Published in final edited form as:
Am J Kidney Dis. 2014 February ; 63(2 0 2): S63–S83. doi:10.1053/j.ajkd.2013.10.047.

New Insights Into Molecular Mechanisms of Diabetic Kidney Disease

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Abstract

Diabetic kidney disease remains a major microvascular complication of diabetes and the most common cause of chronic kidney failure requiring dialysis in the United States. Medical advances over the past century have substantially improved the management of diabetes mellitus and thereby have increased patient survival. However, current standards of care reduce but do not eliminate the risk of diabetic kidney disease, and further studies are warranted to define new strategies for reducing the risk of diabetic kidney disease. In this review, we highlight some of the novel and established molecular mechanisms that contribute to the development of the disease and its outcomes. In particular, we discuss recent advances in our understanding of the molecular mechanisms implicated in the pathogenesis and progression of diabetic kidney disease, with special emphasis on the mitochondrial oxidative stress and microRNA targets. Additionally, candidate genes associated with susceptibility to diabetic kidney disease and alterations in various cytokines, chemokines, and growth factors are addressed briefly.

Keywords

End-stage renal disease (ESRD); diabetes mellitus; diabetic kidney disease; pathogenesis

INTRODUCTION

The mechanisms responsible for the development and progression of diabetic kidney disease remain poorly understood. However, it is known that progression of diabetic kidney disease correlates closely with level of hyperglycemia,[1–3] and improving glycemic control decreases the rate of progression of diabetic kidney disease and loss of kidney function.[3,4] Prolonged hyperglycemia leads to chronic metabolic and hemodynamic changes that modulate various intracellular signaling pathways, transcription factors, cytokines, chemokines, and growth factors.[5,6] The cumulative result of these changes promotes structural abnormalities in the kidney, such as glomerular basement membrane thickening, podocyte injury, and mesangial matrix expansion, plus the occurrence of glomerular sclerosis and tubulointerstitial fibrosis associated with declining glomerular filtration rate (GFR).

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This article is part of a supplement that was developed with funding from Novo Nordisk.

To improve our current understanding of the pathogenesis of diabetic kidney disease, we need to identify the mechanisms by which chronic hyperglycemia results in structural changes in the kidney. Experimental evidence suggests that a myriad of molecular pathways may be implicated in the development of diabetic kidney disease. These pathways include increased oxidative stress, enhanced flux into the polyol and hexosamine pathways, activation of protein kinase C (PKC) and transforming growth factor β (TGFβ)/Smad/mitogen-activated protein kinase (MAPK) signaling pathways and increased formation of advanced glycation end products (AGEs). Studies in mesangial cells, endothelial cells, and podocytes have linked hyperglycemic activation of these critical pathways to increased formation of extracellular matrix (ECM) proteins, cellular hypertrophy, and apoptosis in the kidney. In addition, high glucose levels can activate the proinflammatory transcription factor nuclear factor-κB (NF-κB), resulting in increased inflammatory gene expression, in part through oxidative stress, AGEs, PKC, and MAPKs.[7,8] Finally, hemodynamic changes can be contributing to the pathogenesis of diabetic kidney disease through activation of the renin-angiotensin system (RAS) and vascular endothelial growth factor (VEGF) signaling cascades.[9–12]

This review discusses the latest published data for molecular mechanisms associated with pathologic changes in the kidney during the development of diabetic kidney disease. Although many factors have been implicated in the pathogenesis of diabetic kidney disease, we mainly focus on several specific topics more recently associated with the pathogenesis and progression of diabetic kidney disease, including the role of mitochondrial oxidative stress and mitochondrial fission, microRNAs (miRNAs), epigenetics, and the Rho family of small GTPase proteins.

MITOCHONDRIAL OXIDATIVE STRESS

Mitochondria are best known for producing energy in the form of ATP (adenosine triphosphate) through the concerted actions of approximately 100 proteins located on the inner mitochondrial membrane, which together are termed the electron transport chain. The electron transport chain delivers electrons to oxygen (O_2) by a chain of hydrogen ion (H^+) pumps. These H^+ pumps (complexes I-IV) establish an H^+ gradient across the inner mitochondrial membrane by pumping H^+ down the energy gradient into the space between the inner and outer mitochondrial membranes, and the electrochemical energy of this gradient then is used to drive ATP synthesis. However, other important metabolic reactions occur in mitochondria, including steroid hormone and porphyrin synthesis, the urea cycle, lipid metabolism, glucose sensing/insulin regulation, and cellular calcium ion homeostasis. Mitochondria also are the primary source of reactive oxygen species (ROS) within most mammalian cells.[13–15]

ROS are molecules derived from O_2 that can readily oxidize other molecules. Most intracellular ROS are derived from superoxide ($O_2^{\bullet -}$), which is generated by the 1-electron reduction of O_2. Under physiologic circumstances, ATP synthesis is strictly coupled to O_2 consumption. However, mitochondria can become partially “uncoupled” in a variety of pathologic conditions whereby O_2 is consumed at a greater rate than what is required to make ATP. Under these conditions, free radical production by the electron transport chain dramatically increases. These free radicals also may react with many different molecules in the cell to cause cellular death and damage to cellular components, including proteins, lipids, carbohydrates, and DNA. For example, oxidation of mitochondrial DNA decreases the accuracy of copying by the mitochondrial polymerase, causing deletions, rearrangements, and other mutations. Also, lipids in the mitochondrial and cellular membranes become peroxidized, reducing their flexibility and making them leaky. Proteins also may be damaged and lose proper tertiary structure as a result of oxidation.

Although it initially was assumed that the production of ROS did not have a physiologic function, ROS now are appreciated to also function as signaling molecules to regulate a wide variety of physiology. For instance, mitochondrial ROS have been implicated as key components of angiogenesis, cell division, circadian rhythms, the immune response, and hypoxia-inducible factor (HIF) signaling cascades. [16,17] An extensive discussion of the literature on mitochondrial ROS generation is beyond the scope of this review; however, an outline of ROS sources within mitochondria will aid in understanding how ROS may play a key role in the structural changes in diabetic kidney disease and the consequences of excessive ROS production, both as toxic byproducts of metabolism and their potential role in signal transduction in the diabetic milieu.

In the mitochondrial matrix, tricarboxylic acid (TCA) cycle enzymes generate 2 electron carriers (NADH [reduced nicotinamide adenine dinucleotide] and $FADH_2$ [reduced flavin adenine dinucleotide]), which donate electrons to the electron transport chain. The electron transport chain consists of 4 protein complexes (I-IV), and each complex incorporates multiple electron carriers. Two electrons donated from NADH to complex I (NADH dehydrogenase) or from succinate to complex II (succinate dehydrogenase) are passed first to ubiquinone (coenzyme Q [CoQ]) to produce ubisemiquinone ($CoQH^{\bullet}$) and then ubiquinol ($CoQH_2$). Ubiquinol transfers its electrons to complex III (ubiquinol:cytochrome *c* oxidoreductase), which then passes them to cytochrome *c*. From cytochrome *c*, the electrons flow to complex IV (cytochrome *c* oxidase) and at last to half of an O_2 to produce H_2O.

ROS levels increase when excess electrons are provided to the electron transport chain. The excess electrons are transferred to O_2, which is converted to superoxide. Thus, the primary ROS made by mitochondria is superoxide, which subsequently is converted to hydrogen peroxide (H_2O_2) by mitochondrial matrix enzyme manganese superoxide dismutase (MnSOD [encoded by *SOD2*]) or by the copper/zinc superoxide dismutase (Cu/ZnSOD [encoded by *SOD1*]), which is located in the mitochondrial intermembrane space.

Although there are 8 sites in the mitochondria with the ability to produce $O_2^{\bullet-}$, complex I and III of the electron transport chain traditionally have been considered as important sources of ROS due to the formation of radicals during electron transfer (flavin mononucleotide [$FMN^{\bullet}$] in complex I and $QH^{\bullet}$ in complex III). For complex III, the ubisemiquinone radical intermediate ($QH^{\bullet}$), formed during the Q cycle at the Q_O site of complex III, is the main source of $O_2^{\bullet-}$. The majority of $O_2^{\bullet-}$ from Q_O is made facing the intermembrane space, and its generation is accelerated by complex III inhibitors distal to this site (eg, antimycin A). Complex I also is a source of ROS, though the mechanism involved is less clear than for complex III. Rotenone and other distal complex I inhibitors can cause $O_2^{\bullet-}$ that faces the matrix side of the inner membrane; at this location, MnSOD would convert it to H_2O_2.

Mitochondrial ROS is associated closely with many pathologic conditions, ranging from inflammation to aging and complex diseases, including Parkinson, Huntington, and Alzheimer disease.[18] Oxidative stress also has been identified as a major culprit in microvascular complications of diabetes, especially in the kidneys, where increased oxidative stress has been shown to decrease O_2 tension in the diabetic kidney.[19–21] Some potential mechanisms related to diabetes-induced mitochondrial ROS production are depicted in Fig 1. Intracellular glucose oxidation begins with glycolysis, which produces NADH and pyruvate. Pyruvate can be transported from the cytoplasm into the mitochondria, where it is oxidized by the TCA cycle to produce 4 molecules of NADH and 1 molecule of $FADH_2$. In the diabetic milieu, there is increased flow of the key substrates NADH and $FADH_2$ to the respiratory chain, which overdrives the electron transport system in the mitochondria, resulting in increased superoxide anion production.[22–24]

Although there are still limited insights into the ROS signaling that occurs in diabetic kidney disease with the potential to influence a host of key downstream signaling pathways, a central role for mitochondrial ROS in microvascular complications of diabetes has been long identified.[22,23,25–32]

Brownlee[25] was among the first to suggest that ROS produced by the mitochondrial electron transport chain could be the driving force in the pathogenesis of diabetic kidney disease, whereby hyperglycemia-induced mitochondrial ROS lead to diabetic complications by several seemingly independent pathways, including PKCβ, aldose reductase, AGEs, and the hexosamine biosynthetic pathway.[24–27,29,33–37] However, the evidence for this model comes mainly from experiments on cultured endothelial cells in which the glucose concentration was increased from 5 to 30 mmol/L, which resulted in increased ROS production, as measured by the rate of oxidation of dichlorodihydrofluorescein (DCFH) to dichlorofluorescein (DCF).[22,23,27] This oxidation event was blocked by inhibitors of mitochondrial pyruvate uptake and succinate dehydrogenase, but not by rotenone, a complex I inhibitor, suggesting that ROS generation at complex II may be important. One puzzling observation is that overexpression of mitochondrial MnSOD prevented hyperglycemia-induced DCFH oxidation.[27] DCFH primarily is sensitive to hydrogen peroxide, nitric oxide, or hydroxyl radicals, and it is not directly oxidized by superoxide. The overexpression of MnSOD should have converted the superoxide generated in the mitochondrial matrix to hydrogen peroxide. Therefore, MnSOD overexpression should have enhanced the DCF signal rather than abolished it. A potential explanation for this effect is that MnSOD detoxifies superoxide into hydrogen peroxide within the mitochondrial matrix, preventing its escape into the cytosol. The hydrogen peroxide then is converted to water by glutathione peroxidase in the mitochondria. Thus, cytosolic DCFH oxidation does not occur in the presence of increased MnSOD. This also implies that DCFH oxidation in the cytosol might have been produced within the mitochondrial matrix.

A recent publication examined whether an antioxidant targeted to mitochondria would block progression of diabetic kidney disease in the Ins2(+/)$^{-}$(AkitaJ) mouse model of type 1 diabetes.[38] To test this hypothesis, the authors orally administered a mitochondria-targeted ubiquinone (MitoQ) over a 12-week period and assayed tubular and glomerular function. The treatment improved tubular and glomerular function, but did not have a significant effect on plasma creatinine levels, although it decreased urinary albumin excretion to the level of nondiabetic controls. Of note, interstitial fibrosis and glomerular damage were reduced significantly in the treated animals. These findings provide evidence that mitochondria-targeted therapies may have benefit in treating diabetic kidney disease. Moreover, overexpression of catalytic antioxidants has been demonstrated to protect against diabetic injury. Craven et al[39] showed that compared with nontransgenic mice, diabetic mice transgenic for Cu/ZnSOD had significantly lower urinary albumin excretion, glomerular hypertrophy, and glomerular expression of TGFβ1 and collagen IV protein. The same group also found that overexpression of MnSOD suppresses increases in collagen accumulation that normally occur when mesangial cells are cultured in high-glucose media.[40] Similarly, Du et al[41] showed that overexpression of MnSOD in bovine aortic endothelial cells prevented high-glucose–induced activation of the PKC, NF-κB, hexosamine, and AGE pathways. Finally, Brezniceanu et al[42] demonstrated that renal catalase overexpression in *db/db* mice lessened ROS generation, angiotensinogen, proapoptotic gene expression, and apoptosis in kidneys of diabetic mice.

Recently, in a study by Wang et al,[43] changes in mitochondrial dynamics were shown to contribute to increased mitochondrial ROS and progression of diabetic kidney disease. Recent observations indicate that mitochondria undergo fission, fusion, and intracellular movement on a rapid timescale. Mitochondria can switch their morphology between

elongated interconnected mitochondrial networks and a fragmented disconnected arrangement. The dynamic nature of mitochondrial networks occurs because fission and fusion operate concurrently and act against each other.[44] Mitochondrial fission and fusion are vital for preserving mitochondrial function and are believed to enable rapid repair of damaged mitochondria and allow mixing of DNA and proteins between mitochondria (Fig 2).

An increasing number of studies have investigated changes in mitochondrial dynamics as important parameters for many disease-related processes. Our group recently has investigated the role of mitochondrial dynamics and specifically mitochondrial fission in the context of diabetic kidney disease.[43] In podocytes of kidneys from diabetic mice, we observed condensed fragmented mitochondria, which were associated with changes in the phosphorylation status of the mitochondrial fission protein dynamin-related peptide 1 (Drp1).

The modulation of Drp1 function has been a topic of great interest. Drp1 exists as small oligomers (dimers/tetramers) that can self-assemble into larger multimeric structures at the mitochondrial outer membrane, where they mediate mitochondrial division through a GTP-dependent conformational change. Drp1 primarily is a cytosolic protein and must be recruited to mitochondria for fission to occur. Drp1 seems to trigger fission by first tethering to mitochondria at specific positions known as constriction sites, then forming multimeric spirals around mitochondria that constrict mitochondrial tubules further and result in mitochondrial fission.[45]

So how does hyperglycemia trigger Drp1 translocation to mitochondria, leading to mitochondrial fragmentation and podocyte apoptosis? The study by Wang et al[43] demonstrated that Drp1 is phosphorylated by Rho kinase (ROCK1) and that this posttranslational modification stimulates translocation of Drp1 from the cytosol to mitochondria, thus increasing fission. Whether inhibiting mitochondrial fission and Drp1 phosphorylation in the setting of diabetic kidney disease would be beneficial is still unclear. However, consistent with these preclinical data, mitochondrial dysfunction and abnormalities in mitochondrial biogenesis, number, morphology, and dynamics in both type 1 and type 2 diabetic patients also have been described extensively. For instance, in biopsy specimens of skeletal muscle, individuals with type 2 diabetes have mitochondria of smaller size and number than healthy controls.[46] In addition, mitochondria of the offspring of diabetic individuals are lower in density than those of controls.[47]

Taken together, the mitochondrial respiratory chain represents the main intracellular source of ROS in most tissues. Under normal conditions, these oxidants are kept at nontoxic levels by a number of antioxidant defenses and repair enzymes. The delicate balance between antioxidant defenses and ROS production may play a critical role in diabetic kidney disease, in which the resulting oxidative insult eventually could cause kidney damage. Future examination of the members of the fission and fusion machinery and the development of diabetic kidney disease may enhance our understanding of the role of mitochondrial dynamics in diabetic kidney disease.

NADPH OXIDASE

NADPH oxidase (NOX) is a multiprotein cytosolic enzyme complex initially identified in phagocytes, which generate ROS in response to bacterial infections. As shown in Fig 3, this enzyme is a heme-containing protein complex. The catalytic component of NOX is known as NOX protein family, which consists of 7 members, including NOX1-5 and dual oxidase (Duox)1 and Duox2. Recently, within a short period, many features of the structure, activity, cell biology, and physiology of the NOX proteins have been described. All members of the

family have 6 predicted transmembrane domains, motifs for NADPH and FAD binding, and conserved paired histidines that could ligate heme groups. Other components of NOX include regulatory subunits $p22^{phox}$, $p47^{phox}$ (or its homologue NOXO1), $p67^{phox}$ (NOXA1), $p40^{phox}$, and the major binding partner Rac.[48,49] However, there are conflicting data regarding the contribution of Rac to NOX3 activity, and there is little evidence that Rac figures in the activities of NOX4, NOX5, or Duox proteins. For NOX5 and the Duox proteins, calcium seems to serve as the definitive regulator. The NOX enzyme normally is inactive in the resting state, but with appropriate stimulation, translocation and association of cytoplasmic subunits lead to rapid activation. This activated cytoplasmic complex associates with subunits in the membrane to form a functional enzyme with very specific regulatory mechanisms, tissue and subcellular patterns of expression, downstream targets, and functions.

How does NOX generate ROS? The patriarch of the NOX protein family is NOX2 (also known as $gp91^{phox}$), which serves as the catalytic component of the phagocyte NOX. NOX2 possesses flavin- and heme-binding regions that are involved in the transfer of electrons. Associated with $p22^{phox}$ in the plasma membrane and membranes of selected intracellular compartments, NOX2 operates as an electron transferase, shuttling electrons from NADPH in the cytoplasm to O_2, the electron acceptor, thereby generating superoxide anion (Fig 3). The reduced substrate, NADPH, binds to NOX2 on the cytoplasmic side of the membrane and releases 2 electrons, which are transferred first to FAD, then to the first and second heme groups, and finally accepted by 2 successive molecules of O_2 on the opposite side of the membrane, producing 2 molecules of superoxide radical.[50–54]

Although NOX proteins represent the major non-mitochondrial sources of ROS, identification of the specific NOX protein(s) responsible for ROS production in a particular tissue or organ system has been challenging. In the kidney, it is known that all components of the NOX complex, including $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$, as well as the NOX isoforms 1, 2, and 4, are expressed in a multitude of cell types, including fibroblasts, endothelial cells, vascular smooth muscle cells, mesangial cells, tubular cells, and podocytes. Although NOX activity has been linked to a variety of physiologic functions, including maintenance of vascular tone, surveillance of ambient O_2 tension, and promotion of angiogenesis, the signaling pathways that regulate NOX activity are not well established. Aberrant NOX activity can precipitate endothelial cell dysfunction and contribute to atherosclerosis, hypertension, congestive heart failure, and ischemia-reperfusion injury.

An emerging body of evidence suggests that NOX may play a pathogenic role in diabetic kidney disease. For instance, mRNA expression of essential subunits of NOX, NOX4 and $p22^{phox}$, in the kidneys of streptozotocin-induced diabetic rats was found to be markedly increased compared with control rats.[55] Immunohistochemistry showed that protein levels of NOX4 and $p22^{phox}$ are increased in both distal tubular cells and glomeruli. In the diabetic kidney, insulin treatment for 2 weeks fully restores these components to control levels. Furthermore, pharmacologic inhibition of NOX with apocynin prevents upregulation of $p47^{phox}$ and $gp91^{phox}$ overexpression and retards the mesangial matrix expansion seen in experimental diabetic kidney disease.[56,57] Finally, using antisense oligonucleotides against NOX4, Gorin et al[58] reported a significant improvement in renal hypertrophy and fibronectin accumulation in streptozotocin rats. These results suggest that the expression of NOX subunits NOX4 and $p22^{phox}$ is upregulated in diabetic kidneys, and that NOX4 may play a significant role in the pathogenesis of diabetic kidney disease.

Several other reports have suggested that the expression of $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$ is upregulated in animal models of diabetes[59,60] and patients with diabetes and coronary artery disease.[61] Moreover, NOX-driven superoxide production has been reported to contribute to

vascular dysfunction in an animal model of type 2 diabetes.[59] In addition, at least one report showed that NOX activity also is increased in other microvascular complications of diabetes, including in the retina of diabetic rats.[62]

In summary, these observations suggest that increased oxidative stress in the kidney may have a fundamental role in the development of microvascular complications of diabetes. Although these are exciting times to study the role of ROS in vascular complications of diabetes, many challenges to the diverse group of investigators engaged in their study remain to be addressed, and future studies are needed to translate into therapeutics the role of ROS in patients with diabetes.

MICRORNAS

miRNAs are a class of short (21–24 nucleotides) non-coding RNAs that regulate gene expression at the posttranslational level by targeting mRNAs in their 3′ untranslated regions (UTRs).[63,64] miRNA sequences are highly conserved among animals and plants, and currently more than 1,500 miRNA sequences have been identified in *Homo sapiens*.[65] The target gene prediction databases based primarily on Watson-Crick base pairing (eg, TargetScan [www.targetscan.org], miRanda [www.microrna.org], and PicTar [www.pictar.org]) have suggested that miRNAs may have hundreds of mRNA targets, implying that several mRNAs may be posttranslationally repressed or degraded by a single miRNA, and thus 1 of 3 genes may be regulated by miRNAs. However, several different miRNAs can bind to and cooperatively act on a single mRNA target. Interestingly, single miRNAs also could have multiple target sites in the 3′ UTRs of a particular mRNA in order to increase their repression efficiency.

miRNAs are transcribed initially as independent noncoding genes or are embedded within introns of protein-coding genes. They typically are transcribed by RNA polymerase II into long (up to several kilobases) primary transcripts called pri-miRNA. In the canonical miRNA biogenesis pathway, pri-miRNA processing occurs in 2 steps mediated by 2 members of the ribonuclease III (RNase III) family, namely Drosha and Dicer. Pri-miRNAs initially are processed in the nucleus by the enzyme Drosha to become approximately 70- to 100-nucleotide stemloop precursor strands (pre-miRNA)[66,67] (Fig 4). This precursor then is exported into the cytoplasm, where it is bound to Dicer. Dicer cleaves the precursor miRNA into a mature 22-nucleotide miRNA/miRNA* duplex (comprising the mature miRNA guide strand and an miRNA* passenger strand).[68] While the active or mature strand is retained in the RNA-induced silencing complex (RISC), the passenger strand is removed and degraded.[69–72] For the most part, mature miRNAs downregulate their target genes by recognizing them through base pairing of nucleotides 2–8 of the miRNA (the seed sequence) with complementary sequences within the open reading frame and 3′ UTR of the target mRNA. This base pairing often results in inhibition of the initiation and elongation steps of translation, leading to translational inhibition of the targets. Complete complementarity between the seed sequence and target mRNA is critical, but not required. miRNAs also can inhibit gene expression by sequestrating targeted mRNAs to cytoplasmic mRNA processing bodies (P-bodies), where they are degraded. Besides acting as posttranscriptional regulators, miRNAs also have been implicated in transcriptional gene silencing by targeting of promoter regions.[73]

During the last few years, miRNAs have been implicated in diverse biological and pathologic processes and more recently are proving important to the pathogenesis and progression of diabetic kidney disease. [74–76] Kato et al[74] were the first to describe involvement of a specific miRNA in diabetic kidney disease. The authors showed that miR-192 is upregulated in vitro in mesangial cells and in vivo in glomeruli from

streptozotocin-induced and *db/db* mouse models of diabetic kidney disease. They also convincingly demonstrated in vitro that the target of miR-192 is the E-box repressor Smad-1 interacting protein (SIP1). SIP1 binds E-box enhancer elements in the collagen type 1 alpha2 (Col1α2) gene, and by repressing it, miR-192 may promote collagen deposition in response to TGFβ. More recently, the same group has reported that miR-216a also is upregulated by TGFβ1 in experimental models of diabetic kidney disease.[77,78] Similarly, Wang et al[79] showed that miR- 377 is upregulated in spontaneous and streptozotocin-induced mouse models of diabetic kidney disease and in mesangial cells exposed to high glucose and TGFβ, and its induction through downregulation of MnSOD and p21-activated kinase in mesangial cells could contribute to fibronectin accumulation. Jing et al[80] have reported preliminary work suggesting that miR-23b is involved in kidney diseases by modulating TGFβ1/Smad signaling. In vitro gain- and lossof- function studies have identified several miR-23b targets, including type II TGFβ receptor, Smad3, and TGFβ1 itself; these findings imply that there is a negative feedback loop regulating TGFβ1 signaling. Finally, Dey et al[81] have provided evidence that miR-21 is upregulated in renal cortexes of OVE26 type 1 diabetic mice. miR-21 was shown to negatively regulate PTEN expression and modulate Akt/TORC1 expression, which contributes to fibronectin expression and renal hypertrophy.

More recent studies, including data from our own laboratory, have revealed that miRNAs also play key parts in the homeostasis of podocytes and glomerular endothelial cells in diabetic kidney disease. An initial observation that miRNAs are critical to normal kidney function was made in mice with podocyte-specific deletion of Drosha or Dicer (causing global knockdown of miRNA), because these animals develop significant proteinuria.[82,83] Our group subsequently has shown that 2 miRNAs, miR-93 and miR-29c, play important roles in diabetic changes in podocytes and glomerular endothelial cells in vitro and in vivo. Using comparative miRNA expression profile arrays, both miR-93 and miR-29c were identified as signature miRNAs in hyperglycemic conditions.[75,76] miR-93 levels are lower in glomeruli from diabetic *db/db* mice compared with their control littermates and in high glucose-treated podocytes and renal microvascular endothelial cells. In contrast, miR-29c levels have been found to be significantly increased in glomeruli obtained from diabetic *db/db* mice. Interestingly, VEGF-A was identified as a putative target of miR-93 in the kidney because there is perfect complementarity between miR-93 and the 3′ UTR of *vegf-a* in several species. Using transgenic mice that express a bicistronic mRNA encoding both VEGF and the reporter gene LacZ, we showed that inhibition of glomerular miR-93 by peptide-conjugated morpholino oligomers elicited increased expression of VEGF. Meanwhile, miR-29c was found to induce podocyte apoptosis. Overexpression of miR-29c decreased levels of Spry1 protein, a direct target of miR-29c, and activated Rho kinase. Importantly, use of an antisense oligonucleotide to specifically knock down miR-29c significantly decreased albuminuria and kidney mesangial matrix accumulation in the *db*/*db* mouse model.

As a consequence of the important functions provided by miRNAs in diabetic kidney disease, potential therapeutic approaches that target this pathway recently have attracted much attention. Future studies are needed to establish the roles of miRNA inhibition or replacement strategies to prevent the progression of diabetic kidney disease.

EPIGENETICS

Because DNA is the basic blueprint for all cellular activity, DNA mutations have long been understood to play a role in diabetes and diabetic kidney disease. At the same time, much of a cell's identity is set by modifications to its chromatin, which comprises DNA and histones, the proteins that bind and package it. Epigenetic information provides another layer of

control over gene expression by controlling the interpretation of the underlying genetic sequence defining a cell's identity.[84] In general, the term epigenetics is defined as the study of changes in gene expression that do not result from modifications in the underlying DNA coding sequence. Through epigenetic regulation, genes can be turned on or off as appropriate based on various signaling pathways and environmental stimuli.[85]

The basic functional unit of chromatin is the nucleosome, in which 147 base pairs of DNA are wrapped around a histone octamer comprising a histone H3-H4 tetramer and 2 H2A-H2B dimers followed by a histone H1 linker.[84,85] Epigenetic regulation is a posttranslational modification of histones that allows for the conversion of inaccessible, tightly packaged, and inactive heterochromatin to the accessible euchromatin state that offers a more permissive environment for active transcription of genes.

Although at least 4 different DNA modifications and 16 distinct classes of histone modifications have been described,[86] the main epigenetic changes include DNA methylation (covalent attachment of methyl groups at CpG dinucleotides) and histone modifications, which mainly include acetylation, methylation, and phosphorylation.

DNA methylation usually is a mark of gene repression, whereas postsynthetic modifications of the histone proteins, both at gene control regions (promoters and enhancers) and within the coding regions of genes, can be associated with either active gene expression or repression, depending on the amino acid residues that are modified in each of the histone proteins. The histone posttranslational modifications (eg, methylation, acetylation, phosphorylation, and ubiquitination), also known as "marks," occur at various amino acid residues of each of the 4 core histones that form the octamer core of the nucleosome. These marks form a combinatorial code, known as the "histone code," that serves to specify the activity state of the underlying DNA sequence, that is, whether a gene is recognized in its inactive (heterochromatin) or active (euchromatin) state. Modifications of the 4th, 9th, 27th, and 36th lysine (K) residues of H3 histone seem to be especially important (abbreviated as H3K4, H3K9, H3K36, and H3K27). The enzymes responsible for writing the histone code are, among others, the histone acetyltransferases (HATs) and histone methyltransferases.

HATs are responsible for histone lysine acetylation, a chromatin mark usually indicative of gene activation. Histone deacetylases (HDACs), in contrast, mediate the removal of lysine acetylation. Histone lysine acetylation leads to a more open chromatin structure that is more compatible with recruitment of transcription factors and RNA polymerase II, as required for gene transcription, whereas HDACs are found within repressor complexes and are involved in various signaling pathways.[87,88]

Histone methylation occurs on both lysine and arginine residues and, depending on which residue is modified, is associated with either gene activation or repression.[89] For instance, histone H3 lysine 4 methylation (H3K4me) usually is associated with gene activation.[90–92] In contrast, histone H3 lysine 9 methylation (H3K9me) typically is associated with gene repression.[90,92]

Significant progress in the role of epigenetics in diabetes has been achieved during the last decade, in part because of increased understanding of its basic molecular mechanisms, with several recent observations implicating dysregulation of epigenetic instructions in diabetic kidney disease. Evidence from a recent report reveals that epigenetic silencing of Pdx1, a key transcription factor involved in regulating insulin gene expression and beta cell differentiation, leads to intrauterine growth retardation and type 2 diabetes.[93] Both histone modifications and DNA methylation were implicated. Importantly, DNA methylation recently was shown to be affected by uremic toxins.[94] Stenvinkel et al[94] evaluated blood leukocytes obtained from patients with chronic kidney disease (CKD) and showed that there

was global DNA hypermethylation associated with increased mortality in patients with CKD compared with healthy controls. Several other studies have further implicated a role for chromatin histone acetylation in promoting gene expression in the diabetic environment. For instance, high glucose levels have been shown to lead to increased histone lysine acetylation at the gene promoters for cyclooxygenase 2 (COX-2) and TNF-α, with a corresponding increase in expression of these inflammatory factors.[95] Additional in vitro and in vivo studies have suggested a possible role for HDACs in TGFβ1-mediated ECM production and kidney fibrosis[96] because trichostatin A, an HDAC inhibitor, blocks TGFβ1 induction of key fibrotic genes.[96] Trichostatin A also inhibits TGFβ1-mediated downregulation of E-cadherin and associated epithelial-to-mesenchymal transition in renal epithelial cells.[96,97]

Other studies have detected dynamic changes in the H3K4me2-activation and H3K9me2-repressive marks in cultured monocytes exposed to high levels of glucose.[98] Follow-up studies with blood cells from patients with type 1 diabetes have identified a subset of genes in diabetic lymphocytes with increased H3K9me2. Analysis of the methylated genes has established links with immune and inflammatory pathways frequently associated with the development of diabetes and its complications.[99] Additional in vitro experiments have suggested that the H3K4 histone methyltranferase SET7/9 might coactivate NF-κB transcriptional activity by activating H3K4me promoter in the diabetic milieu.[100]

Although recent studies have established the association of histone modifications with models of diabetic glomerulosclerosis,[101,102] the role of histone methylation in diabetic kidney disease is unknown. Whether particular gene promoter histone methylation patterns are altered in kidney cells under diabetic conditions is an intriguing possibility requiring further study.

RHO FAMILY OF SMALL GTPASES

The Rho family of small GTP-binding proteins forms a subgroup of the Ras superfamily of 20- to 30-kDa GTP-binding proteins. The most extensively characterized members are RhoA, Rac1, and Cdc42. Rho GTPases function as molecular switches, cycling between a biochemically inactive (GDP-bound) and an active (GTP-bound) state[103–105] (Fig 5). The GDP/GTP cycling of Rho GTPases is controlled mainly by 3 distinct functional classes of regulatory proteins: (1) guanine nucleotide exchange factors (GEFs), which promote the formation of active Rho-GTP; (2) GTPase-activating proteins (GAPs), which promote the formation of inactive GDP-bound protein; and (3) guanine nucleotide dissociation inhibitors (GDIs), which inhibit Rho activation by sequestrating cytoplasmic Rho proteins. The proper function and activity of Rho GTPases are critically dependent on their intracellular translocation and recruitment from the cytoplasm to the plasma membrane. [103,106–108] The best characterized downstream effector of RhoA is ROCK, which mediates various cellular functions.[109–114]

ROCK belongs to the AGC family of eukaryotic protein kinases, for which members also include protein kinase A (PKA) and PKC.[109,115–117] Two isoforms of ROCK have been identified: ROCK1 (also known as ROCKβ or p160ROCK) and ROCK2 (also known as ROCKα).[103,118] These 2 proteins are highly homologous, sharing an overall sequence similarity at the amino acid level of 65% and in their kinase domains of 92%.[114–120] Although both isoforms are ubiquitously expressed, ROCK1 mRNA is expressed preferentially in the lung, liver, spleen, kidney, and testis, whereas ROCK2 mRNA is highly expressed in the heart and brain.[115] In general, the catalytic domain of ROCK is located at the amino terminus, followed by a coiled-coil–forming region and a pleckstrin-homology (PH) domain with a cysteine-rich domain (CRD) at the carboxyl terminus (Fig 6). The carboxy-terminal coiled-coil region also includes the Rho-binding domain (RBD). When the

carboxy-terminal portion (RBD and CRD) of ROCK is truncated, the protein is constitutively active; the carboxy-terminal portions themselves act in a dominant-negative manner.[112,121] In the inactive form, the PH and RBD domains of ROCK bind to the amino terminal region of the protein, forming an auto-inhibitory loop. However, in its active form, GTP-activated Rho binds to the RBD, resulting in an open conformation of the kinase.

The RhoA/ROCK pathway has inspired much interest among diabetes researchers for a number of reasons. First, the RhoA/ROCK pathway plays an important part in various signaling pathways that may be implicated in the pathogenesis of microvascular complications of diabetes. For example, our laboratory and a number of others recently have elucidated the role of the RhoA/ROCK pathway in VEGF signaling, a key mediator of microvascular complications of diabetes.[122,123] The second rationale that ROCK has emerged as a popular target is due the suggestion that the pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are mediated, at least in part, by their inhibitory effects on RhoA/ROCK signaling and the resultant inhibition of ROCK.[119,120,124] Several groups already have shown that the inhibitory effects of statins on RhoA/ROCK signaling pathways may be key to their renoprotective effects.[122,123,125,126] Another reason for the recent excitement regarding the RhoA/ROCK pathway arises from the involvement of ROCK in ECM accumulation.[126]

Regarding the role of ROCK1 in diabetic kidney disease, the potential involvement of ROCK in the pathogenesis of diabetic kidney disease initially was described using fasudil, a selective ROCK inhibitor, in animal models of both type 1 and type 2 diabetes in vivo.[127,128] Kikuchi et al[127] demonstrated that a high dose of fasudil (100 mg/kg) given at the onset of diabetes improves metabolic parameters and decreases diabetes-induced proteinuria, glomerulosclerosis, and interstitial fibrosis in a type 2 diabetes rat model. Our own observations also have indicated that low suppressor doses of fasudil (10 mg/kg) given to *db/db* mice, a model of type 2 diabetes, significantly decrease albuminuria, mesangial expansion, accumulation of glomerular type IV collagen, and glomerular basement membrane thickening.[128] Finally, a more recent study has convincingly shown that 18 weeks of fasudil treatment ameliorates diabetes-induced structural changes in the kidney in association with a modest antiproteinuric effect without significant changes in blood pressure in a model of accelerated diabetic kidney disease in which uninephrectomized rats were made diabetic by streptozotocin.[129]

Until recently, one major gap in our understanding of the role of ROCK in diabetic kidney disease was that fasudil cannot distinguish between the effects of the ROCK1 and ROCK2 isomers, and more importantly, at higher concentrations, it may also inhibit other serine/threonine kinases such as PKA and PKC.[130,131] Thus, in a recent publication, the role of ROCK1 in diabetic kidney disease was assessed using a genetic approach.[43] The findings indicate that proteinuric effects of ROCK1 could be related to its pro-oxidative effects on podocytes and glomerular endothelial cells. However, major gaps still exist in our understanding of the role of Rho GTPases in the pathogenesis of diabetic kidney disease. These knowledge gaps will hinder future research with the anticipation that pharmacologic inhibition of the Rho family of small GTPase proteins might broaden the spectrum of treatments available to patients with diabetic kidney disease.

ACTIVATION OF TGFβ SIGNALING

TGFβ and its related peptides, such as activin, inhibin, and bone morphogenetic protein (BMP), form the TGFβ superfamily. They exert pleiotropic effects on a broad array of cellular responses, such as cell proliferation, differentiation, apoptosis, fibrosis, wound repair, and inflammation, in a wide range of target cells.[132–134] There are 3 highly similar

isoforms of TGFβ in mammals: TGFβ1, TGFβ2, and TGFβ3. All 3 isoforms and their receptors are widely expressed in the kidney.[135,136] However, of the 3 isoforms, the role of TGFβ1 in the pathogenesis of diabetic kidney disease has been studied extensively.[135,137]

TGFβ1 is synthesized as an inactive precursor; the amino terminal prodomain, latency-associated protein (LAP), is cleaved in the Golgi but stays noncovalently bound, thus blocking TGFβ1 from binding to its receptors.[135,138] This latent complex of TGFβ1 and LAP associates with latent TGFβ1-binding proteins (LTBPs) before secretion from the cell. After secretion, most TGFβ1/LAP/LTBP complex is stored in the ECM and cross-linked with matrix proteins.[135,138] Extracellular activation of latent TGFβ1 predominantly occurs in response to various stimuli, including changes in pH, ROS, plasmin, cathepsin, integrins, and thrombospondin 1 (TSP-1).[139]

The majority of the conclusions on the contributions of TGFβ1 signaling to diabetic kidney disease are obtained from in vitro studies in cultured glomerular mesangial cells,[138] in which TGFβ1 has been shown to be activated by a myriad of mediators induced by high-glucose conditions, including AGEs, ROS, DAG, PKC, and angiotensin II (Ang II), as well as the physical cyclical stretching of cells.[138,140] Once TGFβ1 is activated, it may modulate the progression of diabetic kidney disease through both the Smad-dependent and Smad-independent pathways.[135,137,138] In canonical TGFβ1 signaling, the interaction of dimeric TGFβ1 ligands with heterotetrameric complexes of type II and type I receptors leads to phosphorylation-dependent activation of the type I receptor kinase, which then results in activation of intracellular Smad2 and Smad3, forming a complex with Smad4, and then translocating into the nucleus, where they regulate the transcription of target genes.[132–134]

Renal expression of TGFβ1 and its receptor is increased in kidneys of various murine models of diabetes and in diabetic patients.[138,141] Although Smad2 and Smad3 are strongly activated in both experimental and human diabetic kidney disease, recent studies from Smad2/3 conditional knockout mice have found differential effects of Smad3 and Smad2 in renal fibrosis under different disease conditions.[135,142,143] For instance, Smad3 seems to be pathogenic, whereas Smad2 is protective, in kidney fibrosis.[135] The expression of connective tissue growth factor (CTGF) and VEGF, as well as epithelial-myofibroblast and endothelial-myofibroblast transition, are inhibited by disruption of Smad3, but upregulated by knockout of Smad2.[135] Smad7 also is inhibitory, negatively regulating the activation of TGFβ1 signaling.[132–134] Levels of Smad7 are decreased significantly in the fibrotic kidney,[135,138] whereas overexpression of Smad7 inhibits Smad2/3-mediated fibrosis in response to TGFβ1, high glucose, AGE, and Ang II. Importantly, overexpression of renal Smad7 attenuates progressive renal fibrosis in a number of disease models, including diabetic kidney disease in vivo.[135,144,145]

BMP-7, another family member of the TGFβ superfamily, opposes the classic profibrogenic effects of TGFβ1.[135,138,146] BMP-7 is highly expressed in the tubular epithelia of the outer cortex and in glomerular podocytes.[138] Renal BMP-7 was shown to be protective against diabetic kidney disease.[147] Transgenic expression of BMP-7 in glomerular podocytes and proximal tubules prevents podocyte dropout and reductions in nephrin levels in streptozotocin-induced diabetic mice. BMP-7 also reduces glomerular fibrosis and interstitial collagen accumulation, as well as collagen I and fibronectin expression.[147]

Another important member of the BMP family of proteins, BMP-4, recently has come into focus as a potential key pathogenic factor in diabetic kidney disease.[148] Mice with inducible transgenic overexpression of BMP-4 manifest many features of diabetic kidney disease, including the development of proteinuria and podocyte effacement.[148] Although the underlying molecular mechanisms of its action are incompletely understood, BMP-4 has

been suggested to act downstream of the AGE pathway to enhance Smad1 activity, leading to increased collagen IV deposition and ECM expansion.[148,149] Importantly, Smad1 has been shown to serve as a potential urinary biomarker for diabetic kidney disease.[150,151] Finally, increased urinary TGFβ1 excretion by itself was shown to correlate with the severity of interstitial fibrosis in patients with diabetic kidney disease, suggesting a link between TGFβ1 expression and progressive diabetic kidney disease.[152]

In summary, accumulating evidence indicates that TGFβ Smad signaling is a prominent pathway leading to the development of diabetic kidney disease. Many mediators of diabetic kidney disease, including high glucose, AGE, Ang II, and PKC, can induce TGFβ expression and activate TGFβ through both Smad-dependent and Smad-independent signaling pathways. Although treatment with a neutralizing antibody toward TGFβ has been shown to prevent glomerulosclerosis in an experimental model of type 2 diabetes, [153] the role of targeting in patients with diabetic kidney disease remains to be elucidated.

HEMODYNAMIC PATHWAYS AND VEGF

There is increasing evidence that the hemodynamic and metabolic pathways are functionally linked in the development of diabetic kidney disease. The “hyperfiltration hypothesis,” a concept put forward by Brenner’s group in the 1980s, has been characterized as one broadly relating to hemodynamic factors that will increase single-nephron GFR, leading to glomerulosclerosis and chronic kidney failure.[154] Of the hemodynamic factors involved, the increase in single-nephron GFR and increases in glomerular pressure appear to be of critical importance. However, important questions remain to be adequately addressed. If elevated single-nephron GFR is the initiating factor, what are the subsequent steps that lead to chronic kidney failure? And what is the initial event that triggers glomerular hyperfiltration?

It now is known that glomerular hyperfiltration is due in part to decreased resistance in both the afferent and efferent arterioles of the glomerulus, with a more pronounced effect on the afferent arteriole.[155] These early hemodynamic changes may lead to albumin leakage from the glomerular capillaries with the subsequent accumulation of albumin and other macromolecules, which may provoke increased synthesis of mesangial matrix material and basement membrane material.

The hyperfiltration hypothesis suggests that increased glomerular pressure would facilitate the increase in single-nephron GFR. This sustained elevation could overburden the remaining nephron units, thereby leading to glomerulosclerosis. This significant alteration in glomerular pressure could contribute to mesangial matrix expansion, altered negative charge on podocyte foot processes, effacement, and subsequent protein loss to the urine.[154,156]

The cellular effects resulting from sheer stress (or stretch) may be the major cause for the increase in glomerular pressure, although other factors also are involved. Cyclic hemodynamic changes in the glomerulus lead to enhanced mechanical stretch of all glomerular structural components, including mesangial cells and podocytes.[157,158] In cultured mesangial cells, for example, mechanical stretch may promote ECM accumulation by increasing the synthesis of ECM components and decreasing the activity of degradative enzymes.[159,160] The main prosclerotic effect of stretch seems to occur because of increases in gene and/or protein expression of ECM components, such as collagen, fibronectin, and TGFβ1, a potent prosclerotic cytokine and a well-known mediator of ECM accumulation in diabetic kidney disease.[159–161]

Mechanical stretch may lead to a decrease in podocyte number through several mechanisms. For instance, stretching podocytes in vitro reduces proliferation by modulating TGFβ1 and cell-cycle proteins.[162] Interestingly, Ang II also produces effects analogous to those seen

upon mechanical stretching, including cell hypertrophy and hyperplasia, accumulation of ECM, and generation of cytokines, chemokines, and growth factors, such as TGFβ1 and VEGF, in mesangial cells.[163] Furthermore, Ang II in podocytes results in enhanced production of α3(IV) collagen by TGFβ and VEGF signaling,[164] and downregulation of nephrin expression. [165] Ang II and stretch have additive effects on the production of VEGF, a strong promoter of vascular permeability that, in this cell type, induces proliferation and enhances collagen synthesis.[163] Of considerable interest is the potential role of VEGF in diabetic kidney disease.

The VEGF family includes 6 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, viral homologues of VEGF collectively named VEGF-E, and placental growth factor (PIGF).[166–168] In humans, VEGF-A is expressed as several isoforms consisting of polypeptides with 121, 145, 165, 189, and 206 amino acids.[169–171] $VEGF_{165}$ is the most abundant and biologically active form. Mouse VEGF isoforms have one fewer amino acid and are designated as VEGF 120, 164, 188, and 205.[169–171] In the kidneys, VEGF expression is localized principally to glomerular podocytes. However, it also is expressed in distal and collecting duct tubular cells[171–173] and possibly in activated mesangial cells.[174,175] VEGF binds to several high-affinity transmembrane tyrosine kinase receptors. The 2 best-characterized VEGF-specific receptors are VEGFR-1 (flt-1 [fms-like tyrosine kinase]) and VEGFR-2 (KDR/flk-1 [fetal liver kinase]).[176–180] These receptors are class III receptor tyrosine kinases within the platelet-derived growth factor receptor superfamily and have 7 immunoglobulin homology domains in their extracellular ligand-binding domain and an intracellular tyrosine kinase domain split by a kinase insert.[178–180] As with other receptor tyrosine kinases, ligand binding induces homodimerization of the VEGF receptors and transphosphorylation of their intracellular domains, allowing for the activation of several signaling pathways. [178–181] Although the affinity of VEGF is about 10-fold higher for VEGFR-1 than for VEGFR-2, the latter seems to be the primary receptor for conveying VEGF-induced signals in many cells.[181–185] Expression of VEGFR-1 and VEGFR-2 and binding of radioiodinated $VEGF_{165}$ have been localized to glomerular and peritubular capillaries and to pre- and postglomerular vessels.[172,186–188] VEGFR-3 expression is largely restricted to the lymphatic endothelium in adult tissues.[189] Another VEGF receptor is neuropilin (NP-1). NP-1 is a non–tyrosine kinase receptor for the $VEGF_{165}$ isoform, which also binds to VEGF-B and VEGF-E. However, the small size of the NP-1 cytoplasmic domain and the lack of known independent functional signaling suggest that NP-1 may act as only a coreceptor.[190–192] A number of cytokines, chemokines, and growth factors can regulate VEGF expression in the kidney. These factors include hypoxia, TGFβ, hyperglycemia, glycation end products, oxidative stress, PKC, and Ang II.[163,193–196] Considering that these same factors have been recognized as the main culprits in diabetic kidney disease, it is not surprising that targeting VEGF might prove to be a useful tool in ameliorating the microvascular complications of diabetes. Several studies have convincingly demonstrated that VEGF is a major mediator of other microvascular complications of diabetes, including proliferative diabetic retinopathy[197,198] and diabetic neuropathy.[199]

However, evidence for a pathogenic effect of VEGF in diabetic kidney disease is less extensive. [12,200–207] Cooper et al[12] were one of the first groups that examined the role of VEGF in streptozotocin-induced diabetes in rats. The authors concluded that VEGF mRNA and protein are upregulated in kidneys of this model. The VEGF-specific receptor VEGFR-2 mRNA also was found to be upregulated after 3 weeks of diabetes. Using a neutralizing anti-VEGF antibody, 2 more recent studies have shown a significant decrease in hyperfiltration, albuminuria, and glomerular hypertrophy in both experimental models of type 1 and type 2 diabetic mice.[205,206] These studies have provided a scientific rationale to target VEGF in early stages of diabetic kidney disease. However, although anti-VEGF therapy has been established as an important part of standard therapy in patients with

diabetic retinopathy, the effect of targeting VEGF in the context of diabetic kidney disease is less known, in part because no effective therapy currently is available to reduce VEGF to its physiologic levels in patients with diabetic kidney disease. This obviously is a critical step because VEGF expression and activity are tightly regulated in the kidney[206,208–211] and VEGF derangement, similar to high VEGF levels, also has been shown to cause significant kidney damage. Agents targeting VEGF and/or its receptors (eg, bevacizumab, a humanized anti-VEGF monoclonal antibody, as well as inhibitors that target several receptor tyrosine kinases, including VEGF receptors) have been shown in several preclinical and clinical studies to be associated with increased risks of hypertension and proteinuria from endotheliosis and thrombotic microangiopathy.[208,212–216] Consistent with these data, deletion and knockdown of podocytespecific VEGF-A in mice have resulted in thrombotic microangiopathy, glomerular endotheliosis, proteinuria, and podocyte effacement.[208,210,217,218]

Interestingly, excessive levels of VEGF and VEGF overexpression have also proved to be detrimental to glomerular homeostasis. Veron et al found that podocyte-specific VEGF overexpression enhances pathogenic features of diabetic kidney disease in the streptozotocin-induced type 1 model of diabetes.[219–221] In line with these results, Eremina et al[210] conditionally overexpressed VEGF[164] in podocytes, which resulted in severe kidney damage with features of collapsing glomerulopathy. Future research clearly is needed to conclusively unravel the role of VEGF in diabetic kidney disease.

GENETIC PREDISPOSITION

Chronic hyperglycemia is necessary but not sufficient for the development of diabetic kidney disease because many patients with uncontrolled diabetes will never develop nephropathy, and conversely, individuals with excellent glycemic control may develop microvascular complications of diabetes. The evidence that genetic factors may contribute to diabetic kidney disease, like many other similar complex diseases, is based mainly on the observation of familial aggregation in epidemiologic studies. The notion that certain individuals with diabetes are at differential risk for developing nephropathy on account of familial aggregation of kidney disease was first reported in 1989, when Seaquist et al[222] examined probands of type 1 diabetic patients with and without diabetic kidney disease and found that 83% of diabetic siblings of probands with nephropathy had evidence of nephropathy compared with only 17% of diabetic siblings of probands without nephropathy. These findings were reproduced in other studies in which significant differences were noted in recurrence risk for nephropathy in siblings with diabetes whose proband had nephropathy, compared with probands without nephropathy.[223] One such study evaluated Pima Indian families in which 2 successive generations had type 2 diabetes and suggested that the likelihood of the offspring developing overt proteinuria was 14% if neither parent had proteinuria, 23% if 1 parent had proteinuria, and 46% if both parents had proteinuria.[224] Segregation analysis in families with type 2 diabetes also suggests that genetic factors are important determinants of urinary albumin excretion levels.[225]

Recently, a genome-wide association study using an Affymetrix array has been completed for diabetic kidney disease in type 1 diabetes; the analysis was based on the US Genetics of Kidneys in Diabetes (GoKinD) Study with 885 case and 820 control individuals. [226] In this study, odds ratios (ORs) for the 2 most promising single-nucleotide polymorphism associations with diabetic kidney disease were as follows: rs10868025 (OR, 1.45; $P = 5.0 \times 10^{-7}$) and rs451041 (OR, 1.36; $P = 3.1 \times 10^{-6}$). These putative markers then were screened in a follow-up study looking for associations between these markers and time to onset of nephropathy in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications. These further analyses showed the 9q single-nucleotide

polymorphism to be associated with both proteinuria and end-stage renal disease (ESRD), whereas rs451041 on chromosome 11p was associated with ESRD alone.

Several genome-wide association studies also have been published on type 2 diabetic patients with nephropathy. One large study was the final report from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-sponsored Family Investigation of Nephropathy and Diabetes (FIND).[227] Results of these studies suggest common linkage peaks on 18q22-23, 7q35-36, 7p15, and 10q26. This replication strongly suggests that causative type 2 diabetic kidney disease genes underlie each peak, and this reduces the likelihood that they are false-positive results. In another large analysis, nearly 26,000 dialysis patients were screened for a family history of ESRD.[228] Independent risk factors for a family history of ESRD in first- or second-degree relatives included earlier age at ESRD onset, female sex, black ethnicity, and diabetic kidney disease.

These epidemiologic studies set the stage for additional efforts to identify diabetic kidney disease susceptibility genes. Obviously, there are still challenging issues in this area of research, but the importance of finding diabetic kidney disease–associated genes is profound. Identification of risk genes could be a powerful means to identify the subset of patients with diabetes that will develop nephropathy and ESRD.

ADDITIONAL FACTORS

There undoubtedly are many other factors that may influence and contribute to the development of diabetic kidney disease. Among these factors are the potential role of PKC, AGEs, the transcription factor Nrf2, and mTOR (mammalian target of rapamycin) signaling. For instance, preclinical and clinical studies have highlighted the contribution of PKC-β to the progression of diabetic kidney disease.[229,230] In a recent preclinical study, PKC-β transgenic mice exhibited many features of diabetic kidney disease through enhancing Ang II activity.[229] Another recent clinical study has shown that selective inhibition of PKC-β with ruboxistaurin is effective in reducing albuminuria and preventing loss of GFR in patients with late-stage diabetic kidney disease.[230] In addition, AGE/RAGE pathways have been implicated as key contributors to diabetic kidney disease.[231,232] A key modulator of oxidative stress, the transcription factor Nrf2 recently has emerged as a potentially viable therapeutic target in several preclinical models of diabetic kidney disease.[233–235] Last, several new preclinical studies have highlighted the role of autophagy and nutrient sensing, which involve the mTOR, AMPK, and Sirtuin signaling pathways, in several experimental models of diabetic kidney disease.[236]

CONCLUSIONS

Despite intense efforts by many investigators and the application of new approaches, our understanding of the underlying mechanisms involved in the development of diabetic kidney disease remains limited. The hemodynamic changes of glomerular hyperperfusion and hyperfiltration can be noted prior to the first detectable clinical signs of nephropathy, but they are not predictive of loss of kidney function. Podocyte foot-process effacement, decrement in podocyte number, thickening of the glomerular basement membrane, and mesangial expansion, among other structural changes, happen alongside the early changes. However, on an individual basis, these features cannot predict disease progression. Hyperglycemia is a key player in a series of damaging effects that are mediated by cytokines and growth factors that produce oxidative stress, abnormal glycosylation, lipid peroxidation, and the production of further inflammatory elements. In the next 2–4 years, studies currently underway that are evaluating the genetics of nephropathy will be completed, which should lead to more complete knowledge of how to incorporate genetic and environmental

susceptibility factors into risk assessment. This information should help clinicians predict at an earlier stage which patients will develop nephropathy, thereby increasing the chances of preventing this devastating disease.

Acknowledgments

Support: The development of this journal supplement was funded by Novo Nordisk. The writing of this manuscript was supported by grants from the NIDDK (RO1DK091310 and RO1DK078900). The authors received no other honoraria or remuneration for this work. Technical editing was provided by Watermeadow Medical, funded by Novo Nordisk. Costs associated with publication were funded by Novo Nordisk.

Financial Disclosure: The authors declare that they have no other relevant financial interests.

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EXECUTIVE SUMMARY

Diabetic kidney disease (DKD) is a major micro-vascular complication of diabetes and the most common cause of chronic kidney failure requiring dialysis in the United States. Prolonged hyperglycemia leads to chronic metabolic and hemodynamic changes that modulate intracellular signaling pathways, transcription factors, cytokines, chemokines, and growth factors, promoting structural abnormalities in the kidney and the development and progression of DKD. This article reviews recent advances in our understanding of the molecular mechanisms underlying the pathogenesis of DKD.

Oxidative stress caused by increased free radical production is believed to play a central role in the development of microvascular complications of diabetes, including DKD. Under physiologic conditions, steady-state concentrations of oxidants are maintained at nontoxic levels by antioxidant defenses and repair enzymes. Disturbance of the delicate balance between reactive oxygen species (ROS) that promote oxidative stress and antioxidant defense systems may play a critical role in DKD pathogenesis. Mitochondria are the main source of ROS, while the NOX protein family, the catalytic component of the multiprotein enzyme complex NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase, represents the primary nonmitochondrial source. Changes in mitochondrial morphology and dynamics under hyperglycemic conditions contribute to increased mitochondrial ROS, while increased ROS secondary to upregulation of certain NOX subunits may underlie microvascular damage and progression of DKD. Animal studies suggest that mitochondrial-targeted therapies may reduce oxidation-induced cell damage, interstitial fibrosis, and glomerular damage and improve tubular and glomerular function. Further examination of mitochondrial machinery, as well as increased understanding of the role of ROS in vascular complications of diabetes, may help identify therapeutic targets for intervention.

Gene expression also plays a key role in the pathogenesis of DKD. Epigenetic factors—those that influence gene expression but not by modification of underlying DNA coding sequence—and genetic predisposition play a key role in determining DKD risk. Through epigenetic regulation, genes can be turned on or off, depending on the needs of the cell, in response to signaling pathways and environmental stimuli, and recent data implicate dysregulation of epigenetic instructions in the development of DKD. We also know from familial aggregation in epidemiologic studies that genetic factors contribute to individual susceptibility and clinical progression of diabetic neuropathy. At the cellular level, microRNAs (miRNAs), a class of short (21–24 nucleotides) noncoding RNAs, regulate gene expression at the posttranslational level and are emerging as critical players in the pathogenesis of DKD by their role in the regulation of renal homeostasis. Dysregulation of certain miRNAs under hyperglycemic conditions has been shown in animal studies to lead to collagen deposition, fibronectin accumulation, and renal hypertrophy, while expression of key miRNAs in vitro and in vivo is associated with pathologic changes in podocyte and glomerular endothelial cell function. Increased understanding of the epigenetic changes associated with gene activation and repression, identification of DKD susceptibility genes, and improved grasp of the role of miRNAs will be key to understanding the environment within which diabetes and its microvascular complications develop. Research efforts then can harness this knowledge to develop strategies that prevent the development and progression of DKD.

A number of small highly active proteins have come under scrutiny as potential therapeutic targets for DKD in recent years. The Rho family of small guanosine triphosphate (GTP)-binding proteins forms a subgroup of the Ras superfamily of GTP-binding proteins. Rho GTPases function as molecular switches, cycling between a biochemically inactive (GDP [guanosine diphosphate]–bound) and an active (GTP-

bound) state according to the actions of regulatory proteins. Along with various downstream effectors, the best characterized being ROCK (Rho-associated coiled-coil containing protein kinase), RhoA plays an important role in signaling pathways implicated in the pathogenesis of microvascular complications of diabetes. The selective ROCK inhibitor fasudil has been shown to improve metabolic parameters in diabetes and reduce proteinuria, glomerulosclerosis, and interstitial fibrosis in animal models. Another protein group of interest is the TGFβ (transforming growth factor β) superfamily of peptides, including TGFβ, activin, inhibin, and BMP (bone morphogenetic protein). These proteins exert pleiotropic effects on a broad array of cellular responses, including cell proliferation, differentiation, apoptosis, fibrosis, wound repair, and inflammation, in a wide range of target cells. Of the 3 TGFβ isoforms, TGFβ1 has been studied most extensively in DKD, and accumulating evidence implicates the TGFβ/Smad signaling pathway in the development of DKD.

There also is increasing evidence that hemodynamic and metabolic pathways are functionally linked in the development of DKD. Of the hemodynamic factors involved, the increase in single-nephron glomerular filtration rate and increases in glomerular pressure appear to be of critical importance. These early hemodynamic changes may facilitate albumin leakage from the glomerular capillaries, leading to accumulation of albumin and other macromolecules and provoking structural changes. We now know that cellular effects resulting from sheer stress and subsequent structural changes may be the primary cause of increased glomerular pressure although other factors, including angiotensin II, acting by VEGF (vascular endothelial growth factor), also contribute to vascular permeability and structural damage.

Although the development of DKD cannot yet be prevented, understanding of the pathogenesis is increasing gradually. Hemodynamic changes become evident before the earliest clinical signs of nephropathy and are followed by structural changes, but disease progression can be understood only in the context of hyperglycemia, which plays a central role in a cascade of damaging effects. As understanding of the relationship between genetic and environmental susceptibility to diabetic neuropathy and its clinical progression improves, clinicians will be better able to predict those at risk and intervene early to limit the damage caused by this devastating disease.

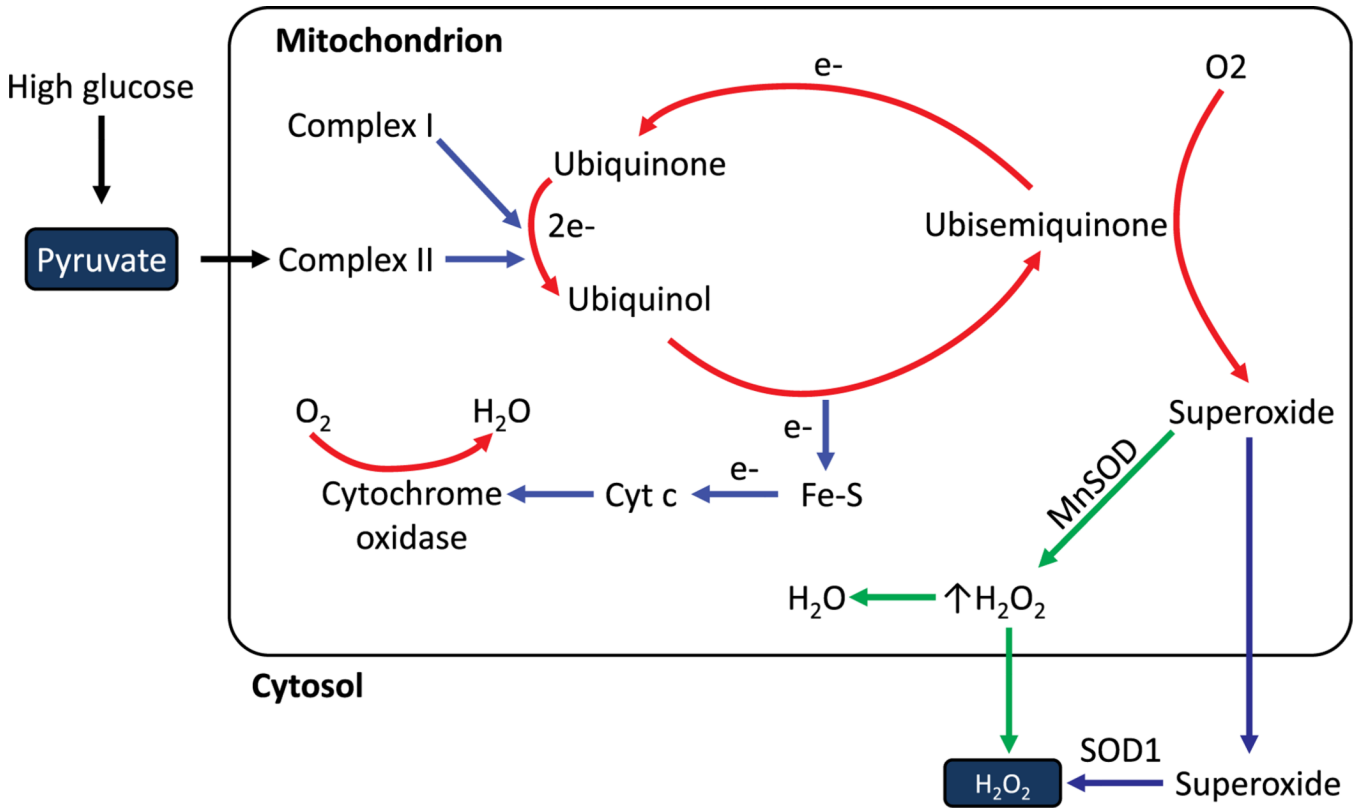

Figure 1.

Mitochondrial ROS. The mitochondrial matrix contains the components of the TCA cycle and the β-oxidative pathway, which provide reduced NADH and $FADH_2$ to the electron transport chain, leading to generation of a proton gradient across the inner mitochondrial membrane. Under high-glucose conditions, pyruvate is shuttled to the mitochondria, where it is oxidized by the TCA cycle to produce 4 molecules of NADH and 1 molecule of $FADH_2$.

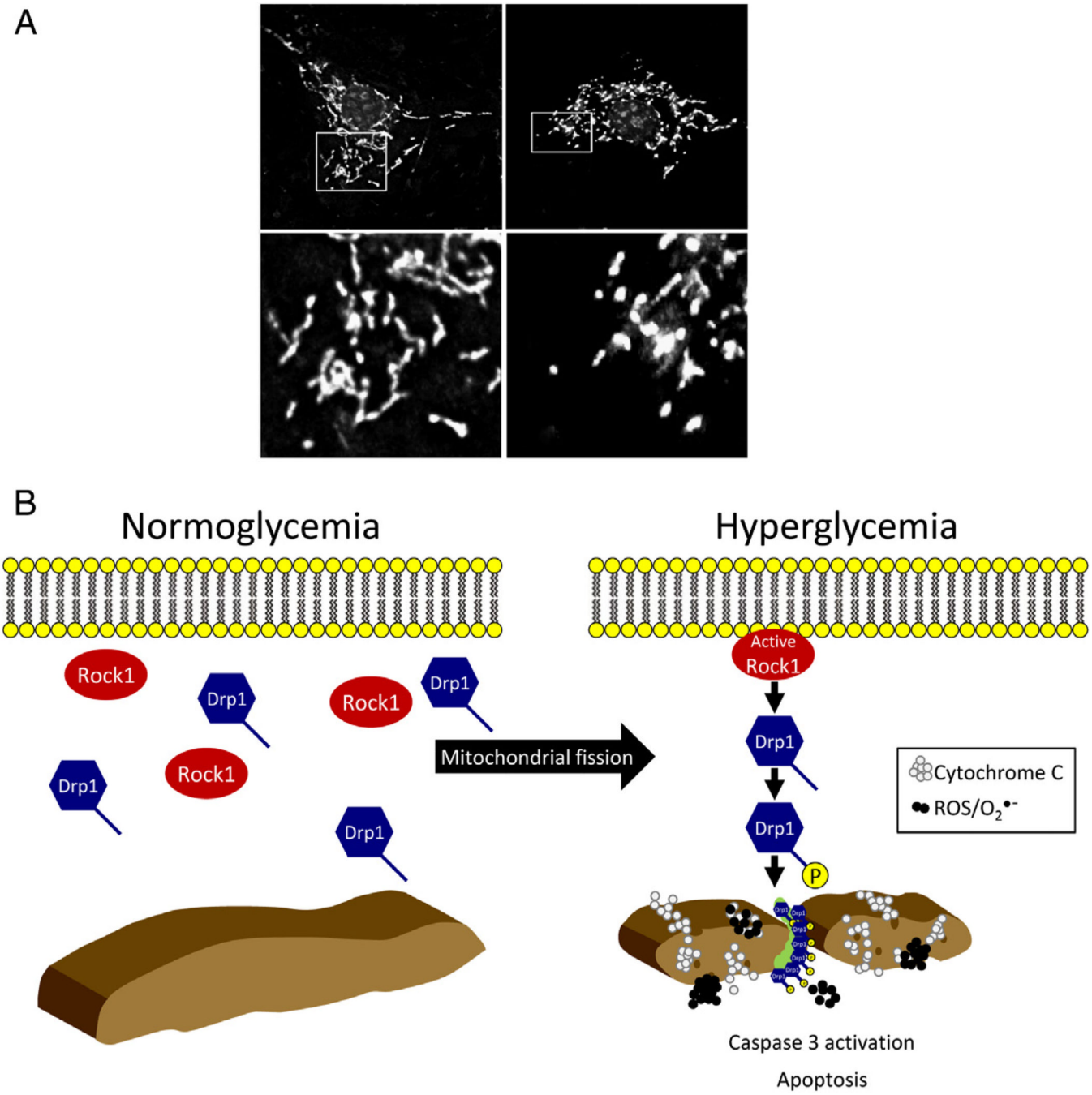

Figure 2.

Mitochondrial fission and fusion. (A) Mitochondrial networks visualized with MitoTracker Red (Life Technologies) fluorescent dye to monitor mitochondrial morphology under (left) normal or (right) high-glucose conditions. Mitochondria appear as long, tubular, and sometimes branched structures that spread throughout the cytoplasm. However, under high-glucose conditions, they appear dense, small, and fragmented. (B) Mitochondrial fission is driven by Drp1, which resides primarily in the cytoplasm. Under hyperglycemic conditions, Drp1 is activated and recruited to the mitochondria. Drp1 then forms spirals around mitochondria at fission sites, which promote the constriction of mitochondria.

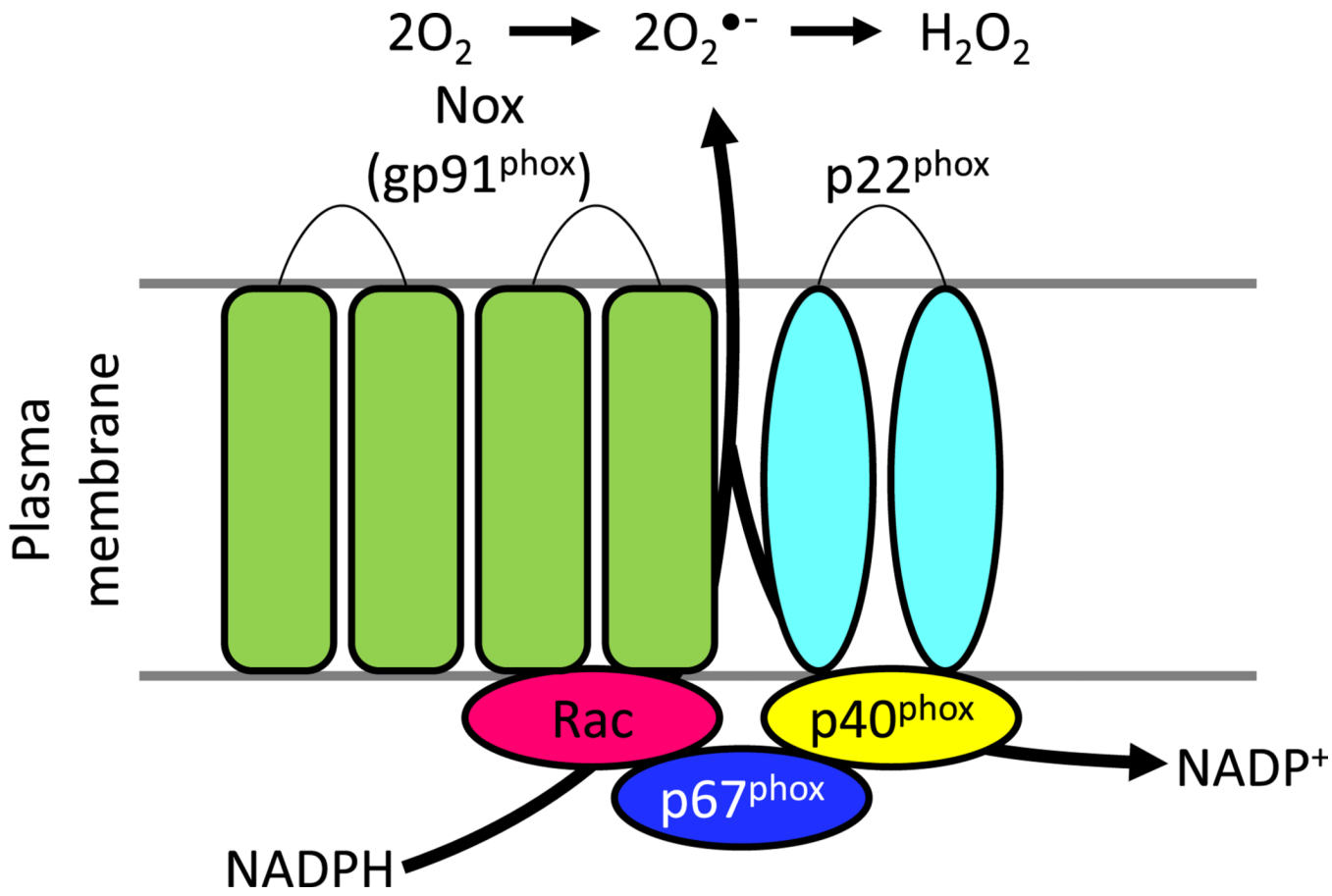

Figure 3.
Assembly and activation of NADPH oxidase. In resting conditions, only heterodimeric NOX-p22phox complex resides in the membrane, whereas the other components of the complex are cytosolic. Activation of the enzyme releases a conformational restriction, which results in association of different components of the complex to the plasma membrane.

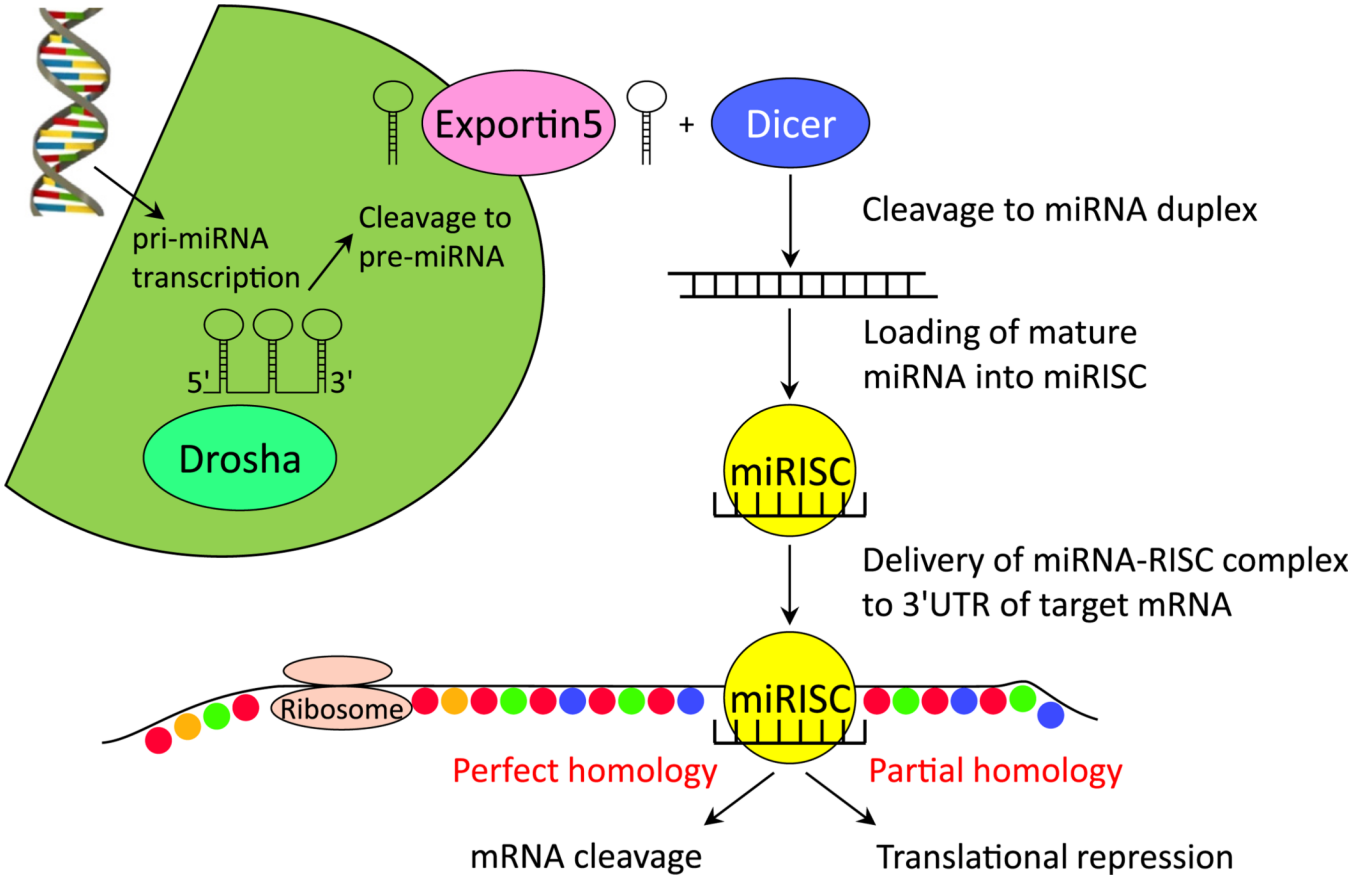

Figure 4.

miRNA biogenesis and function. Pri-miRNA transcription is regulated by RNA polymerase II (Pol II). Pri-miRNA, typically several kilobases long, is converted into pre-miRNA by the RNAse III processing enzyme complex Drosha/DGCR8. This pre-miRNA is exported into the cytosol by the exportin 5/RanGTP complex. Pre-miRNAs are processed further through Dicer to form mature miRNAs that are loaded into the RNA-induced silencing complex (RISC) to perform their gene regulatory functions.

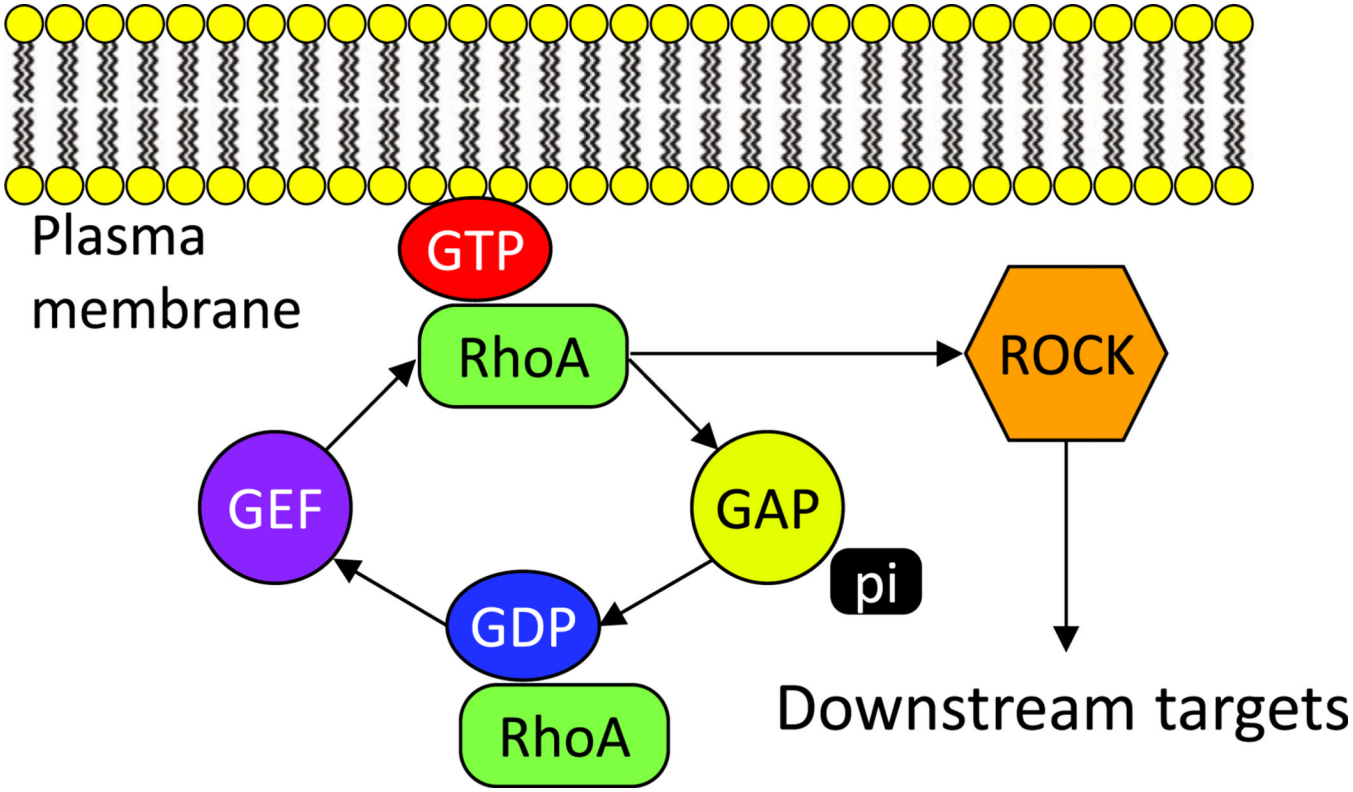

Figure 5.
RhoA/ROCK pathway. Rho GTPases cycle between an inactive (GDP)- and an active (GTP)-bound form.

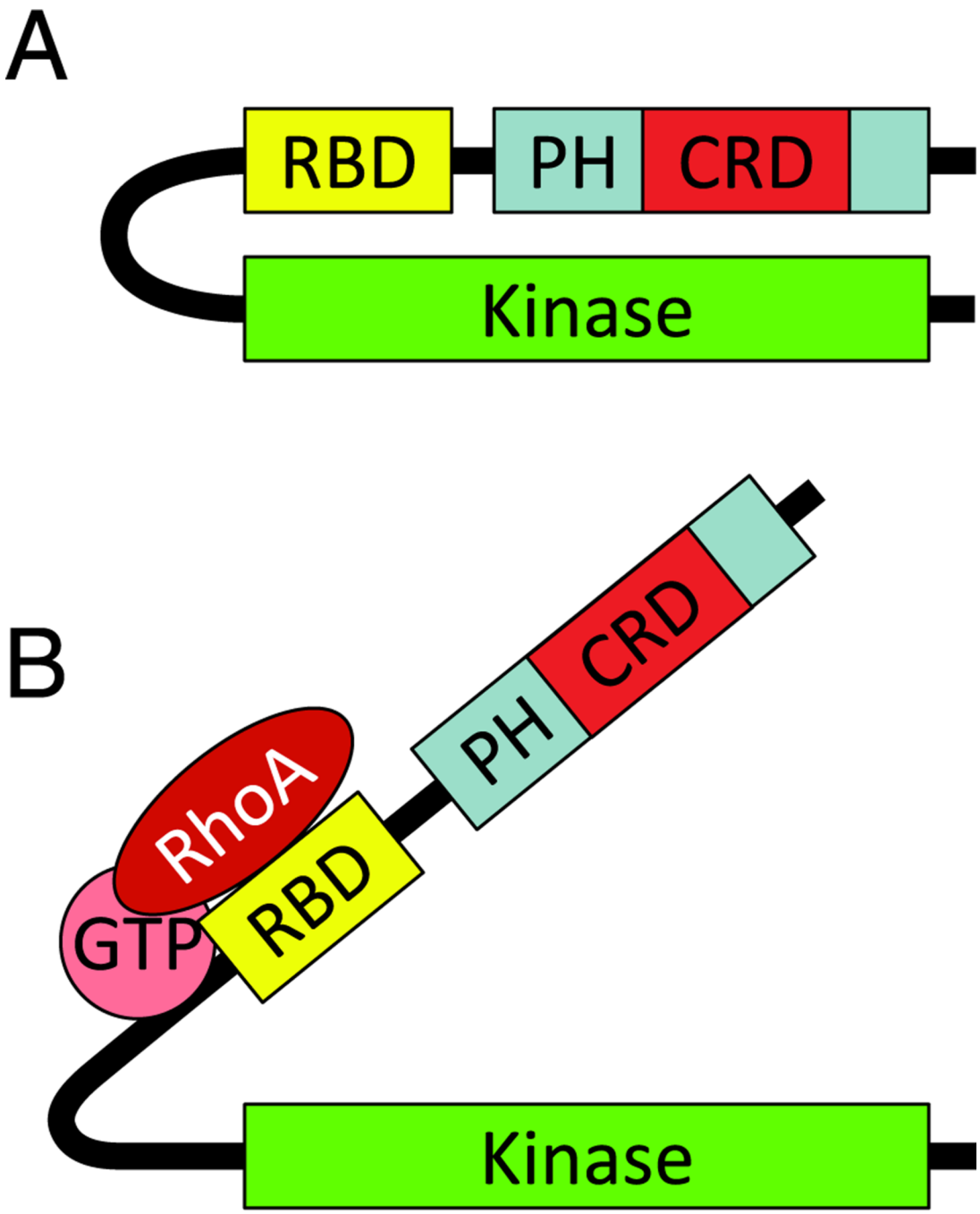

Figure 6.
Regulation of ROCK activation. (A) The catalytic domain of ROCK is located at the amino terminus, followed by a coiled-coil–forming region and a pleckstrin-homology (PH) domain with a cysteine-rich domain (CRD) at the carboxyl terminus. (B) Active Rho binds to the RBD domain of ROCK, resulting in an open conformation and thereby activation of the ROCK.