity of the endothelial PC-receptor (EPCR), resulting in cytoprotective signaling. Hyperglycemia interferes with thrombomodulin-dependent PC activation, resulting in less protection and even promotion of release of apoptosis-inducing factors like cytochrome *c* and Smac/Diablo leading to apoptosis of endothelial cells and podocytes [19, 56].

In summary, data on renal apoptosis in diabetic nephropathy are scarce and scattered, but both the intrinsic and extrinsic apoptotic pathway seem to be involved.

Anti-oxidants and cytoprotective proteins in the prevention of ROS-induced apoptosis of renal cells and diabetic nephropathy

Several studies have targeted oxidative and nitrosative stress, and thus apoptosis of renal cells caused by

hyperglycemia, through administration of anti-oxidants or overexpression of cytoprotective proteins.

Anti-oxidant administration to animals has demonstrated protective effects against the development of diabetic nephropathy. In addition to endogenous anti-oxidant compounds like glutathione, thioredoxin, and biliverdin/bilirubin also dietary anti-oxidants, such as vitamin C and E and beta-carotene may restore the redox balance. The number of apoptotic proximal tubular epithelial cells, proteinuria, glomerular and tubulointerstitial sclerosis, and renal malondialdehyde, as index of oxidative stress, were significantly decreased after anti-oxidant treatment with vitamin C to T2DM rats when compared with untreated T2DM rats [57]. Like vitamin C, vitamin E normalized diabetes-induced renal dysfunction such as glomerular volume and TGF-beta production in STZ-induced diabetic rats [58]. High glucose-induced ROS formation and mesangial and proximal tubular epithelial cell apoptosis could be inhibited in in vitro models, using the anti-oxidant taurine [49, 52].

Unfortunately, translational studies to the administration of solely anti-oxidants did not yet result in potent protection against apoptosis in human diabetic nephropathy as reviewed in [4]. Possibly induction of endogenous cytoprotective proteins (see Table 1) may be more efficiently translated to the clinical situation than administered anti-oxidants. Endogenous cytoprotective proteins may be targeted to the kidney or generated in the kidney, and, therefore, function more efficiently than exogenous added anti-oxidants, since they act within the cell or cellular compartment where ROS are actually generated. Recently, it was shown that ROS production was reduced in a T1DM and T2DM mouse models with transgenic overexpression of the anti-oxidant enzyme catalase in proximal tubular cells [59, 60]. This resulted in reduced pro-apoptotic Bax and active caspase-3 levels. Overexpression of catalase in the proximal tubular cells protected diabetic mice from increased blood pressure, albuminuria, glomerulosclerosis, interstitial fibrosis and tubular apoptosis. These findings are in line with the observations that catalase deficiency in mice renders kidneys more prone to oxidative stress [61], while humans deficient in catalase are predisposed to cumulative oxidant damage leading to T1DM and T2DM [62].

Pharmacological induction or gene transfer of HO-1 in diabetic rats reduced ROS formation and caused an increase in anti-oxidants biliverdin/bilirubin, which resulted in an improvement of vascular and renal function [63]. Induction of HO-1 led to increased levels of the anti-apoptotic phosphorylated AKT (pAkt) and BcL-XL. Additionally, HO-2 deficiency enhanced STZ-induced diabetic nephropathy, possibly via increased levels of superoxide anions [64]. Pharmacological up-regulation of HO-1 by heme or cobalt protoporphyrin in HO-2-deficient T1DM mice resulted in reduced superoxide anions and improved diabetic kidney morphology and function [64]. Selective overexpression of the cytoprotective enzyme HO-1 in the endothelial cell line HMEC-1 was shown to prevent hyperglycemia-mediated O_2^- formation and thus blocked ROS-induced DNA damage and caspase activation [25]. This study indicates that endothelial cell survival after oxidative injury may be enhanced by increased HO-1 expression, thus blocking inflammation and apoptosis.

In isolated lymphocytes of patients affected by T2DM complicated with nephropathy evidence was found of systemic oxidative stress, and the observed induction of HO-1 and thioredoxin reductase-1 was thought to reflect a response in counteracting the intracellular pro-oxidant status [65].

In mice overexpressing SOD, diabetes-induced renal injury was also abrogated, most likely due to reduced

Table 1 Targeted cytoprotective genes and effects on renal cell apoptosis and nephropathy

Targeted genes in diabetic animal models	Effects on diabetic renal cell apoptosis and nephropathy
Transgenic catalase mice	Reduced albuminuria, glomerulosclerosis, interstitial fibrosis and tubular apoptosis [59, 60]
Catalase-deficient mice	Kidneys are more prone to oxidative stress [61]
HO-1 gene transfer in mice or rats	Reduced apoptosis and kidney injury [63]
HO-2-deficient mice	Enhanced STZ-induced diabetic nephropathy, which could be restored by HO-1 gene transfer [64]
SOD transgenic mice	Protection against diabetic nephropathy [68]
Thioredoxin-1 transgenic mice	Diminished albuminuria and tubular apoptosis [67]
Peroxiredoxin-3 transgenic mice	Protection against hyperglycemia and glucose intolerance and reduced fibroblast apoptosis by reducing mitochondrial H_2O_2 production [69]
Glutathione peroxidase-1-deficient mice	No effects on T1DM-induced kidney injury [70]
Mice with a reduction of thrombo-modulin-dependent protein C activation	Aggravated glomerular apoptosis and diabetic nephropathy [19, 56]
Mice expressing a hyper-activatable protein C mutation	Protein C activation prevents glomerular apoptosis and diabetic nephropathy [19, 56]

superoxide–NO interactions [66–68]. SOD overexpression increased anti-apoptotic glomerular Wnt5a/beta-catenin signaling and abrogated diabetes-induced caspase-3 cleavage, DNA damage and subsequent mesangial apoptosis[68]. Overexpression of manganese (Mn)SOD that exclusively is expressed in mitochondria appeared strongly protective against glucose-induced ROS, AGEs and sorbitol formation in endothelial cells [22], corroborating the prominent role of mitochondria in ROS-induced renal cell apoptosis.

Transgenic mice overexpressing thioredoxin-1 were protected against diabetic nephropathy as shown by reduced albuminuria and tubular apoptosis [67]. Furthermore, markers of systemic and renal oxidative stress were attenuated in this model, again indicating a significant role for oxidative stress in the development of diabetic nephropathy. Transgenic mice overexpressing peroxiredoxin-3, a mitochondria-specific thioredoxin peroxidase, caused a reduction of mitochondrial-produced H_2O_2 and an increased resistance to stress-induced fibroblast cell death and apoptosis [69], whereas, surprisingly, glutathione peroxidase-1-deficient animals showed similar levels of diabetes-induced kidney injury as wild type controls [70]. Inhibition of mitochondrial NADPH-dependent isocitrate dehydrogenase activity led to an increase of apoptosis mediated by active caspase-3, PARP and a decreased Bcl-2 expression, whereas overexpression of the enzyme resulted in decreased apoptosis of HEK293 cells associated with a decreased expression in PARP and Bax [71]. Also in other studies it was shown that reduction of oxidative stress by anti-oxidant enzyme activity led to improved renal function after hyperglycemic injury because of a reduction in tubular cell apoptosis [72, 73].

Activated PC mediates protection against mitochondrial apoptosis in the glomerulus, while high glucose levels abrogate activation of PC and its down-stream protective pathways. Interestingly, it has recently been found that increasing activated PC during hyperglycemia avoids oxidative stress and restores activated PC-mediated cytoprotection, and prevents mitochondria-dependent apoptosis of endothelial cells and podocytes, and diabetic nephropathy in T1DM [19, 56].

In summary, anti-oxidants and cytoprotective proteins can counteract ROS-induced apoptosis of renal cells and the development of diabetic nephropathy (see also Table 1, Fig. 1).

Concluding remarks

In the diabetic kidney ROS are produced via different pathways. When cytoprotective and anti-oxidant capacity is insufficient, ROS will induce apoptosis, which may finally result in diabetic nephropathy. Therapeutic options may include restoration of the redox balance by anti-oxidant compounds such as vitamin C and E. Unfortunately, routine anti-oxidants have demonstrated minimal renoprotection in humans with diabetic nephropathy, despite many positive observations in animal models [4]. However, for the treatment or prevention of diabetic nephropathy, the induction of cytoprotective proteins with anti-oxidant activities, such as PC, catalase, SOD and HO-1, constitute a valid alternative strategy. In fact, manipulating cytoprotective proteins and their effector molecules seem to reduce apoptosis in the diabetic kidney, and may therefore be beneficial, as demonstrated in animal models for diabetic nephropathy. These observations warrant further translational approaches addressing cytoprotective proteins in humans with diabetic nephropathy.

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