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# Oxidative Stress in Diabetic Nephropathy

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## Abstract

Diabetic nephropathy is a leading cause of end-stage renal failure worldwide. Its morphologic characteristics include glomerular hypertrophy, basement membrane thickening, mesangial expansion, tubular atrophy, interstitial fibrosis and arteriolar thickening. All of these are part and parcel of microvascular complications of diabetes. A large body of evidence indicates that oxidative stress is the common denominator link for the major pathways involved in the development and progression of diabetic micro- as well as macrovascular complications of diabetes. There are a number of macromolecules that have been implicated for increased generation of reactive oxygen species (ROS), such as, NAD(P)H oxidase, advanced glycation end products (AGE), defects in polyol pathway, uncoupled nitric oxide synthase (NOS) and mitochondrial respiratory chain *via* oxidative phosphorylation. Excess amounts of ROS modulate activation of protein kinase C, mitogen-activated protein kinases, and various cytokines and transcription factors which eventually cause increased expression of extracellular matrix (ECM) genes with progression to fibrosis and end stage renal disease. Activation of renin-angiotensin system (RAS) further worsens the renal injury induced by ROS in diabetic nephropathy. Buffering the generation of ROS may sound a promising therapeutic to ameliorate renal damage from diabetic nephropathy, however, various studies have demonstrated minimal reno-protection by these agents. Interruption in the RAS has yielded much better results in terms of reno-protection and progression of diabetic nephropathy. In this review various aspects of oxidative stress coupled with the damage induced by RAS are discussed with the anticipation to yield an impetus for designing new generation of specific antioxidants that are potentially more effective to reduce reno-vascular complications of diabetes.

### Keywords

Hyperglycemia; reactive oxygen species; renin-angiotensin system; diabetic nephropathy

## INTRODUCTION

Diabetes mellitus is a life threatening disorder since it has a high prevalence of cardiovascular complications, such as, myocardial infarction, stroke and peripheral vascular disease [1]. It is well known that diabetic patients are two to four times more likely to suffer from these macrovascular complications. Of note here is also the fact that the atherosclerosis develops at an early stage and progresses more rapidly compared to non-diabetic patients; which translates into a poor prognosis with high morbidity and mortality in diabetic patients



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[2]. Besides these macrovascular complications, added microvascular injury further compounds the quality of life in such patients. The microvascular injury mainly targets two major organs, *i.e.,* eye and kidney. Its common manifestations include diabetic retinopathy and nephropathy, which incidentally are the leading cause of blindness and end stage renal disease (ESRD) in most of the developed countries [3 – 5]. Since the microvascular injury is usually related to the chronic sustained hyperglycemia, therefore, to control hyperglycemia would be first line of effective measure to seek amelioration from the diabetic complications as concluded from the two large clinical trials, *i.e.,* the Diabetic Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) [6, 7].

The pathogenetic mechanisms for the macro- and micro-vascular complications may be similar with reactive oxygen species (ROS) as the common denominators of various signaling pathways that lead to an assault in various target organ systems [8–11]. The ROS are a family of molecules including molecular oxygen and its derivatives, superoxide anion ($O_2^-$), hydroxyl racial ($HO^{\cdot}$), hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), nitric oxide (NO) and lipid radicals. Many ROS possess unpaired electrons and thus are regarded as free radicals. Excessive amounts of ROS, after surpassing various endogenous anti-oxidative defensive mechanisms, oxidize various tissue biomolecules, such as, DNA, protein, carbohydrates and lipids; and this calamitous state has been commonly referred to as an oxidative stress [8 – 11]. In mammalian cells, potential sources of ROS include mitochondrial respiratory chain, xanthine oxidase, NADH/NADPH oxidases, NO synthase and certain other hemoproteins. Having divergent sources of ROS, conceivably, a number of different mechanisms are operative for their generation under high glucose ambience [11 – 13]. Certainly, their production increases during hyperglycemia and they are considered to play a significant role in the pathogenesis of diabetic complications [13, 14]. The increased production ROS may be so overwhelming that anti-oxidative defense systems are readily exhausted with the emergence of a state commonly referred to as oxidative stress [14]. The oxidative stress is regarded as a common and major factor that couples hyperglycemia with vascular complications *via* two mechanisms, *i.e.,* first, the metabolic modifications of target tissue molecules and second, the alterations in the renal hemodynamics. Accumulating clinical and experimental evidence provide a strong support for this concept, whereby metabolic and hemodynamic (mechanical) assaults have synergistic adverse effects on the target tissues [11]. In this review, current perspectives on the association of hyperglycemia and oxidative stress and the relevant mechanisms by which the latter affects renal homeostasis and ultimately contribute to the development of diabetic nephropathy are summarized.

## MITOCHONDRIAL ORIGIN OF REACTIVE OXYGEN SPECIES (ROS)

It is well known for decades that in majority of the cells the influxed glucose is destined to generate fuel for the production of ATP *via* oxidative phosphorylation in the mitochondrial respiratory chain complex. Following entry into the cells, most of the glucose undergoes glycolysis to form pyruvate, which then enters into the Kerbs cycle to generate ATP, NADH and FADH2. The NADH and FADH2 so generated are transported into the mitochondria from cytosol *via* the malateaspartate or the glycerol phosphate shuttle systems, where they are utilized as electron donors during oxidative phosphorylation. In doing so the electrons from NADH or FADH2 are transferred to molecular oxygen ($O_2$) in the mitochondrial respiratory chains complex I – IV to generate ATP (Fig. 1). During this process, most of the $O_2$ is reduced to water under normal physiological states, and less than 1% of $O_2$ is converted to superoxide anion, $O_2^-$. However, under mitochondrial dysfunctional or hyperglycemic states there is an excessive leakage of electrons at two major sites, one at the complex I and another is at the interface between coenzyme Q and complex III. Thus, quantitatively, the mitochondrial respiratory chain has been regarded as the major source of

superoxide generation in the mammalian cells [15 – 18]. Intriguingly, certain target cells, including glomerular mesangial cells, retinal capillary endothelial cells and neuronal cells are unable to regulate intracellular glucose concentrations adequately in the diabetic *milieu* [11]. These cells are particularly susceptible to hyperglycemic assault since they are unable to avert intracellular high glucose ambience in states of systemic rise in blood glucose levels. As a result, these target cells with increased flux of glucose in diabetic state have accelerated oxidative phosphorylation and excessive leakage of $O_2^-$, and thereby are subjected to extraordinary ROS-mediated oxidative stress.

The ROS can induce DNA damage by causing breaks in the single- or double strands, or by disrupting the base (histones) or sugar moieties [19, 20]. Since mitochondrial DNA (mtDNA) is deficient in histones, it is believed to be more susceptible to oxidative damage. Moreover, it has been demonstrated that mtDNA damage is more extensive and persists longer than nuclear DNA damage in human cells following an oxidative stress. In this regard, the oxidative mtDNA damage has been linked to the onset of various human diseases, such as, aging, cardiovascular and neuronal degenerative diseases [19, 20]. Conceivably, the mtDNA damage impairs electron transport in the respiratory chain, which leads to mitochondrial dysfunction and thus giving rise to further generation of mitochondria-derived ROS. This repetitive-cyclic production of ROS is likely to damage various enzymes of the mitochondrial respiratory chain, which ultimately is expected to lead to blunted ATP synthesis, perturbation in the mitochondrial permeability transition, cytochrome C translocation, activation of various caspases and thus predisposing the cell to undergo apoptosis or necrosis [21 – 23].

## PENTOSE PHOSPHATE PATHWAY

Pentose phosphate pathway is primarily an anabolic pathway in which 6 carbons of glucose are utilized to generate 5 carbon sugars and reducing equivalents. Among the major pentoses synthesized includes ribose and reducing equivalents, such as, NAD(P)H. The NAD(P)H from $NADP^+$ are produced in a series of reactions confined to the first or oxidative stage of pentose phosphate pathway. This stage is initiated by a rate limiting enzyme, glucose-6-phosphate dehydrogenase (G6PDH), which shunts glucose-6-phosphate from the glycolytic cycle into this pentose pathway. Activation or upregulation of G6PDH activity results in an increase of NAD(P)H in failing heart or ischemic cardiomyopathy [24]. Increased availability of NAD(P)H thus in turn fuels the NAD(P)H oxidase to generate increased amounts of ROS production and depletion of glutathione. The potential of this pathway as a source of ROS production in diabetic nephropathy has yet to be elucidated.

## POLYOL/SORBITOL PATHWAY

During the first step in the polyol pathway aldose reductase, an intracellular enzyme, converts glucose to sorbitol, which in subsequent series of steps is channeled to form fructose-3-phosphate (F-3-P) and 3-deoxyglucosone (3-DG). Normally, very little amounts of glucose is diverted into the polyol pathway; however, in states of hyperglycemia more than 10-fold glucose is channeled into this accessory pathway [25]. The metabolites of F-3-P and 3-DG leads to the formation of advanced glycation end products (AGEs), the latter upon interaction with their receptor, RAGE, generate ROS. In addition to the AGE:RAGE interactions, there are other intrinsic steps in the polyol pathway which facilitate the production of ROS. For instance, during the initial conversion of glucose to sorbitol, NAD(P)H is consumed as a cofactor for this reaction. Since NAD(P)H is required for the glutathione reductase reaction in which glutathione disulfide is reduced to glutathione, the consumption of NAD(P)H will eventually reduce the levels of cellular glutathione and ultimately also the antioxidant activity. Although in the second step of polyol pathway the

sorbitol is oxidized to fructose by sorbitol dehydrogenase, still the excessive amounts of sorbitol would accumulate in the cytosol in states of hyperglycemia [26, 27]. Since the cell membrane is impermeable to sorbitol, accumulation of sorbitol increases intracellular osmotic pressure and this would likely perturb overall cellular functions that are amenable to reduce oxidative stress.

## NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE REDUCED {NAD(P)H} OXIDASE

NAD(P)H oxidase is a multi-subunit enzyme that catalyzes generation of $O_2^-$ by reduction of $O_2$ using either NADPH or NADH. The prototype of this enzyme was originally found in neutrophils and phagocytic cells [28 – 30]. In these cells this enzyme plays a pivotal role in host defense and innate immunity by production of large (millimolar) quantities of superoxide, $O_2^-$. The enzyme has five units: membrane-associated $p22^{phox}$ and $p91^{phox}$ (also termed as NADH Oxidase 2 “NOX2”), cytosolic subunit $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and GTPase Rac1 or Rac2. Upon stimulation, $p47^{phox}$ gets phosphorylated and the two cytosolic subunits form a complex. The complex then translocates to the plasmalemma, where it associates with membrane subunits to form an active NAD(P)H oxidase multi-subunit complex, which transfers electrons to $O_2$ with the formation of $O_2^-$. It should be noted here that *Rac1* or *Rac2* are also required to form an active NAD(P)H enzyme complex [29].

Besides neutrophils or macrophages, NAD(P)H oxidase has been identified in various cell types in the kidney. These are mesangial cells and proximal tubular epithelia, vascular smooth muscle cells, endothelia and interstitial fibroblasts [31 – 37]. Other homologues of Nox2 include Nox3, Nox4, and Nox5, and these have been found to be expressed in these renal cells. In these non-phagocytic cells, the quantity of $O_2^-$ production from the NAD(P)H oxidase system is much smaller than in phagocytic cells (*vide supra*). Nevertheless, ROS generated in these cells play a vital role by serving as an intracellular second messengers, and thus mediate diverse biological functions. In all these scenarios, the first step is the activation of NAD(P)H oxidase that is initiated following ligand:receptor interactions. The nature of ligands can vary from various cytokines (tumor necrosis factor-α and interleukin-1) to growth factors (platelet-derived growth factor, epidermal growth factor and transforming growth factor-β) to G protein-coupled receptor agonists (Angiotensin II, Ang II; serotonin, thrombin, bradykinin and endothelin); all these are amenable of activating NAD(P)H oxidase followed by an increase of intracellular concentration of $O_2^-$ and $H_2O_2$ [38]. In addition to the receptor-mediated activation, the mechanical forces, such as, stretch and shear stress, and metabolic factors like hyperglycemia, free fatty acid, and advanced glycation end products (AGEs), are conceivably capable of modulating the activity of NAD(P)H oxidase.

Among the various ligands alluded above, Ang II is considered to be most important modulator of NAD(P)H oxidase [39, 40]. The Ang II following its interaction with $AT_1$ receptor activates NAD(P)H oxidase *via* stimulation of signaling pathways including c-Src, protein kinase C, and phospholipase $A_2$/D. It is worth mentioning here that Ang II also affects NAD(P)H oxidase activity through transcriptional regulation of its subunits [31, 41 – 43]. The various downstream signaling events and biophysiological processes modulated by NAD(P)H oxidase-derived ROS include cell growth, proliferation, migration and differentiation. The pathobiological processes modulated by ROS include inflammation, cell proliferation, extracellular matrix accumulation, fibrosis, endothelial dysfunction and aberrant angiogenesis [41 – 43]. In these processes, a multitude of other redox responsive signaling molecules and transcription factors, including mitogen-activated protein kinases (MAPKs), tyrosine kinases and phosphatases, NF-κB, AP-1 and SP-1 also participate and

dictate the final outcome of cellular injury [44]. The renal injury related to ROS happens to be a frequent occurrence, as has been reported in states of ischemia-reperfusion injury and diabetic nephropathy [45 – 48]. In the latter, the expression of subunits of NAD(P)H oxidase has been reported to be increased in experimental models of diabetic nephropathy; and more importantly, the inhibition of NAD(P)H oxidase with apocynin or diphenylene iodinium, leads to an attenuation in the renal ROS production and amelioration in the morphological changes and functional abnormalities that are seen under high glucose ambience or hyperglycemia [45 – 48]. These observations indeed highlight the relevance of NAD(P)H-derived ROS as one of the major mediators in the pathogenesis of diabetic nephropathy.

## UNCOUPLED NITRIC OXIDE SYNTHASE (NOS)

There are three major isoforms of the nitric oxide synthase (NOS), inducible NOS (iNOS, type1), neuronal NOS (nNOS, type2), and endothelial NOS (eNOS, type 3). All the isoforms are expressed in the kidney tissues [49 – 51]. Both nNOS and eNOS are constitutively expressed, but iNOS is inducibly expressed in immune-cells, such as, macrophages. Expression and activity of iNOS is regulated *via* the signaling pathway including redox-sensitive transcription factor NF-κB. Enzymatic activity of eNOS is regulated by the intracellular $Ca^{+2}$ concentration, and also is affected by the availability of its substrate L-Arginine and cofactors. The eNOS requires cofactors, such as, tetrahydrobiopterin ($BH_4$), flavinmononucleotide (FMN) and calmodulin to generate NO [52 – 55]. Under normal conditions, NO is produced from L-Arginine through a “five” electron oxidation step in the presence of sufficient amounts of cofactors. However, if the availability of cofactors is restricted, eNOS enzyme is unable to completely catalyze oxidation of L-Arginine to NO [56, 57]. Interestingly, even with the deficiency of cofactors like $BH_4$ the eNOS still receives electrons from NADPH, stores them in bound flavins, then donates them to $O_2$, resulting in an “one” electron reduction to form superoxide anion, $O_2^-$ [58 – 63]. This process is known as NOS uncoupling and under such cofactor deficiency, addition of the L-Arginine generates $0_2^-$ instead of NO. Alteration of renal NO production, availability and function of NOS and their possible involvement in the pathophysiology of a wide variety of diseases has been extensively studied. However, it is still difficult to make an unequivocal statement as to its precise role in these diseases because of the complexity of the issue and confusing and contradictory available literature reports and paradoxes as alluded to below in the following paragraphs [64].

The major subject matter that is paradoxical and needs to be discussed relates to renal functional and hemodynamics that are conceivably modulated by NO. Increase of glomerular filtration rate (GFR), renal plasma flow (RPF) and filtration fraction FF), along with renal hypertrophy are regarded as the physiological hallmarks of early stages of diabetic nephropathy [65, 66]. Usually, hyperfiltration precedes the development of microalbuminuria and the histological changes in diabetic nephropathy, both in humans as well as in animal models of diabetes. Increased production of NO has been speculated to be responsible in the pathogenesis of hyperfiltration since there is an upregulation of almost all forms of NOS accompanied with increased urinary excretion of NO metabolites in diabetic models. The fact that treatment of NOS inhibitor ameliorates hyperfiltration in diabetes lends further support to this idea. In addition, enhanced NADPH diaphorase staining, reflecting constitutive NOS activity, is detected in the afferent arterioles, suggesting possible involvement of increased NO production in the development of hyperfiltration (assessed by creatinine clearance) in diabetic rats, and these changes are normalized with the treatment of NOS inhibitor, such as, L-NAME [67 – 70].

On the other hand, there are abundant reports indicating the presence of endothelial dysfunction and defective endothelium-dependent NO production in the diabetic kidney,

although there is good evidence that hyperfiltration directly relates to the increase of NO production [69, 70]. This discrepancy appears to be attributable to several factors, such as, difficulties in measuring NO production or bioavailability, inconsistent experimental conditions, such as, presence or absence of insulin treatment, and finally duration of the study in a given model. There may be other confounding factors as well. For instance, in many studies L-NAME, non-specific NOS inhibitor, was used to assess the effect of NOS inhibition. In this regard, Komers *et al.* used an isoform-specific inhibitor of NOS and demonstrated that increased activity of neuronal NOS (nNOS) and NO derived from this pathway plays an important role in the hemodynamic changes seen in diabetes [71, 72]. The rate of NO synthesis is often evaluated by measurement of urinary excretion of NO metabolites, *i.e.*, nitrite and nitrate. Although this is a widely accepted index of NO production, the validity of this method has not been confirmed. Suto *et al.* found that NOX is reabsorbed extensively in the proximal tubule, which clouds the possibility of a clear relationship between urinary NOX excretion and the systemic and/or renal production of NO [73]. Another factor that may well influence the outcome of these studies is the duration of diabetes. Pieper suggested that the length of duration of diabetes is an important determinant for NO production in the vascular system. Initially, there may be an increased synthesis, followed by a period of normal production, and then decreased NO production during the later stages of the disease process [74]. The experimental data also indicates that a sustained chronic exposure of high glucose ambience suppresses NO bioavailability. In addition, the balance between production and bioavailability is continuously modified by metabolic control modulated by presence or lack of insulin [64].

Other factors that may well modulate the eNOS activity are post-translational modifications. In addition to synthesizing NO, NOS catalyzes $O_2^-$ formation in states of tetrahydrobiopterin ($BH_4$) or L-Arginine deficiency [75 – 77]. In such “uncoupled state,” electrons flowing from the NOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-Arginine. Thus NOS acts as an enzyme with dual functions, *i.e.*, capable of producing both NO and ROS. The duality of the eNOS is related to the fact that it is a homodimer containing a reductase and an oxygenase domain that are linked to calmodulin binding domain; and it has been proposed that the formation of a dimer is crucial for the NO production by NOS. The relevance of this dimer configuration was elucidated in studies by Satoh *et al.* where NAD(P)H oxidase and uncoupled NOS were found to be the major source of renal superoxide production in rats with experimental diabetic nephropathy [78]. They also demonstrated that the glomeruli of these animals were under excessive amount of oxidative stress since there was an increased accumulation of lipid peroxidation products and 8-hydroxydeoxy guanosine (8-OHdG). Concomitantly, a decreased bioavailability of NO in the same kidney tissue, as assessed by fluorescent dyes and confocal laser microscopy was noted. Although expression of isoforms of NAD(P)H as well as of eNOS was increased; nevertheless, the bioavailablility of NO was decreased, suggesting thus marked dysfunctions of eNOS which were apparently responsible for the generation of ROS in experimental diabetic nephropathy.

## RELEVANCE OF TETRAHYDROBIOPTERIN ($BH_4$) AND DIHYDROBIOPTERIN (BH2) IN eNOS PATHO-BIOLOGY AND ENDOTHELIAL DYSFUNCTIONS

The plasma levels of $BH_4$ in the diabetic rats are usually lower than that in the control rats [78]. The underlying reason for the decreased $BH_4$ bioavailability in diabetic nephropathy has not been fully understood but there are speculations that it may be related to the impaired synthesis or increased catabolism or its oxidation to $BH_2$, as described in other systems [79]. Excessive peroxynitrite, related to accumulation of nitrotyrosine in the tissues,

has been shown to uncouple eNOS by oxidation of $BH_4$. Oxidization of NOS cofactor $BH_4$ leads to eNOS uncoupling (monomerization), and results in increased ROS and reduced NO production by this enzyme. Therefore, the initial oxidative loss of $BH_4$ in response to increased ROS production by NADPH oxidases seems to result in the amplification of oxidative stress due to the resulting loss of NO production and increased NOS-dependent $O_2^-$ generation. In support of this speculation are the studies demonstrating beneficial effect of $BH_4$ administration in diabetic nephropathy [80]. Also, short-term BH4 treatment restores plasma levels of $BH_4$ and eNOS dimeric state associated with increased NO and decreased ROS production in the diabetic glomeruli. In this regard, transgenic mice with endothelial-targeted overexpression of guanosine triphosphate-cyclohydrolase I (GTPCH-1), a rate-limiting enzyme in $BH_4$ *de novo* synthesis, had normalization of NO-mediated endothelial functions under hyperglycemic states [81]. Importantly, several clinical studies have reported beneficial effects of $BH_4$ administration on the endothelial functions in patients with cardiovascular risk factors, such as, hypercholesterolemia, smoking, hypertension, diabetes and coronary artery disease [82, 83]. Other therapeutic measures that can prevent the oxidation of $BH_4$ include supplementation of vitamin C [84]. Recently, it has also been observed that folate supplementation is capable of $BH_4$-dependent potentiation of eNOS functions *in vitro* and improvement of NO-mediated endothelial function *in vivo* [85]. Interestingly, recent studies also have shown that statins, in addition to augmenting eNOS expression, may also potentiate GTPCH-1 gene expression and $BH_4$ synthesis, thereby improving eNOS functions [86]. Thus, the mechanisms that modulate $BH_4$ functions in human vascular disease, including diabetic nephropathy, represent promising targets for future therapeutic interventions. Although, the short-term $BH_4$ treatment corrects NOS uncoupling the malondialdehyde (MDA) and 8-OHdG expression, indicative of oxidative stress, however, it was not found to be different in glomeruli of diabetic rats versus those treated with $BH_4$-treated diabetic rats [78]. The effect of long-term $BH_4$ treatment in diabetic nephropathy remains to be investigated.

In addition to oxidative inactivation to $BH_2$, $BH_4$ bioavailability can be potentially influenced at transcriptional levels by the downregulation of GTPCH1 [87, 88]. Recently Chalupsky *et al.* described a role of dihydrofolate reductase in the transcriptional regulation of $BH_4$ and NO bioavailability in the endothelium [89]. Endothelial NAD(P)H oxidase-derived $H_2O_2$ downregulated the dihydrofolate reductase expression, which resulted in $BH_4$ deficiency and uncoupling of eNOS. Moreover, Xu *et al.* demonstrated that diabetic hyperglycemia activates 26S proteosome *via* peroxynitrites and results in the ubiquitination and degradation of GTPCH1 [90]. Based on these studies, one can propose the following working model for the effects of hyperglycemia on the oxidative stress and decreased bioavailability of NO in the diabetic glomeruli. First, high glucose levels increase the formation of $O_2^-$ through NAD(P)H oxidase activation. NO, produced by NOS, and $O_2^-$ combine to form peroxynitrite anion. Oxidation of $BH_4$ by ROS, such as, peroxynitrite results in the formation of $BH_2$, which inactivates eNOS cofactor functions, suggesting that reduction in availability of $BH_4$ uncouples NOS, leading to a decrease in bioavailable NO and further boost the formation of $O_2^-$ in diabetic glomeruli. This may mean that the initial oxidative loss of $BH_4$ by ROS production *via* NADPH oxidases may lead to the amplification of oxidative stress that is overtly seen in later stages [88]. This paradigm thus suggests that even small initial changes in ROS production gets amplified and are later modulated *via* interactions between different biological systems pertaining to various oxidases.

Besides supplementation of vitamin C or $BH_4$ to improve modality from oxidative stress, the Angiotensin II receptor blocker (ARB) has also been described to reduce such a stress in human essential hypertension associated with diabetic nephropathy and in mouse models of type 1 and 2 diabetic nephropathy [91 – 93]. Essentially, the ARB treatment ameliorates

NAD(P)H oxidase activity and reduces oxidative stress in these diabetic models [32, 94]. As a result, ARB restores $BH_4$ bioavailability in diabetic nephropathy by reducing catabolism and oxidation of $BH_4$ to $BH_2$. The reduced $BH_4$ oxidation by ARB leads to eNOS recoupling and results in increased NO and reduced ROS production. Moreover, ARB treatment restores GTPCH1 expression levels in diabetic kidney. Here, it is tempting to speculate the use of GTPCH1 as a potential therapeutic target to restore eNOS uncoupling in diabetes, as supported by two novel observations. First, the statins have been reported to potentiate GTPCH1 gene expression and $BH_4$ synthesis, thereby improve eNOS function [86]. Second, the calcium channel blocker, benidipine, has been also reported to be effective in preventing $BH_4$ deficiency by activating GTPCH1 in type 2 diabetic rats [95].

Finally, with respect to overall pathobiology eNOS it is of interest that restoration of endothelial dysfunction (ED) is accompanied with reduction of albuminuria, one of the hallmarks of glomerular damage in diabetic nephropathy. Microalbuminuria may reflect potential involvement of endothelial dysfunction in the alteration of permselectivity across the glomerular capillary wall in diabetes. Theoretically, ED may cause albuminuria both directly, by increasing glomerular pressure and glomerular basement membrane permeability, and indirectly, by influencing mesangial cell and podocyte functions in a paracrine fashion, *e.g.*, through inflammatory mechanisms. However, the precise molecular pathways by which ED causes (micro)albuminuria have yet to be worked out.

## XANTHINE OXIDOREDUCTASE (XOR)

Xanthine oxidoreductase (XOR), an enzyme that catalyzes the oxidation of xanthine and hypoxanthine to uric acid, is another possible generator of ROS [96]. The XOR exists in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. The former reduces $NAD^+$, whereas the latter prefers molecular oxygen as an electron acceptor, leading to the production of both $O_2^-$ and $H_2O_2$. Under physiological conditions, there is very little activity of xanthine oxidase. Although, XOR-derived ROS have been implicated in the oxidative stress in diabetic state [96, 97], its contribution to the pathogenesis of diabetic complications is somewhat controversial.

Several lines of evidence indicate that hyperuricemia is a risk factor for renal complications in diabetes. Cirillo *et al.* reported that fructose increases intracellular uric acid by XOR, and that in turn results in the induction of monocyte chemotactic protein-1 (MCP-1) [98]. Other investigators have demonstrated that lowering uric with xanthine oxidase inhibitor, allopurinol, reduces albuminuria and ameliorates tubulointerstitial injury [96]. These effects were associated with a reduction in ICAM-1 expression by tubular epithelial cells and subsequent decrease in inflammatory cell infiltration. In this regard, uric acid has been described to directly induce ICAM-1 expression in the human proximal tubular cell *in vitro* [98]. These data indicate that hyperuricemia may lead to an increase in oxidative stress which could play a role in the pathogenesis of tubulointerstitial injury associated with diabetic nephropathy.

## ADVANCED GLYCATION END PRODUCTS (AGEs)

Non-enzymatic glycation of proteins with the formation and then accumulation of advanced glycation end-products (AGEs) occurs ubiquitously and irreversibly in patients with diabetes [99,100]. Nonenzymatic glycation occurs through the covalent binding of aldehyde groups of glucose to free ε-amino groups of proteins, forming a labile Schiff's base. The initial Schiff's base undergoes rearrangements to a more stable ketoamine, called Amadori's product. These initial Amadori's products can generate a variety of other reactive carbonyl compounds, which react with amino groups of other proteins to form intermediate glycation products, such as, 3-deoxy-glucosone, glyoxal and methyl-glyoxal. These initial and

intermediate glycation products undergo a complex series of further chemical rearrangements to yield irreversible AGEs, including: 2-(2-furoyl)-4(5)-furanyl-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrroles (AFGPs), N- -carboxy-methyl-lysine (CML), pyrraline and pentosidine.

These AGEs can accumulate in tissues and generate ROS [101]. Serum and tissue AGEs' levels seem to correlate with the severity of diabetic complications [102]. It has been hypothesized that accumulation of AGEs plays a causal role in the development of diabetic complications, including nephropathy [103 – 105]. AGEs are involved in the development of renal complications *via* two general mechanisms, non-receptor-dependent and receptor-mediated interactions [106]. The former mechanism includes modifications of various components of extracellular matrix (ECM) molecules, such as, collagens. Glycation of collagens yields cross-linking of these molecules which as a result acquire extreme degree of resistance to collagenase digestion and thereby perturbed metabolic turnover of ECM [107]. Glycation of proteins also impairs the integrity of ECM by disrupting self-assembly of their components and eventually affects cellular functions. This non-enzymatic AGE modification is not only confined to proteins but it also occurs in DNA or lipids [101].

With regard to the receptor mechanisms, several AGE-binding proteins have been identified, including lactoferrin, oligosaccharyl transferase complex protein 48 (AGE-R1) and 80K-H protein (AGE-R2), galectin-3 (AGE-R3), and the receptor for AGE (RAGE) [108, 109]. The RAGE is a newly identified member of the immunoglobulin superfamily of cell-surface associated molecules [110, 111]. The protein has an extra cellular segment that comprises a V(variable)-type immunoglobulin domain that is inclusive of two putative N-linked glycation sites, followed by two C(constant)-type domains and a short cytoplasmic tail; and these domains are stabilized by internal disulfide bridges between cysteine residues. RAGE is expressed on the surface of a variety of cell types, including endothelia, smooth muscle cells, lymphocytes, monocytes and neuronal cells [111]. Interaction of AGEs with RAGE triggers the generation of ROS [112]. NAD(P)H oxidase has been incriminated to mediate generation of ROS during AGE:RAGE interactions. The role of ROS in AGE:RAGE-mediated effects has been supported by studies demonstrating the inhibitory effect of antioxidants, such as, N-acetylcysteine, probucol, vitamin E and pyrrolidine dithiocarbamate on diabetic complications [113, 114].

So far from the above discussion one can say that the oxidative stress certainly plays a central role in the pathophysiological mechanisms underlying the development of various complications of diabetes [112]. Also, one can conclude that the oxidative stress is caused by an increased ROS production *via* multitude of mechanisms. But it is noteworthy to point out that the oxidative stress is enhanced if there is a decreased or inefficient removal of ROS. This is supported by the fact that the activity of superoxide dismutase (SOD) and the levels of vitamin E, a lipid soluble antioxidant belonging to the tocopherol family, have been found to be decreased in diabetes [113, 114]. Although diverse mechanisms have been alluded above for the pathogenesis of diabetic nephropathy, it is worth mentioning here that there are many other redox sensitive signaling pathways and various transcription factors that as well contribute to the oxidative stress in diabetic *milieu* and these deserve some discussion.

## REDOX REGULATION OF TRANSCRIPTION FACTORS

Hyperglycemia induces a vast variety of genes that are relevant to a wide variety of pathobiological processes, including extracellular matrix (ECM) accumulation, cellular hypertrophy, inflammatory responses, prothrombosis, hyper-coagulabiity and accelerated apoptosis. A range of transcription factors have been implicated in the induction of these

genes. They include nuclear factor kappa-B (NF-κB), activator protein-1(AP-1), cAMP response element binding protein (CREB), nuclear factor of activated T cells (NFAT) and stimulating protein 1 (Sp1) [115 – 117].

Among them, NF-κB plays a central role in the initiation of immune and inflammatory responses and apoptosis in a hyperglycemic *milieu*, and in this regard redox regulation of NF-κB has been extensively investigated [118, 119]. In general, NF-κB remains in the cytosol as an inactive heterodimeric form, composed of p50 and p65 subunits, associated with its inhibitory protein, IκB. NF-κB is activated with a common pathway of phosphorylation and subsequent proteasome-mediated degradation of IκB. Dissociation from IκB allows NF-κB heterodimer to translocate into the nucleus, where it binds to κB-responsive elements in the target sequences, and thus activates gene transcription. A large number of genes contain κB-responsive elements in their regulatory DNA sequences, including in VEGF, IL-6, TNF-α, RAGE and cell adhesion molecules. In the common pathway alluded above, a cascade of serine kinases induce IκB phosphorylation upon cellular stimulation by inflammatory cytokines, free fatty acid hyperglycemia. This IκB kinase (IKK) is also a heterodimeric complex made up of catalytic subunits, IKKα and IKKβ and an inhibitor subunit IKKγ. It is noteworthy to mention here that IKK is activated mainly by the upstream IKKβ serine kinase [44, 120].

ROS participate NF-κB activation by several different mechanisms [121]. ROS modify IκB so that it becomes susceptible to proteasomal degradation. Treatment with antioxidant or overexpression of gluthathione peroxide inhibits IκB degradation and subsequent activation of NF-κB by the cytokine activation. ROS is also reported to activate IKK and enhance the phosphorylation of downstream substrate, IκBα. On the other hand, oxidative stress also negatively regulates the transcriptional activity of NF-κB *via* oxidation of thiol residues that are confined to the binding region of NF-κB. Oxidation of these sites attenuates binding activity of NF-κB to negatively charged target DNA of various genes. This oxidative stress effect on the NF-κB activation may sound contradictory, but the effects are too elaborate and these differences can be reconciled. In this regard, the net effects of oxidative stress on transcriptional activity of NF-κB differ depending upon the levels of redox state. This contention derives a certain degree of indirect support from the experiments with interleukin-2 (IL-2). The production of IL-2 is enhanced by exposure of T-cells to relatively low concentration of $H_2O_2$ (10 mM), whereas at high concentration (100 mM) it is inhibited [122]. The downstream effects of NF-κB include an increase in the expression of pro-inflammatory genes, including that of the monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-8 [123, 124]. These chemokines are responsible for recruiting and activating neutrophils, macrophages, and T-cells. Finally, the importance of NF-κB in the pathogenesis of diabetic nephropathy lies in the fact that it is activated by the hyperglycemic oxidative stress in renal mesangial cells, endothelial cells and proximal tubular epithelial cells [125].

AP-1 is another major redox-sensitive transcriptional factor that may be relevant to the biology of diabetic nephropathy [117, 126]. AP-1 is a heterodimer complex, typically composed of c-Fos and c-Jun. ROS stimulate mRNA expression of both the genes. AP-1 exerts diverse biological effects upon binding with the corresponding TPA-responsive elements (TREs), also known as AP-1 sites that have been localized in a wide variety of genes. The AP-1 activation leads to cellular proliferation, hypertrophy and differentiation *via* the modulation of a wild variety of genes, including the cell-cycle regulators, such as, cyclins, p53, p21 and p16 [126].

## CELLULAR SIGNALING PATHWAYS AFFECTED BY THE OXIDATIVE STRESS

The mitogen-activated protein (MAP) kinases (MAPKs) are a superfamily of serine/threonine kinases consisting of extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38MAP kinase and c-Jun NH2-terminal kinase (JNK) [127]. These MAPKs regulate diverse cellular activities ranging from cell proliferation, differentiation, motility, apoptosis and survival. The MAPKs are activated by a vast variety of different stimuli. For instance, ERK1/2 are preferentially activated in response to growth factors, while the JNK and p38 MAP kinases are more responsive to exogenous and endogenous stress stimuli ranging from oxidative stress, osmotic shock, ionizing radiation to cytokine stimulation. Therefore, JNK and p38MAP kinases are known as “stress-activated protein kinase” (SAPK). The MAP kinases including p38MAP kinase are implicated in the pathogenetic mechanisms relevant to diabetic nephropathy [128]. Increased activity of MAP kinases is detected in the kidney of rodent models of diabetes. A prolonged activation of p38MAP kinase is associated with influx of inflammatory cells, increased ECM synthesis and cellular proliferation - the processes that are integral to the pathogenesis of diabetic nephropathy.

## HEMODYNAMIC ALTERATIONS IN THE RENAL MICROCIRCULATION RELATED TO OXIDATIVE STRESS

Glomerular hyperfiltration is a well recognized early feature of diabetic nephropathy [65, 66]. Glomerular filtration rate (GFR) is well maintained even during fluctuations in the systemic blood pressure by autoregulatory mechanisms operative at level of the glomerular afferent arterioles, which make fine adjustments in the intraglomerular transmural pressure. Using micropuncture techniques to measure intra-glomerular pressure, Brenner and his colleagues elucidated that the autoregulatory mechanisms are impaired and glomerular capillary pressure is increased in a wide range of disease models including in diabetes [129]. Apparently, hyper-glycemia impairs the autoregulatory adjustments in the afferent arterioles *via* multiple mechanisms, such that the renal plasma flow (RPF) and GFR are tremendously increased from the basal levels [130]. First, since these effects are abolished with the treatment of furosemide it would suggest that hemodynamic changes induced by hyperglycemia are mediated *via* tubuloglomerular feedback [131]. Secondly, derangements in the voltage-gated L-type $Ca^{2+}$ channels and eicosanoid synthesis have been reported to impair myogenic responsiveness of the afferent arterioles in diabetes [132]. Thirdly, hyperglycemia has been shown to activate renin-angiotensin system (RAS), in particular at the local or tissue levels, resulting in vasoconstriction of efferent arterioles. Since the latter are much more sensitive to the vasoconstrictive effect of Ang II than afferent arterioles, this would eventually lead to intraglomerular hypertension and an increase in the capillary pressure. These changes are ameliorated by angiotensin converting enzyme (ACE) inhibitor, a RAS inhibiting agent, and Ang II receptor blocker (ARB). The renoprotective effects of these inhibitors have been well-documented by micropuncture studies in animals where reduction in intraglomerular pressure was observed [133]. Similar renoprotective effects of ACE inhibitors have been described in various clinical studies, where reduction in proteinuria without any significant change in the systemic blood pressure was observed [134–136]. Likewise, ARB reduced the rise of creatinine, degree of proteinuria, GFR decline and progression to ESRD in patients with type 1 or 2 diabetes [137–140]. Also, reduction in urinary excretion of angiotensinogen, oxidative stress markers and inflammatory cytokines following ARB administration in patients with type 2 diabetic nephropathy has been observed [141]. Intriguingly, although RAS inhibition exerts renoprotective effect, the plasma renin activity (PRA) usually is low in diabetic patients.

Nevertheless RAS of local renal tissue has been demonstrated to be disproportionately activated in diabetes [142].

The primary cause and exact mechanism of local RAS activation in diabetes have not been fully elucidated, but local RAS are presumed to be regulated independently from systemic RAS. In this regard, the intrinsic cells of the glomerulus, *e.g.*, mesangial cells and podocytes, have been reported to express Ang II and $AT_1$ receptors. These receptors may contribute to the regional activation of RAS with ensuing mechanical pressure-induced damage and thereby an accentuation and perpetuation of the hyperglycemia mediated oxidative or glycative stress injuries to the kidney [143, 144]. With respect to mesangial cells, studies by Yamamoto *et al.* and Kagami *et al.* carried out more than a decade and half ago demonstrated that these cells respond to Ang II stimulation with excessive production of various ECM proteins *via* the induction of TGF-β, a potent fibrogenic cytokine that is heavily incriminated in the genesis of glomerular lesions seen in diabetic nephropathy [145, 146]. More recently, Liebau *et al.* reported that human podocytes express all the components of RAS to generate Ang II [147]. They also showed that Ang II induced a concentration-dependent increase in cytosolic $Ca^{2+}$ *via* $AT_1$ receptors in podocytes. Secretion of Ang II from podocytes in the cultured medium was also detected. Interestingly, Ang II generation by the podocytes was not influenced by mechanical stress or inhibitors of ACE, renin or of any other signaling kinase, and thus the local regulation of RAS need to be further investigated to get at a clearer picture in regard to contribution of podocyte to the oxidative stress in diabetic nephropathy. Although as indicated above that the plasma renin activity (PRA) levels are low in diabetic patients, surprisingly the prorenin levels have been found to be high, suggesting prorenin:(pro)renin receptor interactions may play an important role in the pathophysiology of various complications in diabetes [148 – 150]. This ligand:receptor interaction stimulates intracellular signaling pathways, including phosphorylation and activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2), which may enhance the oxidative stress (*vide supra*). Indeed synthetic inhibitory peptide decoy of (pro)renin receptor have been found to exert an ameliorative effect on diabetic nephropathy [151]. The precise mechanisms as to how this prorenin:(pro)renin receptor interaction contributes to the oxidative stress are unclear; nevertheless, the RAS has been demonstrably implicated in the development of oxidative stress; and a cross talk signaling between hemodynamic changes (capillary pressure load) and oxidative stress adversely impact the progression of diabetic nephropathy [152, 153] (Fig. 2).

Another recently described new signaling mechanism includes the role of GPR91, a G-protein-coupled receptor (GPCR) in the activation of RAS. The GPR91 responds to citric acid cycle intermediaries, such as succinate and α-ketoglutarate, with a consequential rise in system blood pressure, which apparently gets negated in GPR21 deficient mice [154]. The events of this new paracrine signaling transduction pathway leads to the changes in cytosolic $Ca^{2+}$, NO and $PGE_2$ in the endothelium of juxtaglomerular apparatus (JGA) and and synthesis of Ang II [155]. These elegant studies by Peti-Peterdi and his colleagues clearly establish that high glucose *via* succinate receptor, GPR91, mediates the release of renin which then induces the generation of Ang II and thus modulates the RAS [156].

Finally, the subject matter of pressure load as a major inducer of cardiovascular complications relevant to diabetic nephropathy need to be stressed. It has been suggested that Ang II-Ang II type 1($AT_1$) receptor system plays an important role in such complications [157]. In this regard, a novel mechanism of mechanical stress-induced activation of the $AT_1$ receptor independent of Ang II has been recently described [158]. It has been shown that the mechanical stress activates ERKs and increases phosphoinositide production and association of the $AT_1$ receptor with the cytosolic signaling proteins, *e. g*, Janus kinase 2 without the involvement of Ang II. Similar Ang II independent $AT_1$ receptor

activation and downstream signal transduction events have been reported in the mesangial cells. Conceivably, these signaling cascades would eventually boost further the generation of ROS *via* NADPH oxidase activation and ERK phosphorylation, and thus catachysmically worsening the progression of diabetic nephropathy [159].

## CONCLUDING REMARKS

The above discussion highlights that a number of pathways and molecules are involved in the induction of oxidative stress in diabetic nephropathy. The major sources seem to be mitochondria and NAD(P)H oxidase complex, and both have received much attention in recent years. There are several other pathways or molecules that also participate in the generation of reactive oxygen species (ROS), the important ones include AGEs, transcription factor NFκB and the process of eNOS uncoupling. Since the ROS appear to be the common denominator for the causation of renal injury they may be the most suitable targets for developing novel therapeutic agents to ameliorate reno-vascular complications of diabetes. The amelioration of diabetic nephropathy can be achieved to a certain extent by interrupting other cellular signaling pathways leading to the production of ROS and Ang II, the most relevant among them being inhibitors of PKC, MAPK, TGF-β and of citric acid cycle intermediaries. The subject matter of therapeutic agents in the reno-vascular complications of diabetes has been thoroughly discussed in some of the most recent reviews, and the reader is referred to these articles to gain their in-depth knowledge [160 – 163]. Nevertheless, at present one can say with certain degree of confidence that dampening the activity of renin-angiotensin system (RAS), which contributes to the generation of ROS, has a remarkable reno-protective effect in shielding the patient from progression to diabetic nephropathy besides instituting a strict normoglycemic control.

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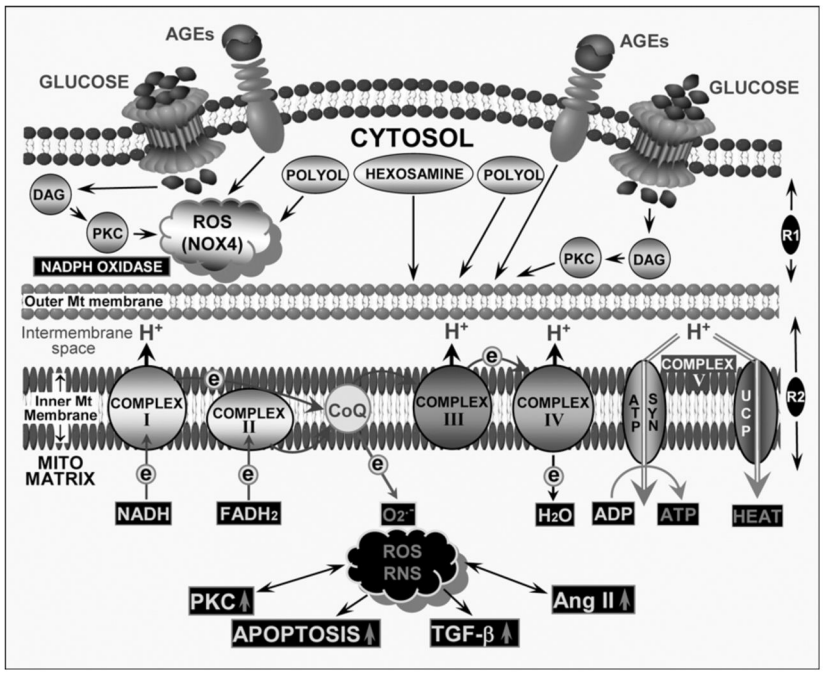


**Fig. 1.**

Summary of events leading to the generation of cytosolic ROS (R1) and mitochondrial ROS (R2). Extra-mitochondrial cytosolic ROS generation occurs following increased glucose flux and activity of polyol pathway and AGEs:RAGE interaction and *via* NADPH oxidase system, such as NOX4. The mitochondrial matrix ROS and reactive nitrogen species (RNS) are generated *via* the well-characterized electron transport chain Complex I to IV redox carriers localized in the inner mitochondrial membrane. During hyperglycemia there is an increased donation of electrons (e) by powerful NADH and $FADH_2$ reducing agents of respective Complex I and II with pumping of protons ($H^+$) into the inter-membrane space, giving rise to an increased mitochondrial membrane potential. As a result the electron transport at complex III is inhibited with consequential backing up of the system and prolonged half life of free-radical intermediates of Coenzyme Q (CoQ), leading to an increase in the reduction of $O_2$ to superoxide ($O_2^{\cdot -}$) and production of ROS. The generated ROS/RNS release Cytochrome C, activate caspases and induce apoptosis. They also modulate the activity of Ang II, PKC and TGF-β, which would ultimately affect the synthesis of ECM. Intriguingly, the PKC and Ang II *via* feedback loop lead to the generation of ROS/RNS (double headed arrow), and this sets up a vicious cycle resulting in perpetuation of renal injury.

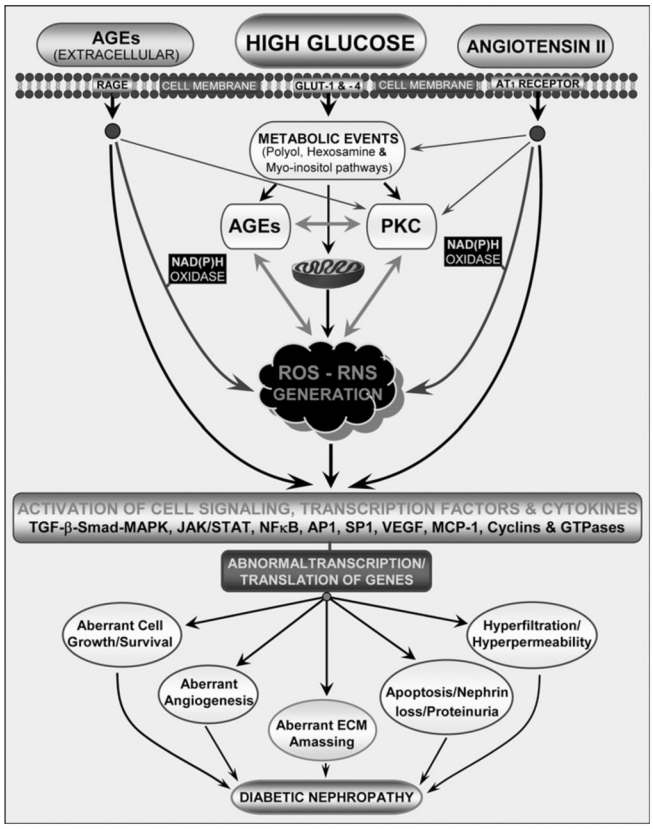


**Fig. 2.**

An overview of different signaling events induced by exposure of renal cells to high glucose concentrations, with resulting altered expression of various genes and cellular abnormalities leading to diabetic nephropathy. The schematic drawing also highlights the hypothetical cross-talk between the AGE-RAGE and renin-angiotensin system (RAS); and the reciprocal-cyclical modulation of the events between AGEs, ROS and PKC, with ROS as the central mediator. AGEs – advanced glycation end products, RAGE – receptor for AGEs, ROS – reactive oxygen species, RNA – reactive nitrogen species, PKC – protein kinase C, ECM – extracellular matrix.