Diabetes-Induced Alterations in Renal Medullary Microcirculation and Metabolism

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Abstract: Diabetes-induced renal complications, i.e. diabetes nephropathy, are a major cause of morbidity and mortality. The exact mechanisms mediating the negative influence of hyperglycemia on renal function are unclear, although several hypotheses have been postulated. Cellular mechanisms include glucose-induced excessive formation of reactive oxygen species, increased glucose flux through polyol pathway and pentose phosphate shunt, formation of advanced glycation end-products and activation of protein kinase C and NADPH oxidase. However, the renal effects *in vivo* of each and every one of these mechanisms are less clear, although recent studies have shown several major alterations predominantly in the renal medulla as a result of sustained hyperglycemia.

Already during normal conditions, the renal medulla has a remarkably low oxygen tension (PO_2) and a high degree of non-oxygen dependent energy metabolism. Alterations in either blood perfusion or oxygen delivery to the medullary region will have significant effects on both regional metabolism and total kidney function. Recently, sustained hyperglycemia has been shown to induce a pronounced reduction in preferentially renal medullary PO_2.

This review will present the current knowledge of diabetes-induced alterations in renal medullary metabolism and function, but also discuss future targets for prevention of diabetic nephropathy.

Keywords: Diabetes, Renal medulla, Oxygen metabolism, Oxygen tension, Microcirculation, Renal function, Reactive oxygen species.

INTRODUCTION

In land mammals, a pivotal role of the kidneys is to concentrate urine in order to maintain water and electrolyte homeostasis and allow survival in a dry environment. Thus, the blood flow to the renal medulla is derived through the *vasa recta*, acting as a counter current system in order to maintain the high osmotic gradient necessary for formation of concentrated urine. Other pivotal functions of the kidneys are long-term regulation of arterial blood pressure and excretion of metabolic waste products and water soluble toxins. Numerous hormones necessary for maintaining control of the internal milieu are produced within the different renal structures. The kidney also produces significant amounts of arginine, alanine and serine, and during certain conditions even glucose [1].

RENAL MEDULLARY METABOLISM, FUNCTION AND OXYGEN DELIVERY

Even though the kidney only is about 0.5% of total body mass, it accounts for approximately 10% of total oxygen consumption. The major consumer of energy within the renal structure is the basolaterally located Na^+/K^+-ATPase in proximal tubular cells within renal cortex. About 80% of the oxygen consumed in mammalian kidneys is attributed to active transport of electrolytes by tubular cells [2], while only 3-18% of the total oxygen consumption can be attributed to basal metabolism [1].

The metabolism within different parts of the kidney is highly heterogeneous, and is likely to reflect local energy demand and milieu of that specific region. The metabolism in renal cortex has been found to be highly dependent on oxygen availability, i.e. aerobic metabolism. Glucose oxidation in renal cortex is relatively low compared to that of renal medulla, indicating a high glycolytic rate in the renal medulla. High medullary glycolytic rate, together with relatively low oxygen consumption, indicates presence of anaerobic metabolism in renal medulla [1]. Basically, cells in the medulla work under hypoxic conditions even in normal physiology. The energy metabolism is heterogenous also within renal medulla, with higher glucose oxidation and higher oxygen consumption in the outer part of medulla, while the deeper situated inner medulla is highly dependent on anaerobic metabolism, i.e. almost absent oxygen consumption, but high glycolytic rate [3].

Renal blood flow equals approximately 25% of total cardiac output. This perfusion, however, is mainly directed towards renal cortex, with only about 10% of total renal blood flow perfusing the deeper situated medullary structures. Renal medullary blood flow is low in order to ensure an osmotic gradient, thereby optimizing urinary concentration capacity [4]. Blood flow to renal medulla is derived through *vasa recta*. The close vicinity of ascending and descending *vasa recta* results in a counter current system necessary for maintain a high osmotic gradient used when concentrating the urine. The counter current system allows

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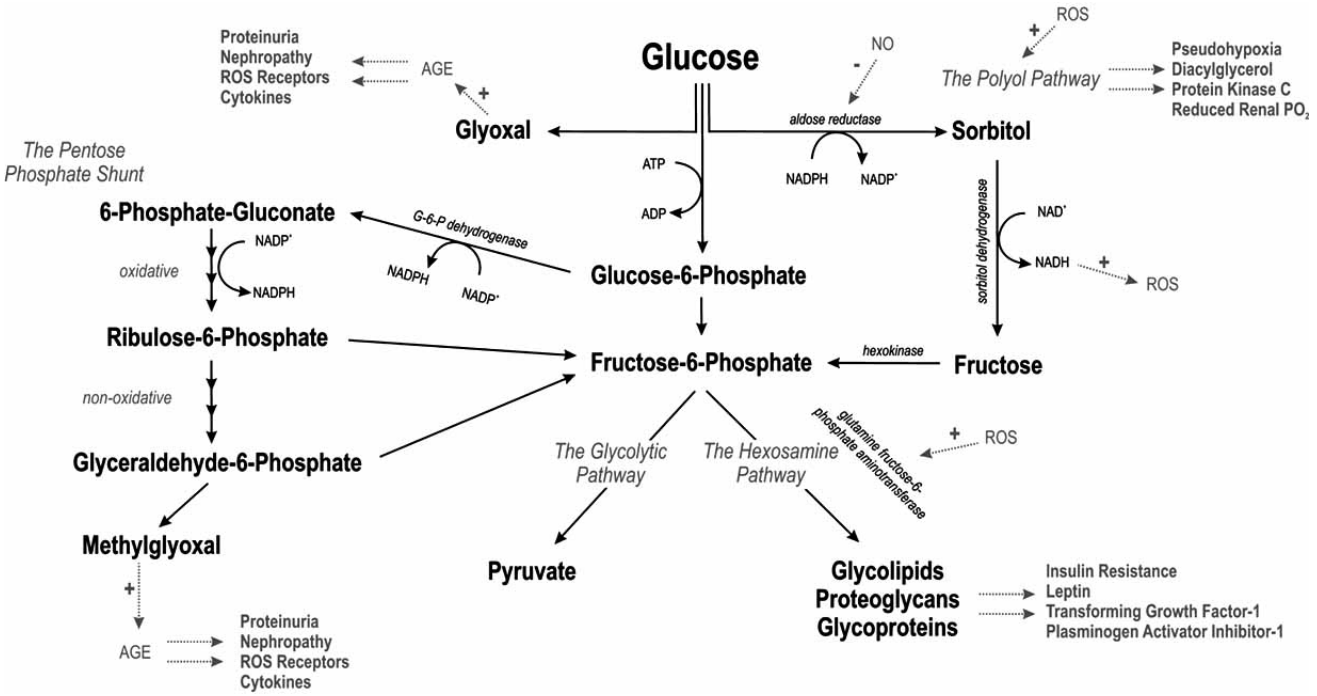

Fig. (1). Hyperglycemia-induced alterations in biochemical cellular pathways believed to contribute to the development of diabetic nephropathy. See text for further details. ROS - reactive oxygen species, NO - nitric oxide, AGE - advanced glycation end-products.

electrolytes to be re-circulated from ascending to the closely located descending *vasa recta*. However, oxygen is shunted in opposite direction, resulting in a low delivery of oxygen to the medullary region [5, 6]. In fact, hypoxia occurs in renal medulla as an obligatory part of kidney function even under normal conditions [7]. Conditions which alter shunting of oxygen, with concomitant alterations in medullary oxygen delivery, have the potential to influence medullary oxygen tension (PO_2) [8]. Insufficient oxygen delivery to renal medulla will lead to anoxic conditions, resulting in tubular damage and reduced kidney function.

Several studies have targeted various defense mechanisms developed by renal medulla to prevent ischemic and hypoxic injury [9, 10]. Long-term hyperglycemia, e.g. diabetes mellitus, is known to significantly increase the risk of developing progressive renal dysfunction [11]. The exact mechanism accounting for the increased risk is presently unknown, but it has been suggested that aggravated low PO_2 in renal medulla may cause progression of nephropathy during several pathological conditions [12].

HYPERGLYCEMIA-ACTIVATED CELLULAR PATHWAYS AND ALTERATIONS IN MEDULLARY METABOLISM

Numerous cellular pathways are activated by increased glucose levels. The pathways believed to be most involved in the development of diabetic nephropathy are summarized in Fig. (**1**).

Polyol Pathway Activation

When Gabbay published his results in Science in 1966, polyol pathway was the first pathobiological phenomenon to be discovered in basic diabetes research [13]. Aldose reductase is located in the cytosol [14]. Within renal tissue, aldose reductase has predominantly been found in medulla, while there seems to be little enzymatic activity in cortex [15]. Interestingly, nitric oxide (NO) has been found to possess inhibitory effects on aldose reductase due to modification of a cystein residue in the active region of the enzyme [16].

Increased intracellular glucose concentrations have been shown to increase flux through polyol pathway *in vivo*, preferentially in tissues with insulin-independent glucose uptake. During normal conditions, the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme aldose reductase inactivates toxic aldehydes by reducing them to alcohols. However, during conditions with high glucose concentrations aldose reductase reduces glucose to sorbitol, concomitantly consuming NADPH [17]. Sorbitol is further oxidized to fructose by sorbitol dehydrogenase, reducing NAD^+ to NADH. Activation of this pathway has been shown in both clinical and experimental studies during manifest diabetes [18, 19].

Aldose reductase has a very low affinity for glucose (K_m = 70 mmol/l), resulting in a glucose metabolism via polyol pathway accounting for a very minute part of total glucose usage during normal conditions. However, elevated intracellular glucose concentration will result in increased flux through polyol pathway. Diabetes-induced activation of polyol pathway has been demonstrated in numerous tissues [20-22], including renal medulla [23]. Increased activity through the polyol pathway alters the cellular redox-state, mainly due to increased NADH/NAD^+ ratio [24]. This will concomitantly shift the equilibrium between pyruvate and

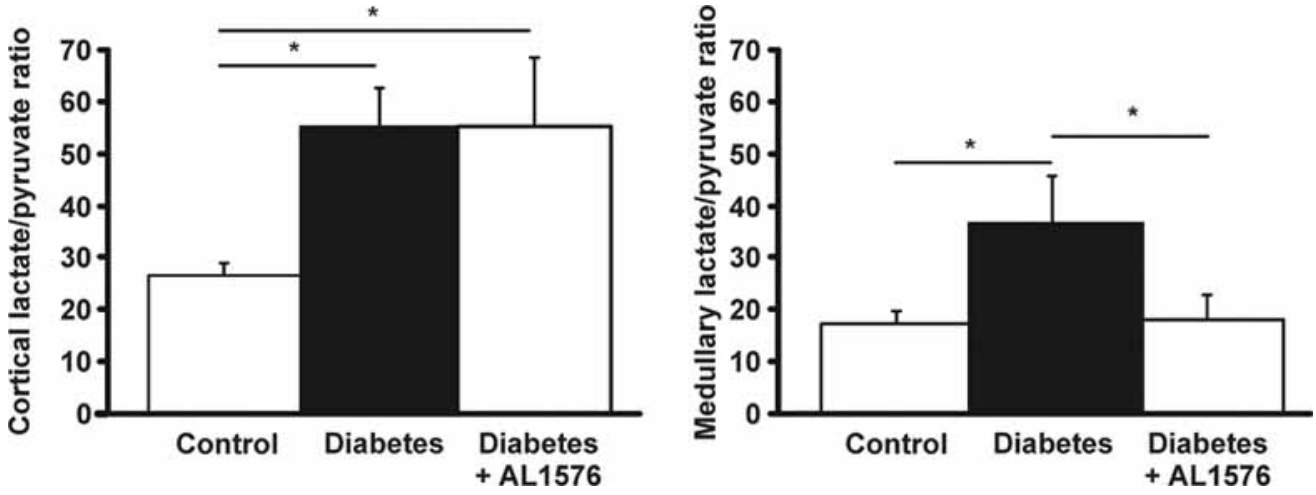

Fig. (2). Lactate/pyruvate ratios in renal cortex and medulla of normoglycemic control and streptozotocin-induced diabetic animals with and without treatment with the specific aldose reductase inhibitor AL-1576. Modified from [23].

lactate, resulting in an increased lactate/pyruvate ratio, predominately as a result of increased lactate concentration [18]. Long-term diabetes has been shown to increase lactate/pyruvate ratios in both renal cortex and medulla, but inhibition of polyol pathway only prevents increased lactate/pyruvate ratio in the medullary region [23] Fig. (**2**). This finding is consistent with the almost exclusive presence of aldose reductase in the medullary region [25].

It is important to note that increased formation of lactate is not a result of hypoxia, since tissue levels of purine-base metabolites (adenosine, inosine and hypoxanthine) did not increase after onset of hyperglycemia [23]. Increased lactate/pyruvate ratio is known to occur during sustained hyperglycemia even though oxygen supply is sufficient for full mitochondrial respiration. Increased NADH/NAD^+ ratio will result in cellular abnormalities similar to those seen during hypoxia, despite the fact that the available oxygen is well above hypoxic threshold. This state is commonly referred to as "pseudohypoxia" and is a result of altered intracellular redox status [18].

The increased formation of lactate found in renal medulla of diabetic animals results in a significantly lower extracellular pH in this region [23] Fig. (**3**). An increased lactate concentration will decrease pH and protons will re-circulate in medullary structures due to the counter current mechanism in *vasa recta*.

NADPH is crucially involved in regenerating antioxidants, and decreased levels of NADPH will make tissues

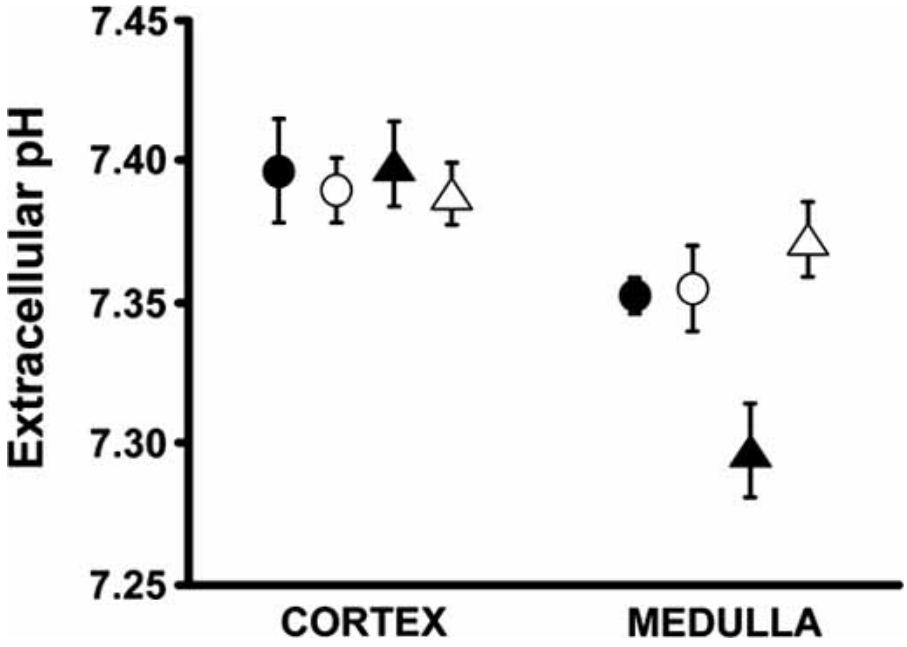

Fig. (3). Extracellular pH in kidneys of control (empty circles) and diabetic animals (triangles) with and without treatment with the aldose reductase inhibitor AL1576 (empty and filled symbols, respectively). Modified from [23].

more susceptible to oxidative damage. Cellular alterations induced by increased polyol pathway activity include activation of pentose phosphate pathway, diacylglycerol (DAG) and protein kinase C (PKC) [18]. Furthermore, a direct link between increased polyol pathway activity and reduced renal PO_2 has been established [23].

Hexosamine Pathway

Unlike polyol pathway, the hexosamine pathway is a relatively new addition to the group of pathways shown to be involved in development of diabetic nephropathy. The hexosamine pathway is also induced by shunting of excess intracellular glucose [26]. The main part of all glucose that enters a cell will be metabolized through glycolysis. However, a few percents of the intracellular glucose enter the hexosamine pathway. Fructose-6 phosphate is metabolized into *N*-acetyl-glucosamine and thereafter metabolized to glycolipids, proteoglycans and glycoproteins [17]. The rate limiting enzyme in hexosamine biosynthesis is glutamine:fructose-6-phosphate amidotransferase (GFPT). Increased concentration of GFPT in mesangial cells will enhance production of cytokines [27], and it has been proposed that variations in genes encoding this enzyme contributes to the susceptibility to diabetic nephropathy [28]. Studies suggest that hexosamine pathway is involved in increasing the expression of growth factors and leptin, something that promotes development of insulin resistance and diabetic complications [29]. Formation of proteoglycans and glycoproteins result in increase transcription of transforming growth factor (TGF)-α, TGF-β1 as well as plasminogen activator inhibitor-1 [30]. In cell cultures, high glucose concentrations lead to induction of TGF-β1 [31], and it is known that TGF-β1 is a pro-sclerotic agent, as well as a causative factor for development of diabetic complications. Addition of either glucose or glucosamine to cultured tubular cells results in a time- and dose-dependent stimulation in TGF-β1 transcriptional activity [32]. Taken together, hexosamine pathway has a regulatory function acting as a sensor of plasma glucose level [29, 33].

Activation of Protein Kinase C

There are eleven known isoforms of PKC. Nine are activated by DAG, which is formed from excess glyceraldehyde-3-phosphate. Increased glucose concentration results in increase amount of DAG, which activates PKC [34]. PKC activation has been shown to mediate changes in renal blood flow [35], perhaps by inducing a decrease in production of NO [36, 37]. Also, inhibition of PKC decreases mesangial expansion, albuminuria mice and GFR, as well as increases pro-inflammatory gene expression and vascular permeability in several models of experimental diabetes [17, 35, 38, 39]. Results obtained from isolated epithelial cells indicate that PKC regulates the activity of several active transporters. Among these are $Na^+/K^+/2Cl^-$-cotransporter, Na^+/H^+-exchanger, Na^+/Pi-cotransporter and Na^+/K^+-ATPase [40].

Several activators of PKC have been discovered, e.g. increased glucose levels, angiotensin II, low density lipoprotein and thromboxane, which all increase either TGF-β1 activity or mRNA expression [41]. Nishikawa *et al*. have shown that normalization of mitochondrial superoxide production will prevent hyperglycemia-induced activation of PKC [42].

Pentose Phosphate Shunt

The pentose phosphate shunt is a NADPH-regenerating system acing as an intracellular mechanism controlling cellular sorbitol synthesis sorbitol concentration and intracellular glutathione. Intracellularly, NADPH is the principal reductant protecting against oxidative stress. Production of NADPH is mainly dependent on the activity of glucose-6-phosphate (G6P) dehydrogenase.

Hexokinases binding to mitochondria results in increased formation of both adenosine diphosphate (ADP) and G6P. ADP enters the mitochondria which stimulates mitochondrial oxidative phosphorylation. G6P is an important intermediate for energy metabolism, facing a switch position between glycolysis, glycogen synthesis and the pentose phosphate shunt. When plasma glucose increases, mitochondrial oxidative phosphorylation accelerates, coupling glycolysis to mitochondrial metabolism [43].

The enzymes of the pentose phosphate shunt provide an antioxidant function within the cell consisting of an initial oxidative part, where G6P dehydrogenase activity is rate limiting. Thus, a decrease in G6P dehydrogenase activity results in decreased NADPH levels, thereby sensitizing cells to oxidant damage. Certain conditions can cause changes in the activity of G6P dehydrogenase and other enzymes in the pentose phosphate shunt. For instance, a high dietary protein intake can give long-term adaptive responses of the dehydrogenases in the pentose phosphate shunt by increasing intracellular levels of these enzymes [44].

Studies show that chronic metabolic acidosis, such as untreated or suboptimally treated diabetes, activates both G6P dehydrogenase and 6-phosphogluconate dehydrogenase [45, 46]. Kinetics of the renal pentose phosphate shunt enzymes are altered also in animal models of experimental diabetes [46]. In another study of the effect on enzyme activity during different periods of diabetes, the oxidative segment and glucose flux displayed increased activity during the first 7 days after onset of diabetes. Hereafter, enzyme activity returned towards normal levels, but with a persisting functional activity of this pathway. However, transketolase activity was decreased during the early phase [47]. These alterations have been implicated in development of renal hypertrophy [46]. Also, in studies performed on isolated rat glomeruli, elevation of glucose levels increased G6P, fructose-6-phosphate, total triose phosphates, lactate, lactate/pyruvate ratio, sorbitol and fructose, but did not affect sn-glycerol 3-phosphate or pyruvate levels [48].

In a study performed on streptozotocin (STZ)-induced diabetic rats, maximal aldose reductase activity was increased 4-fold in renal medulla, while sorbitol synthesis only was increased about 1.3-fold. An explanation could be that NADPH/$NADP^+$ ratio is decreased in STZ-diabetic rats with concomitant alteration of the cytosolic redox state, which may alter the control of sorbitol synthesis [49].

Many studies have faced the problem of finding means of reducing the effects of diabetes on the pentose phosphate

shunt. A more reduced redox state induced by increased glucose level was completely normalized when aldose reductase inhibitors were added to the diet prior to isolation of the tissue. This observation support the hypothesis that metabolic imbalances associated with a reduced ratio of cytosolic NADH/NAD^+ plays an important role in mediating glucose-induced glomerular dysfunction [48]. Steer *et al*. have shown that somatostatin analogs have a normalizing effect on pentose phosphate pathway in diabetic rats with acute renal hypertrophy. Diabetes-induced increases in G6P dehydrogenase and 6-phosphogluconate dehydrogenase were less pronounced after treatment in this study. Furthermore, kidney weight increased less in treated diabetic animals [50].

Formation of Advanced Glycation End-Products

In diabetic renal glomeruli, there is an excess amount of sugar-derived advanced glycation end-products (AGE) [51], that were discovered in the 1970s [17]. Studies conducted shortly thereafter found increased levels of AGE in urine of diabetic patients [52]. AGE are formed through autooxidation of glucose to glyoxal, decomposition of Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone, and conversion of glyceraldehyde-3-phosphate to methylglyoxal, which all produce reactive dicarbonyls. These dicarbonyls (glyoxal, methylglyoxal, and 3-deoxyglucosone) can all react with amino groups of various proteins to from AGE [53]. When AGE are formed intracellularly , they will induce pathologic alterations in gene expression and protein function, also in extracellular matrix and blood [17].

AGE accumulate in diabetic tissue as a function of time and sugar concentration and induce permanent abnormalities in extracellular matrix function, stimulate the production of cytokines and ROS specific receptors, but also modify proteins [54]. Increased AGE formation has multiple negative consequences since posttranslational modifications of proteins can lead to mitochondria-induced oxidative stress in kidneys of both diabetic patients and diabetic rats [55]. As previously stated, a normalization of this superoxide production, e.g. by a reduction in plasma glucose [56] blocks at least three pathways of hyperglycemic damage, including formation of AGE [42]. In a study performed by Horie *et al.*, different AGE were found to be co-localized with diabetic renal glomerular lesions [51]. AGE in the plasma can bind to albumin and other plasma proteins. These AGE-modified plasma proteins will bind to AGE receptors, activating proinflammatory genes, cytokines and growth factors, causing vascular pathology [53, 57].

It has been shown that pharmacologic inhibition of AGE in long-term diabetic rats prevents both structural and functional manifestations of diabetic nephropathy [54, 58, 59]. Recently, similar results were obtained also in a placebo-controlled, double-blind multi-centre trial with 690 type-1 diabetic patients displaying overt nephropathy. The AGE inhibitor aminoguanidine was shown to lower proteinuria and slow progression of nephropathy [60]. Taken together, AGE may play an important part in progression of diabetic nephropathy and inhibitors may come to prove useful in reducing the incidence of diabetes-related complications in patients.

Reactive Oxygen Species-Induced Alterations

Accumulating evidence suggests that diabetes-induced production of reactive oxygen species (ROS) is an important pathogenic mechanisms responsible for development of diabetic complications [61, 62]. Experimental, as well as clinical evidence support ROS to be crucially involved also in development of diabetic nephropathy [51, 63].

Hyperglycemia is closely associated with increased production of radicals, which has been linked to increased levels of NADH [61]. Cytosolic glucose is oxidized to pyruvate, which is either converted to lactate or enter the mitochondrial tricarboxylic acid (TCA) cycle. NADH transfers electrons to NADH:ubiquinone oxidoreductase, complex I in the electron transfer chain. Further electron transfer includes complex III, IV and finally molecular oxygen. Electron transport through complex I, III and IV generates an electrochemical potential due to a gradient of protons across mitochondria membrane. A high potential stabilizes superoxide-generating intermediates in electron transport chain, resulting in increased formation of ROS [42].

When formation of ROS exceeds the antioxidant defense mechanisms of the tissue, cellular injury and dysfunction will develop. ROS can inactivate enzymes and damage DNA, and are thought to regulate different transcription factors, e.g. AP-1 complex, nuclear factor (NF)-κβ, hypoxia-inducible factor (HIF)-1 and p50. Hereby, ROS regulate the activity of various oxygen-sensitive genes. Out of these transcription factors, HIF-1 has emerged as a pivotal molecular mechanisms mediating adaptation to low PO_2 in renal medulla under physiological conditions [64].

There are several ways to prevent increased formation of hyperglycemia-induced ROS in cell cultures. The use of inhibitors for complex I, complex II and uncoupler for oxidative phosphorylation that abolishes the mitochondrial membrane potential, have all been shown to prevent formation of ROS during hyperglycemia [42]. Hyperglycemia-induced formation of ROS activates at least three major pathways involved in development of diabetic complications, namely activation of PKC, formation of advanced glycation end-products and increased flux through the polyol pathway [42]. Inhibition of hyperglycemia-induced excessive formation of ROS at the level of mitochondrial membrane, results in complete lack of activation of hexosamine pathway (and PKC), polyol pathway, NF-κβ, and DAG, but also prevents formation of AGE. As a summary; the discovery of a unifying mechanism, by which ROS activate several classical pathways involved in development of diabetic complications, provides a whole new framework for future studies.

Activation of NADPH Oxidase

Our entire antioxidant system is dependent on a sufficient supply of the energy-containing agent NADPH, which is the main intracellular reductant for all cells. NADPH is formed during glycolysis or oxidative phosphorylation, and exerts antioxidant activity by regenerating glutathione and lipoic acid. Glutathione and lipoic acid act as important intracellular antioxidants by reacting with ROS and organic

peroxides [65, 66]. Thus, reducing the levels of NADPH will result in reduced antioxidant defense.

In renal vessels, macula densa, thick ascending loop of Henle, distal tubules, collecting ducts, interstitial fibroblasts, and in glomerular podocyte and mesangial cells, the enzyme NADPH oxidase is a significant source of production of superoxide radicals [67]. In phagocytes, NADPH oxidase consists of two membrane bound units ($p22^{phox}$ and $gp91^{phox}$; the latter is also referred to as Nox2) and four cytosolic subunits ($p40^{phox}$, $p67^{phox}$, $p47^{phox}$ and Rac) [68]. In kidneys, $gp91^{phox}$ is expressed in glomerular cells and distal tubules [69], and its homologue Nox4 (and to some extent Nox1) are present in high levels in distal tubular cells and mesangium in renal cortex [67, 70, 71]. $p47^{phox}$ is expressed in distal tubules, cortical renal glomerular cells, thin limbs of the loop of Henle and medullary collecting ducts [69, 72]. For activation of NADPH oxidase, assembly of the subunits and translocation of $p47^{phox}$ to the membrane is necessary. NADPH oxidase generated superoxide radicals can react with NO forming peroxynitrite, which is a potent oxidant and nitrosing agent. Furthermore, this reaction can cause NO deficiency. NO normally regulates tubuloglomerular feedback and renal blood flow, and is involved in regulation of natriuresis. The NO deficiency can be worsened by the fact that oxidative stress promotes activation of vasoconstrictors [73]. Thus, NO deficient animal models develop glomerulosclerosis and proteinuria, as well as hypertension and renal failure [73].

Oxidative stress has been implicated in pathogenesis of diabetic renal injury. The total degree of oxidative stress is determined by the balance between ROS production and the antioxidant defense system, e.g. superoxide dismutase (SOD). This implies that increased production of ROS could be involved in pathogenesis of diabetic nephropathy, for instance by increased activity of NADPH oxidase [69, 72]. The effects of NADPH oxidase inhibition on diabetic nephropathy are not fully elucidated [74], but its subtypes have been shown to be induced during early diabetic proteinuria, as well as in spontaneously hypertensive rats [67, 69, 72]. The few data available on expression of NADPH oxidase subunits suggest that $p22^{phox}$ is unrelated to development of nephropathy [75]. However, expression of $p47^{phox}$ is increased in podocytes from diabetic rats, as well as in glomeruli, cortical distal tubules, loops of Henle and medullary collecting ducts [69, 72]. Also, renal tissue abundance of the catalytic subunit $gp91^{phox}$ is elevated in chronic renal failure [76, 77], and there are indications that $gp91^{phox}$ is responsible for regulating renal vascular tone [78]. In addition, over-expression of Nox4 leads to increased superoxide production in cultured cells and decreases growth rate, making Nox4 a possible renal oxygen sensor and cell growth regulator [71]. Both expression of $gp91^{phox}$ and activation of $p47^{phox}$ are decreased by the NADPH oxidase inhibitor apocynin in diabetic rats, which also prevented proteinuria [69]. Renin-angiotensin-aldosterone system (RAAS) inhibitors also normalize $p47^{phox}$ expression [72], while Nox1 and $p22^{phox}$ are increased in renal cortex after prolonged infusion of angiotensin II [79]. These findings are supported by the finding that lipoic acid prevents diabetic renal damage in experimental settings [80, 81].

In a recent study, high salt intake increased renal activity of NADPH oxidase, simultaneously diminishing renal expression of SOD, which enhances superoxide generation [82]. Also, acidity seems to influence NADPH oxidase activity in murine mesangial cells, leading to an uncoupling of NADPH oxidation. This resulted in decrease NO formation [83]. Increased NADPH oxidase activity will decrease $NADPH/NADP^+$ ratio, causing oxidative stress. However, an increased $NADPH/NADP^+$ ratio can also be damaging since this can increase production of ROS by the TCA cycle enzyme complex α-ketoglutarate dehydrogenase [22, 84].

INFLUENCE OF DIABETES ON RENAL OXYGEN TENSION AND OXYGEN CONSUMPTION

Reduced Renal Medullary Oxygen Tension

The *in vivo* PO_2 in any tissue is the result of net delivery of oxygen and oxygen consumption within that specific tissue. Altering any of these two parameters will undoubtedly affect the PO_2. Decreased PO_2 in renal medulla has been proposed as a mechanism involved in progression of nephropathy [12]. Diabetes-induced increase in renal medullary hydrogen ion concentration will increase the Bohr effect when acidic blood in ascending *vasa recta* comes in the vicinity of arterial blood in descending *vasa recta*. Shunting of oxygen (from descending to ascending vessels) will increase, and the net result will be an even further reduced oxygen delivery to renal medulla. Decreased oxygen delivery to medullary structures will occur despite the fact that total blood perfusion might be unaffected, or even increased.

Nishikawa and co-workers [42] showed that hyperglycemia induces excessive formation of ROS from the electron transport chain located in the mitochondrial membrane. Hyperglycemia-induced increase in substrate available for the electron transport chain will increase electrochemical potential gradient over across the mitochondrial membrane. This will stabilize superoxide-generating intermediates in electrode transport chain, resulting in increased formation of ROS [42]. Formation of ROS leads to reduced renal PO_2 [85] Fig. (**4**).

Treatments with antioxidants such as α-tocopherol, pycnogenol, β-carotene, and α-lipoic acid have been shown to affect oxidative stress [86]. Antioxidant treatment reduced the diabetes-induced increase of the antioxidant enzyme hemeoxygenase-1 and increased the activity of glutathione and glutathione redox enzyme [63, 87, 88]. It should be noted that mechanisms which mediated these alterations are highly dependent of the nature of the antioxidant used [86]. In one of our recent studies, treatment of diabetic animals with the free radical scavenger α-tocopherol fully prevented diabetes-induced decrease in renal PO_2 [85]. Furthermore, it was shown that increased levels of ROS were accompanied by increased mitochondrial respiration.

Increased Oxygen Consumption

A previously reported adaptation of the renal medulla to a low PO_2 is a decrease in enzyme activity, which rapidly and profoundly decreases metabolic activity, thereby de-

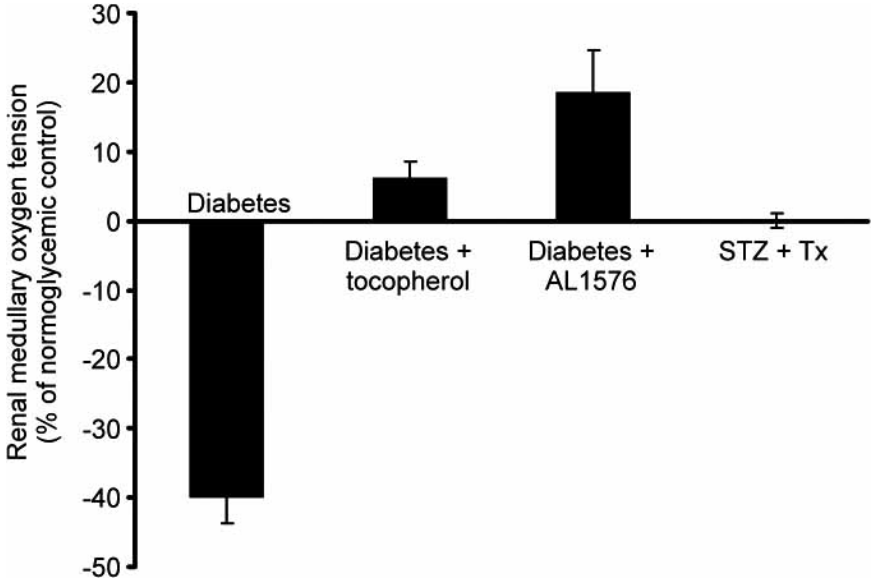

Fig. (4). Diabetic rats have decreased renal medullary PO_2. Antioxidant treatment with α-tocopherol or inhibition of polyol pathway with AL1576 fully prevented the reduction of medullary PO_2. Animals receiving streptozotocin and 24 hours later transplanted with islets of Langerhans (STZ+Tx) display unaffected PO_2. Modified from data published in [23,85,162].

creasing oxygen demands [10]. In a recent study, however, renal oxygen consumption was found to be increased in cells isolated from diabetic animals [23]. This finding is in conjunction with previous reports and is linked to increased Na^+/K^+-ATPase, since the ouabain sensitive oxygen consumption was significantly increased [89]. It is well known that glomerular hyperfiltration occurs during the early onset of hyperglycemia, both in diabetic patients and in animal models of experimental diabetes [90, 91]. Increased glomerular filtration will increase tubular load of electrolytes, resulting in increased tubular reabsorption and increased oxygen consumption [89]. This is certainly a contributing mechanism, but a large part of the diabetes-induced increase in medullary oxygen consumption is unrelated to active transport since oxygen consumption is independent of glomerular filtration rate (GFR) [92]. This suggests that other mechanisms apart from an altered tubular sodium load are involved in the increased oxygen consumption. Increased flux through the Na^+/glucose-linked transporters (SGLT), as a result of excessive tubular load of glucose due to the increased blood glucose concentration, has been shown to increase Na^+/K^+-ATPase activity [93]. Another possible mechanism is a decreased inhibition of the oxygen consumption by NO, which would occur in a dose-dependent manner [94, 95]. The ROS-induced increase in cellular oxygen consumption is at least partly due to increased degradation of NO [96].

Sustained hyperglycemia is known to induce enhanced expression of uncoupling protein-2, increase gluconeogenesis and also increase fatty acid metabolism, all resulting in increased oxygen consumption [97]. During normoglycemic conditions there are no insulin independent Na^+/glucose transporters in the renal medulla, but the low-affinity GLUT-2 has been found in significant amounts all along the nephron [9, 10]. A challenging speculation is that increased medullary tubular glucose load can induce protein expression of SGLT, resulting not only in increased intracellular glucose concentrations, but also in increased cellular energy demand and subsequent oxygen consumption.

DIABETES-INDUCED ALTERATIONS IN THE NITRIC OXIDE SYSTEM

Production and Bioavailability of Nitric Oxide

In the renal medulla, NO is predominately synthesized by nitric oxide synthase (NOS) in thick limb of Henle [98] Except for dilating *vasa recta*, NO also inhibits tubular transport and counteracts the constricting activity of norepinephrine, angiotensin II and antidiuretic hormone [92]. NO bioavailability is of importance not only for hemodynamic regulation and delivery of oxygen to renal medulla, but also for regulation of oxygen consumption [99]. Koivisto and coworkers showed that NO inhibits mitochondrial respiration in proximal tubular cells, and that inhibition is proportional to inverse square of oxygen concentration [100]. Furthermore, if mitochondrial NO reacts with superoxide radicals peroxynitrite will form, which induces oxidative stress and cause irreversible changes and modifications of mitochondrial targets [83].

Even during normal physiological conditions, the influence of NO inhibition of mitochondrial respiration is likely to be significant in renal medulla due to normally low PO_2. It has been demonstrated that NO bioavailability in STZ-diabetic animals is markedly lower compared to normoglycemic animals [101]. The reason for the decreased NO activity is a reduced plasma L-arginine concentration, which limits NO production. Intravenous administration of L-arginine caused a pronounced increase in bioavailable NO specifically in diabetic animals. This might influence the mitochondrial oxygen consumption rate and, thus, the renal PO_2.

Acidosis is thought to influence NO production. A study by Prabhakar *et al*. showed an 80% reduction of inducible NOS (iNOS) activity in mesangial cells during low pH, despite close to normal NOS mRNA and protein levels. This decrease could not be reversed by L-arginine supplementation, but NO production resumed when pH was returned to normal [102].

Density and distribution of NOS binding sites are decreased in kidneys from diabetic rabbits, implying a role in pathogenesis of renal disease [103]. Renal cortex from STZ-treated diabetic rats displays a 50% reduction in total NOS activity despite increased protein levels of endothelial NOS (eNOS) [99]. Levels of neuronal NOS (nNOS), however, were decreased in macula densa as well as in renal cortex. All alterations in NOS expression and activity were totally restored by intense insulin treatment. In another study, performed on bovine aortic endothelial cells, hyperglycemia and treatment with glucosamine reduced eNOS activity. Blocking the hexosamine pathway via inhibition of its rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase, reversed these changes. This chronic impairment of eNOS activity may partly explain the accelerated atherosclerosis seen in diabetic patients [99].

Recently, mitochondrial NOS (mtNOS) has been reported [104]. Data from experiments performed on renal cells are still scarce, but mtNOS has been reported to regulate oxygen consumption and mitochondrial transmembrane potential in intact isolated hepatic mitochondria [105].

Endogenous Dimethylarginines

Asymmetric dimethyl arginine (ADMA) is an endogenous L-arginine analog, competitively inhibiting NOS activity [100]. It may play an important role in regulation of NO production, and kidneys are crucial in metabolizing ADMA. Although symmetric dimethylarginine (SDMA) does not directly inhibit NOS activity, it has been proposed that it interferes with NO production by competing with L-arginine by cellular uptake by the cationic amino acid transporters [106, 107]. Both ADMA and SDMA could be involved in regulation of NO levels in the kidneys. However, in STZ-induced diabetic rats we consistently find lower levels of ADMA and SDMA [108], whereas both ADMA and SDMA have been reported to increase in patients with chronic renal disease and diabetic neuropathy [109]. This increase can be detected early on in the course of the disease. In patients with end-stage renal disease (ESRD), plasma concentrations of ADMA strongly predicts both cardiovascular disease and overall mortality [64]. It is likely that accumulation of ADMA interferes with pivotal physiological functions by blocking NO production, thus making it not a marker, but rather a causative mechanism involved in development of renal and cardiovascular failure.

ENDOGENOUS SUBSTANCES MEDIATING DIABETES-INDUCED ALTERATIONS

Many systems with seemingly similar functions are up-regulated and more abundant in renal medulla compared to cortical regions. A common factor for many of them is that they are regulated by HIF-1, a transcription factor responsible for obtaining oxygen homeostasis and adapting renal medulla to low PO_2 [110-112]. When hypoxia occurs in a tissue or cell reduced degradation of HIF-1α activates genes involved in maintaining oxygen homeostasis, e.g. iNOS, vascular endothelial growth factor, erythropoietin, and hemoxygenase-1 [113].

Prostaglandins are Increased in Diabetes

Prostaglandin E2 (PGE_2) acts as a vasodilator in *vasa recta*, increasing oxygen delivery at the same time as inhibiting Na^+/K^+-ATPase [114]. Synthesis of PGE_2 occurs mainly in inner medullary collecting duct cells and renal medullary interstitial cells. Not surprisingly, the concentration of PGE_2 is significantly higher in renal medulla compared to renal cortex [115]. Cellular productions of PGE_2 and other eicosanoids are increased in diabetic animals. This increase may contribute to glomerular hyperfiltration, commonly found in the early onset of diabetes mellitus. Furthermore, thromboxane A2 have been implicated in the pathogenesis of glomerular basement membrane thickening, mesangial matrix expansion and albuminuria commonly associated with development of diabetic nephropathy [116].

Increased Effects of Endothelins

Endothelin-1 (ET-1) is a pro-fibrotic vasoconstrictor for renal vessels involved in cell proliferation and extracellular matrix accumulation, giving it a possible role in the pathogenesis of chronic renal failure. Furthermore, kidneys from STZ-induced diabetic rats display elevated ET-1 mRNA levels [117, 118], and ET-1 receptor expression is increased in renal glomeruli from alloxan-treated rabbits [118]. Increased production of endothelins occurs during increased renal acidity and oxidative stress [119-121]. It has been shown that addition of AGE-albumin to bovine endothelial cells increases ET-1 mRNA, ascribed to increased ROS production [122]. The interaction between ROS and endothelin has been studied in mesangial cells, which showed that ROS alters production of ET-1 [117]. Furthermore, production of ET-1 is also stimulated by TGF-β and angiotensin II [123]. In an acidic environment, endothelins cause increased urine acidification via Na^+/H^+-exchanger activation [124].

Beneficial Effects of Adenosine?

The effects of adenosine are more pronounced in the renal medulla than in the cortex. Diabetic patients with reduced renal function have markedly increased risk to develop acute contrast media-induced kidney injury, and adenosine has been proposed to be involved [125]. More recently, administration of specific adenosine receptor blockers has been shown to reduce these damages [126].

In kidneys from diabetic rats, region-specific changes in adenosine receptor expression have been reported, but these changes occurred predominately in renal cortex [127]. Awad and Huang recently demonstrated anti-inflammatory effects and attenuation of histological and functional renal injury in diabetic nephropathy after activation of adenosine 2A-receptors, supporting the theory that this receptor possesses renoprotective properties [128].

HYPERGLYCEMIC MEMORY

Hyperglycemic memory is normally referred to as the phenomenon that diabetic micro vascular alterations progress even during prolonged periods of normoglycemia following a hyperglycemic event. Even though the different groups in the Diabetes Control and Complications Trial had close to identical blood glucose levels after completion of the trial, both the prevalence as well as the severity of complications were still in line with the treatment the group received during the trial [129]. The patients receiving intense treatment benefited from this treatment even after four years compared to group receiving less intense treatment. The most commonly used explanation is that ROS cause mutations in mitochondrial DNA, which results in activation of classical diabetes-induced pathways.

CLINICAL TREATMENT OF DIABETIC NEPHROPATHY

Diabetic patients with chronic renal complications are ever increasing. Treatment strategies today include controlled blood pressure, good glycemic control, regular exercise, quit smoking, and interference with RAAS [130-133]. For numerous patients, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) improve both life expectancy and quality of life. There are no creatinine level limitations when treating patients with ACE inhibitors or ARB. Rather, patients with more advanced nephropathy may benefit more than those displaying only mild nephropathy [134, 135].

Inhibition of RAAS

The RAAS inhibitors have advantageous effects in addition to their blood pressure lowering effects [129], and are recommended to normotensive diabetic, microalbuminuric patients, as well as hypertensive patients [136-138]. Reduced blood pressure is normally accompanied by a reduction in GFR, as well as decreased proteinuria and microalbuminuria [139]. These inhibitors reduce glomerular hydrostatic pressure through an increase in the diameter of the efferent, and to some extent afferent arteriole, preventing loss of glomerular membrane permselective function proceeding microalbuminuria and morphological changes [82, 140, 141]. This additional nephroprotective effect has, however, recently been questioned [72, 79], and the exact mechanism mediating these beneficial effects is unclear. One possible target for RAAS inhibitors is the NADPH oxidase. It has been reported that NADPH oxidase activity is increased by high salt intake, as well as after long-term infusion of angiotensin II [78, 142-144]. Activation of angiotensin receptor subtype 1 increases NADPH oxidase activity, which stimulates superoxide formation [72]. Several studies have implicated NADPH oxidase in development of hypertension and nephropathy [72]. As previously mentioned, recent studies show a normalization of $p47^{phox}$ by ACE inhibitors and ARB. Therefore, one mechanism for a renoprotective action of RAAS inhibition may be decreased NADPH oxidase activity, thereby limiting ROS formation [115]. Drugs reducing the formation of ROS clearly have the potential to prove effective for the prevention of developing diabetic nephropathy. A study performed by Onozato *et al.* showed that diabetes-induced eNOS activity can be prevented by RAAS inhibition in rats with STZ-induced experimental diabetes mellitus [145]. This might be yet another mechanism by which RAAS inhibitors protect against diabetic complications. ACE inhibitors have also shown to reduce diabetes-induced ET-1 activation [146]. There are no clear advantages using any one type of RAAS inhibitor [147, 148], although dual therapy is emerging as superior in controlling both hypertension and microalbuminuria than either therapy alone [149]. This dual therapy advantage, though, may be applicable also with other combinations, not affecting solely RAAS [149].

Dialysis

As with all patients with ESRD, diabetic patients with ESRD need to receive renal replacement therapy. The most common renal replacement therapy for these diabetics is hemodialysis, but there are several clinical problems related to this procedure, e.g. vascular access and increased frequency of intradialytic hypotension [150]. Although there is probably no difference in outcome between hemodialysis and peritoneal dialysis for these patients, the latter is associated with progressive increase in peritoneal permeability, loss of ultrafiltration and increased peritoneal fibrosis, which all are related to the hyperglycemic state [151]. However, diabetic patients on renal replacement therapy do worse with respect to both survival and medical rehabilitation compared to corresponding non-diabetic patients [152]. Furthermore, diabetic patients have higher incidence of left ventricular hypertrophy and ischemic heart disease upon admission to renal replacement therapy. The conclusion is still that tight glycaemic control is the best preventive measure to increase the survival of diabetic patients receiving dialysis.

Future Therapeutic Targets for Prevention of Diabetic Nephropathy

Today, there are very few clinically available treatments other than RAAS inhibitors for treatment of diabetic nephropathy. However, diabetic dogs treated with aldose reductase inhibitors for five years prevented the deterioration of nerve conduction velocity [153]. If these effects would prove to be true in tissues other than nerves, aldose reductase inhibitors could be a valuable treatment preventing diabetes complications. Unfortunately, a similar five-year study showed that aldose reductase inhibition did not prevent albuminuria or renal structural changes [154].

Since the discovery of aminoguanidine as a potent inhibitor of AGE formation, analogs have been proposed as potential drugs for preventing chronic diabetic complications [155]. Furthermore, oxidative stress and decreased renal PO_2 play major parts in development of diabetic complications. Antioxidants could prove to be valuable candidate drugs for therapeutic strategies to prevent diabetes-induced tissue damage. It should be noted that the greatest beneficial effects of antioxidant treatments in experimental settings are achieved in preventing renal complications, while reversal of already existing structural and functional changes is limited. It could also be of value to investigate therapies reversing impaired NO production.

Diabetic complications have long been considered irreversible. However, there are emerging possibilities of reversal of diabetic renal injury, since leptin treatment alleviates glomerular injury and proteinuria in an experimental mouse model of diabetic nephropathy [156-160], and pancreas transplantation reduces diabetic lesions after ten years of normoglycemia [161]. The latter brings about a need for further studying the metabolic properties of proinsulin C-peptide, a molecule believed to influence both eNOS and insulin signaling [23]. Not the least, more effort must be directed towards investigating the mechanisms accounting for the regression of kidney injury in more than 50% of the microalbuminuric patients in a prospective, randomized trial [23].

SUMMARY

During conditions with experimental diabetes mellitus, it is evident that several alterations in renal function occur, including increased mitochondrial respiration and increased lactate accumulation. Consequently, these alterations will contribute to decrease the interstitial PO_2, preferentially in renal medulla as a result of increased mitochondrial respiration. A multi-targeting therapeutic strategy ranging from inhibitors of RAAS and AGE to scavenging of free radicals and ET-1 inhibiting drugs could prove to be a valuable strategy in future treatment of diabetic nephropathy.

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