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Novel mechanisms of protein synthesis in diabetic nephropathy —role of mRNA translation

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Abstract

Ambient protein levels are affected by both synthesis and degradation. Synthesis of a protein is regulated by transcription and messenger RNA (mRNA) translation. Translation has emerged as an important site of regulation of protein expression during development and disease. It is under the

control of distinct factors that regulate initiation, elongation and termination phases. Regulation of translation occurs via signaling reactions, guanosine diphosphate–guanosine triphosphate binding and by participation of non-coding RNA species such as microRNA. Recent work has revealed an important role for translation in hypertrophy, matrix protein synthesis, elaboration of growth factors in *in vivo* and *in vitro* models of diabetic nephropathy. Studies of translation dysregulation in diabetic nephropathy have enabled identification of novel therapeutic targets. Translation of mRNA is a fertile field for exploration in investigation of kidney disease.

Keywords

Protein synthesis; MicroRNA; Signaling reactions; Insulin; Glucose; Angiotensin II; Vascular endothelial growth factor

1 Introduction

Regulation of gene expression culminating in protein synthesis is complex and tightly regulated consistent with the fundamental role of proteins as important executors of cell function [1]. While considerable attention has been paid to the regulation of transcription as a site of regulation of protein synthesis, our understanding of the role played by messenger RNA (mRNA) translation in this process is rudimentary. Changes in mRNA levels are frequently thought to be reliable indicators of changes in the levels of the corresponding protein. This approach should be changed as studies in several cell types have shown discordance between the two parameters in a large number of proteins [2, 3]. However, in evaluating differences between mRNA and protein contents to make a case for translation regulation, changes in degradation rates of protein should also be taken into consideration [4]. Thus, it is essential to complement studies on transcription with investigation of synthesis, function and degradation for a comprehensive understanding protein metabolism [5]. For optimal function of an organ such as the kidney, it is essential to have the right amount of proteins as both deficiency or excess can lead to atrophy, or, hypertrophy and fibrosis, respectively. The latter processes are particularly relevant to diabetic nephropathy.

The biography of a protein begins with the generation of mRNA from the complimentary DNA sequences by the process of transcription. This is followed by transport of the mRNA out of the nucleus to the cytoplasm where it interacts with the ribosomes to make new peptide. The process by which the ribosomes generate a polypeptide from the genetic message present in the mRNA is called mRNA translation. Following synthesis of the full-length peptide there can be post-translational modifications such as glycosylation, acetylation and phosphorylation. The protein is then sorted to the target compartment in the cell or secreted to the pericellular environment. In time, the protein undergoes degradation. Of these steps, the process of mRNA translation is thought to be rate limiting in protein expression [6]. An important role for mRNA translation has been found in control of development, differentiation, tumorigenesis, inflammation, apoptosis and cell survival [7]. In this review, we will focus on recent developments in understanding the role of mRNA translation in diabetes-associated kidney injury.

1.1 Participants in eukaryotic translation

RNA exists in multiple forms that serve functions beyond coding for proteins. While only 2% of the human genome codes for mRNA involved in protein synthesis, nearly 60–70% of genome codes for non-coding RNAs such as the ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA, small nucleolar RNA, microRNA (miRNA) and lesser known species such as vault RNAs, Y RNAs and rasi-RNAs and piRNAs [8]. In this review we will focus on mRNA and its function.

The structural features of mRNA that are of importance in translation are as follows. At the 5' end of the mRNA is the m7 cap that is made of methylated guanosine triphosphate. The sequence of bases from the cap to the first codon (usually AUG) is called the 5' untranslated region (5'UTR) and these nucleotides do not code for amino acids; however, it is a site for regulation of mRNA translation. The 5'UTR is followed by the sequence of bases that carry the genetic message for amino acids in the form of triplet codons (coding sequence of mRNA); the end of peptide synthesis is signaled by a termination codon at the 3' end of the coding sequence. There is another segment of nucleotides that do not code for amino acids that form the 3'UTR and this is another site of regulation of mRNA stability and translation efficiency. Following 3'UTR, several adenosine bases are seen forming the polyA tail which is believed to stabilize the mRNA [9], and, provide binding sites for proteins that control translation efficiency, e.g., polyA binding protein (PABP) [10].

Translation of mRNAs requires the participation of tRNAs and rRNAs. The aminoacyl tRNAs bring specific amino acids for addition to the nascent polypeptide chain. Most of RNA in a cell is made of rRNA. rRNAs are important constituents of ribosomes. There are two ribosomal subunits that participate in eukaryotic translation: the 40S subunit made of 18S rRNA and 33 proteins, and, the 60S subunit made of 28S, 5.8S, and 5S rRNA and 49 proteins [5]. During translation, the two ribosomal subunits unite to form the 80S unit.

1.2 Process of mRNA translation

There is controversy regarding the steps and regulation of the translation process [4]. Eukaryotic translation occurs in three stages: the initiation stage, elongation stage and the termination stage. The goal of initiation stage is to locate the ribosomal complex on the codon for the first amino acid on the open reading frame, usually AUG for methionine. During the elongation stage, peptide synthesis occurs according to the genetic message in the mRNA. The fully synthesized peptide is released from the ribosome-mRNA complex in the termination stage. Each step is tightly regulated by eukaryotic initiation (eIFs), elongation and release factors. We will focus on the initiation stage, as most of the control on translation is exerted there [11].

Initiation stage—There are two ways the process of mRNA translation initiation can be achieved: scanning for the first AUG by participation of eIF4E, and, and the internal ribosome entry system (IRES) which eschews the need for eIF4E but employs other initiation factors. Most of the eukaryotic mRNAs are translated by the scanning mechanism. We will present a brief summary of the consensus view; the reader is referred to more detailed reviews [5, 7].

Scanning model—The early events in the initiation stage include formation of two multimeric complexes, i.e., 43S ribosomal complex and eIF4F. Several eIFs, i.e., eIF1, eIF1A, eIF3, eIF5 and the 40S ribosomal subunit are brought together in a complex. eIF2. Guanosine triphosphate (GTP) and initiator methionyl tRNA combine to form a ternary complex. eIF2 has three subunits, alpha, beta and gamma. Activation of eIF2 alpha requires exchanging the associated guanosine diphosphate (GDP) for GTP, a step catalyzed by eIF2B, a guanidine nucleotide exchange factor consisting of 5 subunits [12]. The ternary complex joins the eIF1, 1A, 3, 5, 40S ribosomal subunit to form the 43S preinitiation complex. The other important early event in the initiation step is the formation of eIF4F complex by the binding of eIF4G, a large scaffolding protein, with the mRNA cap binding protein, eIF4E and, a DEAD box protein, eIF4A. eIF4G has binding sites for eIF3, which as a component of the 43S preinitiation complex, may serve as a bridge between the 43S preinitiation complex and eIF4F complex [13]. eIF4G also has a binding site for PABP which binds to the polyA tail at the 3' end of the mRNA. This interaction serves to circularize the mRNA, which may augment the efficiency of translation [14]. The preinitiation complex binds to the mRNA close to the cap [11]. Any impediment to scanning for the first AUG codon by the ribosomal subunit posed by complexities in the 5' UTR is resolved by the helicase activity of eIF4A, probably assisted by eIF4B. The 43S preinitiation complex scans for the first AUG in an ATP-dependent manner during which GTP bound to eIF2 also undergoes hydrolysis. This is associated with release of eIF1 and eIF2.GDP. The next step consists of recruitment of the 60S ribosomal subunit and release of other eIFs, a step facilitated by eIF5B.GTP. The final product of initiation step consists of methionyl tRNA bound to AUG site on mRNA which is associated with 40S and 60S ribosomal subunits forming the 80S complex (Fig. 1).

IRES model—Translation of some mRNA species can occur even when the cap-dependent process is inhibited as occurs during cell cycle progression or during cell stress. This involves binding of the ribosome to sites on the mRNA called the IRES. Although this process is independent of eIF4E, it is facilitated by other eIFs. Other proteins called the IRES-interacting proteins are also thought to facilitate IRES-mediated translation [15]. Several viral mRNAs and a few eukaryotic mRNAs (e. g., vascular endothelial growth factor (VEGF), ornithine decarboxylase) are believed to undergo translation by this model, although it has been criticized as being inefficient [4]. The ribosomal binding sites on the IRES driven mRNAs do not share a specific sequence; however, the structural conformation of ribosomal binding sites on the mRNA is believed to facilitate interaction between the mRNA and the ribosome. IRES mRNAs may contain multiple AUG start sites.

Elongation stage—The objective of translation of synthesizing the peptide is achieved during the elongation stage. The first step in elongation stage is the arrival of the amino acyl tRNA bearing a specific amino acid corresponding to the codon on the mRNA [5]. The recruitment of aminoacyl tRNA to the A (aminoacyl) site on the 80S ribosomal complex is facilitated by eEF1A.GTP. The next step is helped by eEF2 which is active when dephosphorylated on Thr56. Dephosphorylated eEF2 is believed to facilitate movement of the ribosomal complex exactly three bases in the 5' to 3' direction such that the position of the aminoacyl tRNA now corresponds to the P site [1, 16]. At the P site, a peptide bond is

created between the previous amino acid and the one that has arrived newly and releases tRNA from the previous amino acid. The released tRNA can then be reused to deliver the specific amino acid as dictated by the codon sequence on the mRNA.

Termination stage—The synthesis of the peptide ceases when the ribosomal complex comes to the termination codon on the mRNA. Ribosomal release factor assists in the process [17]. At the end of peptide synthesis, 80S ribosomal complex dissociates into 40S and 60S subunits, a process assisted by eIF6 [18]. The subunits can be reused for the next cycle of peptide synthesis.

Regulation of translation—Several different modes of regulation exist in controlling the process of translation. They include phosphorylation of participating proteins, changes in amounts of those proteins, binding interactions among proteins, and, binding RNAs which are newly discovered small RNAs which associate with mRNA and influence outcome of translation.

One of the features of mRNA translation is the rapidity with which it occurs following application of a stimulus. It is logical to anticipate that the regulatory reactions be rapid as well. This is made possible by phosphorylation reactions by specific kinases which can be activated in a matter of seconds. Activity of regulatory factors can be affected in a positive or negative direction by phosphorylation. The dimeric complex of eIF4E and 4E-BP1 is a good example. Upon receiving a stimulus for increasing translation, 4E-BP1 is phosphorylated rapidly allowing it to dissociate from eIF4E. The newly freed eIF4E forms a complex with eIF4G and binds to the cap structure of the mRNA to promote its translation [19]. Conversely, in some instances, dephosphorylation can augment activity of a translation factor. In the resting cell, eEF2 is kept inactive by phosphorylation of Thr56. Upon stimulation of translation, eEF2 is activated by dephosphorylation of Thr56 [20]. Activity of some of the regulatory proteins in translation is controlled by their binding molecules. Thus, eIF2 alpha, eIF5 and eEF1A are activated by association with GTP and inhibited by association with GDP. There are distinct guanine nucleotide exchange factors that dictate these reactions. The association between eIF4E and 4E-BPs, is another prime example of regulation by binding proteins, and was described above. Binding interactions among the factors that regulate initiation phase can also affect efficiency of translation. For example, binding of eIF4G, which interacts with the 5' end of the mRNA, with PABP, which binds the polyA sequence at the 3' end of the mRNA, serves to promote circularization of the mRNA; this is believed to improve the efficiency of translation [14].

Recent advances have shown that the process of translation can be regulated by small interfering RNAs (siRNAs), miRNAs, and piRNAs. The siRNAs are between 20–25 nucleotides long and are derived from double stranded RNAs [21, 22]. The precursor double stranded RNAs are digested by Dicer, a cleaving enzyme. The fragmented double stranded RNAs bind to proteins which contain double stranded RNA binding domains, e.g., Ago protein, belonging to the Argonaute family, and form a complex called the RNA-induced silencing complex (RISC) [21]. The RISC helps in processing the double stranded RNA into a single functional strand and the complex binds to the mRNA in areas of strict complementarity. This results in degradation of the mRNA and inhibition of its translation.

The second class of RNAs, miRNAs, are 15–18 nucleotides long. They combine with proteins like the Ago to form microribonucleoprotein (miRNP), also called miRNA-induced silencing complexes. The mature miRNA binds with roughly complimentary sequences in the target mRNA. The miRNP can promote not only fragmentation of mRNA but also inhibit mRNA translation [23, 24]. It appears this inhibition applies to translation of capped mRNAs with poly A tails [25] and not to IRES containing mRNAs [23, 24].

The role of signaling reactions in regulation of renal mRNA translation is discussed in detail in the context of diabetic renal disease.

1.3 mRNA translation in diabetic nephropathy

Cardinal aspects of diabetic nephropathy include hypertrophy evident in early stages and matrix accumulation and renal fibrosis that occur with longer duration of disease. Both these events require increase in contents of structural proteins and extracellular matrix proteins, which is achieved by a combination of increased synthesis and decreased degradation. Kidney injury in diabetes is mediated by several growth factors that are synthesized in higher amounts, e.g., angiotensin II (Ang II), TGF beta, VEGF, connective tissue growth factor. Until recently, increase in synthesis of proteins was attributed almost solely to augmented transcription. Recent investigations from many laboratories including ours have revealed an important role for mRNA translation as an independent or coordinated site of regulation of protein synthesis. There is an additional reason for translation to be a target of regulation in diabetes. The process of translation consumes large amounts of cell energy. Diabetes is clearly a state of altered energy regulation due to lack of physiological transport of glucose into the cells. It is inevitable that such major energy consuming reactions as translation be affected in the diabetic state.

Hypertrophy in diabetic nephropathy—Hypertrophy is characterized by cell enlargement due to increase in protein and RNA content per cell with little change in DNA content. Kidney growth in diabetes is mostly due to hypertrophy although a brief period of hyperplasia, mostly involving mesangial cells, has been reported [26–28]. Although renal hypertrophy is encountered in unilateral nephrectomy and following high protein diet consumption or steroid administration, progressive renal disease is not seen with these conditions; however, renal hypertrophy in diabetes is associated with progressive renal injury [29]. Renal hypertrophy is the earliest structural abnormality in diabetes and is coincident with high GFR. There are contesting schools of thought on the mechanism of hypertrophy in diabetes that support glomerulocentric and tubulocentric schemes [29, 30]. In addition to the aforementioned growth factors, insulin like growth factor (IGF-I), hepatocyte growth factor and epidermal growth factor, have also been implicated in diabetes-induced renal hypertrophy [29]. IGF-I expression is augmented in renal cortex at the time of renal hypertrophy in rodents with type 1 or type 2 diabetes, suggesting a role for IGF-I in stimulation of protein synthesis [31]. Mechanism of IGF-I stimulation of protein synthesis in renal proximal tubular epithelial cells was mediated by the induction of 4E-BP1 phosphorylation in a PI3-kinase-Akt-mammalian target of rapamycin (mTOR) signaling pathway [31].

Cell cycle events are closely regulated in renal hypertrophy in diabetes. Thus, p27 Kip1, a cyclin-dependent kinase inhibitor, is stimulated in diabetes-associated kidney growth, diverting renal cells to increase protein synthesis rather than progress toward cell division; thus, in mice deficient in p27 kip1 renal hypertrophy is inhibited following 6–12 weeks of diabetes induced by streptozotocin [32, 33].

AMP activated protein kinase in diabetic renal hypertrophy—Since increase in protein synthesis contributes to hypertrophy, we have studied the regulation of mRNA translation in models relevant to diabetic nephropathy. High glucose induced hypertrophy of glomerular epithelial cells *in vitro* [34]. This was associated with increase in phosphorylation of Thr37/46 on 4E-BP1 and decrease in Thr56 phosphorylation of eEF2 indicating both the initiation phase and elongation phases of translation were stimulated. Since translation consumes large amounts of cellular energy and energy status is dysregulated in diabetes, we studied the role of AMP-activated protein kinase (AMPK) in high glucose induced glomerular epithelial cell hypertrophy. AMPK is a heterotrimeric protein with distinct alpha, beta and gamma subunits; Thr172 phosphorylation of alpha subunit is required for the kinase activity [35]. AMPK activity is stimulated in energy deficient states resulting in stimulation of energy generating reactions and inhibition of energy consuming reactions [36]. AMPK phosphorylation on Thr172 of the alpha subunit and its kinase activity were reduced in glomerular epithelial cells incubated with high glucose. Stimulation of AMPK activity with metformin or AICAR resulted in inhibition of protein synthesis induced by high glucose. Streptozotocin-induced diabetes in the rat was associated with 31% increase in the kidney to body weight ratio demonstrating hypertrophy. This was associated with increase in phosphorylation of Akt, mTOR, p70S6 kinase and 4E-BP1 in renal cortical homogenates. Administration of metformin and AICAR resulted in inhibition of renal hypertrophy in diabetic rats without reducing the plasma glucose levels [34]. Stimulation of AMPK with metformin and AICAR reversed changes in high glucose induced changes in phosphorylation of 4E-BP1 and eEF2, both *in vivo* and *in vitro*, suggesting AMPK regulated initiation and elongation phases of translation. Analysis of signaling pathway was performed to locate AMPK in high glucose-induced signaling pathways that control initiation and elongation phases of translation. High glucose induced the activity of phosphatidylinositol 3 kinase (PI 3 kinase), a lipid kinase, as shown by the increased generation of phosphatidylinositol 3, 4, 5, tris phosphate. This was associated with increase in phosphorylation and activation of PI 3 kinase downstream target, Akt, a serine threonine kinase, and its downstream target, mTOR (Fig. 2). The activation of mTOR was demonstrated by increase in phosphorylation of its substrates 4E-BP1 and Thr389 on p70S6 kinase. Stimulation of AMPK by metformin and AICAR resulted in inhibition of diabetes and high glucose-induced mTOR activation but without affecting the increase in Akt phosphorylation. These data suggested that AMPK was interposed between Akt and mTOR in the renal cells in diabetes [34]. Studies in non-renal cell types have shown that AMPK is downstream of Akt and phosphorylation of AMPK by Akt suppresses the activity of the latter [37]. AMPK phosphorylates tuberous sclerosis protein, tuberin, (TSC-2) and stimulates its activity [38]. In the resting cell, TSC-1 and TSC-2 form a heterodimer; TSC2 is a GTPase activating protein that suppresses activation of (Ras homolog enriched in brain) Rheb[39]. Rheb when associated with GTP stimulates mTOR activity [40]. Thus, it appears

that AMPK-TSC pathway suppresses Rheb and mTOR and keeps protein synthesis in check. Upon stimulation, AMPK activity is inhibited by induction of PI 3 kinase-Akt axis resulting in inhibition of TSC2/TSC-1 and release of Rheb-mTOR activity (Fig. 2). The latter leads to stimulation of initiation and elongation phases of translation by phosphorylation of 4E-BP1 and p70S6 kinase, respectively [34]. These pathways are shown in a schematic in Fig. 2. It is important to note that mTOR induction of 4E-BP1 phosphorylation is also important for compensatory kidney hypertrophy induced by uninephrectomy [41].

Resveratrol, a phytochemical present in grapes, was also found to inhibit high glucose-induced protein synthesis in the glomerular epithelial cells and renal hypertrophy in mice with streptozotocin-induced diabetes, by promoting AMPK activity [42]. Thr172 phosphorylation of AMPK is under the control of LKB1, a serine threonine kinase [43]. High glucose reduced AMPK phosphorylation at Thr172 by inhibiting activity of LKB1 in glomerular epithelial cells; this was associated with increase in acetylation of LKB1. Resveratrol augmented LKB1 activity by deacetylating the kinase. Since resveratrol is known to stimulate Sirt1 deacetylase [44], we examined its role in resveratrol action; Sirt1 did not appear to be involved in resveratrol-induced deacetylation of LKB1 in glomerular epithelial cells, suggesting involvement of some other deacetylase or inhibition of the acetylating enzyme. Stimulation of AMPK activity with resveratrol resulted in reversal of high glucose-induced decrease in eEF2 phosphorylation in association with inhibition of high glucose-induced protein synthesis. These data suggested that inhibition of protein synthesis by resveratrol involved regulation of elongation phase of translation mediated by AMPK. Based on these data, ability of resveratrol to inhibit diabetes-induced renal hypertrophy was examined. Resveratrol administration to mice with Streptozotocin-induced diabetes did not affect hyperglycemia but inhibited renal hypertrophy significantly; this effect was associated with restoration of diabetes-induced reduction in AMPK activity in the renal cortex to normal levels. Resveratrol is being widely investigated as an agent to promote weight control and improve insulin sensitivity in obesity [45, 46]. Further work is needed to explore if it can ameliorate long-term kidney injury in diabetes.

Another agent that stimulates AMPK is adiponectin. Reduction in plasma levels of adiponectin, an adipose tissue-derived cytokine, correlates with insulin resistance [47]. In a series of elegant studies, Sharma and associates have recently described that genetic deficiency of adiponectin in mice results in podocyte injury and albuminuria [48]. This was associated with disordered distribution of the tight junction protein, ZO-1, in the podocyte membrane. Furthermore, deficiency of adiponectin was associated with reduction in AMPK activation and increase in oxidative stress mediated by NAD(P)H oxidase 4, which is abundantly present in the kidney, particularly in podocytes; stimulation of AMPK with a pharmacologic agent or by administration of adiponectin reduced oxidative stress, ameliorated podocyte injury and albuminuria in the adiponectin deficient mice with or without diabetes. Thus, AMPK activation by adiponectin is important for maintenance of the integrity of podocyte structure and selective permeability function of the glomerular capillary wall. These studies by Sharma et al demonstrate the presence of an adipose tissue-renal axis.

We mentioned above that high glucose increases PI 3 kinase activity in the GEC. Inhibition of PI 3 kinase activity with LY294002 abolished high glucose-induced increase in 4E-BP1 phosphorylation and augmented protein synthesis, showing hyperglycemia-promoted GEC hypertrophy was PI3 kinase-dependent [34]. Cells express an inhibitor of PI 3 kinase called phosphatase and tensin homolog (PTEN). Expression of PTEN is reduced in the hypertrophic renal cortex in streptozotocin-induced diabetes. In mesangial cell hypertrophy induced by high glucose or TGF β , PTEN expression and activity are reduced, mediated by TGF β ; restoration of PTEN gene expression prevented mesangial hypertrophy induced by high glucose and TGF β [49]. Thus, PI 3 kinase axis participates in diabetes-induced renal hypertrophy.

Angiotensin II in diabetic nephropathy—The importance of renin-angiotensin II-aldosterone axis in the pathogenesis of diabetic nephropathy has been established by numerous studies employing *in vitro* and *in vivo* models [50, 51], and success of the antagonists of these proteins in ameliorating clinical manifestations of diabetic nephropathy [52]. In addition to its direct action on the hemodynamics of the kidney and direct modulation of biology of renal cells, Ang II is well known to recruit other mediators, e.g., TGF β and connective tissue growth factor [53]. Expression of VEGF is increased in diabetic nephropathy in both type 1 and type 2 diabetes [31], and, clinical indices of kidney injury in diabetic rodents are improved by neutralizing antibodies against VEGF [54, 55], suggesting VEGF has a pathogenic role in diabetic nephropathy. Feliers and associates have examined if synthesis of VEGF is also under the control of Ang II in the diabetic kidney. Physiologic concentrations of Ang II (1 nM) rapidly augmented synthesis of VEGF by proximal tubular epithelial cells, starting at 5 min and lasting for up to 1 h [56]. VEGF increment was not due to increase in its mRNA, or decrease in its half life, suggesting a non-transcriptional mechanism. Polysome analysis showed that angiotensin II augmented multiple ribosomes to associate with VEGF mRNA, providing direct evidence of increase in efficiency of initiation phase of translation. Angiotensin II-induced VEGF synthesis was associated with rapid phosphorylation of 4E-BP1 on Thr37/46 which required activation of PI 3 kinase and Akt. Experiments employing cells stably expressing Thr37/46 to Ala37/46 mutation showed that Ang II induction of VEGF synthesis required phosphorylation of 4E-BP1 on these residues [56]. The rapid induction of VEGF mRNA translation by Ang II was found to be dependent on generation of reactive oxygen species originating in the NAD(P)H oxidase system rather than the mitochondrial system [57].

Feliers et al explored if in addition to the events taking place in the 5' terminus of the mRNA such as phosphorylation of 4E-BP1, whether events occurring in the 3' end of the mRNA during translation also had a regulatory role in angiotensin II induction of VEGF synthesis. They explored the role of heterogeneous nuclear ribonucleoprotein K (hnRNP K). The hnRNPs are known to bind the 3' UTR of mRNA and generally they are thought to inhibit mRNA translation [58]. Induction of VEGF translation by angiotensin II was associated with phosphorylation and activation of hnRNP K, and augmented binding to VEGF mRNA [59]. Reduction in hnRNP K expression by siRNA led to inhibition of VEGF translation induced by angiotensin II, suggesting that hnRNP K contributes to Ang II stimulation of VEGF translation. Angiotensin II also promoted phosphorylation of hnRNP K

on both tyrosine and serine residues; however, temporally only the serine phosphorylation correlated with increased binding (ibid). Src, a nonreceptor tyrosine kinase, was activated by angiotensin II and was required for stimulation of hnRNP K tyrosine phosphorylation. Surprisingly, inhibition of Src not only inhibited tyrosine phosphorylation of hnRNP K but also the serine phosphorylation, indicating that Src activated another kinase, which led to serine phosphorylation. In a subsequent study, these investigators identified the hnRNP K serine kinase to be PKC delta [60]. These studies have revealed that events occurring at both the 5' and 3' ends of the mRNA play regulatory role in VEGF translation induced by angiotensin II.

Feliers and associates have initiated experiments examining the role of angiotensin II receptors in VEGF expression in the early stages of diabetes in the rat. In association with renal hypertrophy, renal cortical VEGF protein expression was increased two fold; however, its mRNA content was unchanged suggesting translational mechanism (Feliers and Kasinath, unpublished data). Polysome analysis confirmed that increased VEGF expression was due to stimulation of the initiation phase of translation. Reduction in Thr56 phosphorylation on eEF2 suggested stimulation of elongation phase of translation in the diabetic renal cortex. Administration of specific inhibitors showed that activation of AT2 receptor, and not AT1 receptor, was involved in increased VEGF expression in the kidney in the diabetic rats. This is the first demonstration that AT2 receptors are involved in VEGF translation *in vivo* in the kidney in diabetic rats.

TGFb plays an important role in kidney injury in diabetes [61]. High glucose-stimulated increment in TGFb expression is regulated at the level of transcription in proximal tubular epithelial cells; however, a permissive action of platelet derived growth factor may be needed for the successful translation of the mRNA transcripts of TGFb into protein [62].

Extracellular matrix accumulation in diabetic nephropathy—Prolonged and uncontrolled diabetes leads to gradual accumulation of proteins in glomerular and tubulointerstitial extracellular matrices [63]. Although hyperglycemia is an obvious pathogenic factor, we have also examined the role of hyperinsulinemia in pathologic changes in the kidney in type 2 diabetes. Elevation of plasma insulin levels precedes hyperglycemia and persists following the onset of hyperglycemia in both rodent models and humans with type 2 diabetes [64, 65]. In the db/db mouse with type 2 diabetes, insulin resistance accompanies obesity and precedes appearance of type 2 diabetes, similar to humans [66]. The phase of insulin resistance and elevation of plasma insulin coincides with onset of hyperglycemia-associated hypertrophy and matrix accumulation in the kidney [67]. *In vitro*, insulin promotes protein synthesis by stimulating initiation phase of mRNA translation mediated by the PI 3 kinase-Akt-mTOR and Erk signaling systems [68]. Detailed examination of renal cortices in the early (4 weeks of diabetes) and established (3 to 4 months of diabetes) stages of type 2 diabetes in the db/db mouse showed increase in tyrosine phosphorylation of the beta chain of the insulin receptor and insulin receptor substrate 2, increase in the tyrosine kinase activity of the insulin receptor and increase in PI 3 kinase activity associated with the insulin receptor [69]; however, liver tissue from the same mice displayed insulin resistance. Activation of insulin receptor has also been reported in the retina and the vessel wall in a state of insulin resistance and in type 2 diabetes [70, 71].

Activation of insulin receptor in the kidney tissue in db/db mice demonstrated that unlike the liver, a site of insulin resistance in type 2 diabetes, renal cortex is insulin-sensitive and therefore, susceptible to pathologic effects of hyperinsulinemia in type 2 diabetes. Role of hyperinsulinemia and hyperglycemia in matrix metabolism in type 2 diabetes has been studied using laminin as an example. Laminin is a heterotrimeric protein with distinct chain composition in each of the extracellular matrix compartments of the kidney [72]. Ha et al reported that accumulation of beta1, gamma1 and alpha5 chains of laminin in the renal cortex of db/db mice occurred without corresponding increments in the respective mRNA content [67]. The non-transcriptional regulation of laminin content could be due to increase in efficiency of mRNA translation and decrease in degradation rates. The former possibility was further examined. Mariappan and associates showed that incubation of proximal tubular epithelial cells *in vitro* with 1 nM insulin or 30 mM glucose, levels seen in db/db mice [67], or both together, resulted in rapid increment in laminin beta 1 synthesis without changes in its mRNA [73]. Abolition of this effect by cycloheximide but not actinomycin D suggested translational regulation. High glucose- and high insulin-induced increase in laminin beta 1 chain synthesis was found to be dependent on increase in Thr37/46 phosphorylation of 4E-BP1 and accompanied by dissociation of eIF4E/4E-BP1 complex and formation of eIF4E-eIF4G dimeric complex, two events of critical importance in the initiation phase of translation. Initiation phase stimulation was found to be dependent on recruitment of PI 3 kinase, Akt, mTOR signaling pathway (Fig. 2) [73].

Both initiation and elongation phases of translation are under the control of mTOR. Activity of mTOR is exerted in the form of two complexes. mTOR complex1 (mTORC1) is formed by the association of mTOR with raptor and GbetaL whereas mTORC2 is formed by binding of mTOR with rictor and GbetaL [74]. Rapamycin is able to inhibit mTORC1 and not mTORC2. The actions of mTORC1 include phosphorylation of 4E-BP1 and p70S6 kinase, facilitating protein synthesis. Although the full spectrum of activity of mTORC2 is not fully understood, it may include reorganization of cytoskeleton [74]. Recent studies have revealed a novel interaction between mTORC2 and mTORC1 in mesangial cells; reduction in rictor by shRNA augmented phosphorylation of mTORC1 substrates, 4E-BP1 and p70S6 kinase. These data suggest that at basal level, rictor may impose a tonic inhibition on activity of mTORC1 [75].

Mariappan et al showed importance of initiation phase regulation in translation of laminin beta 1 synthesis by high glucose and high insulin [73]. Studies were extended to examine if high glucose and high insulin also regulated elongation phase of translation. Sataranatarajan and associates reported recently that high glucose and high insulin promoted Thr56 dephosphorylation of eEF2, a major event in the translocation step of elongation [76]. This was found to be dependent on mTOR activation by employing rapamycin, a specific inhibitor of the kinase. Further studies were done to elucidate the regulation of the elongation phase by mTOR. Activation of p70S6 kinase by mTOR enables the former to catalyze phosphorylation of Ser366 on eEF2 kinase [77], thereby inactivating it (Fig. 3). There may be input from Erk via p90rsk at this step. This could contribute to decrease in Thr56 phosphorylation of eEF2. In addition, a phosphatase such as PP2, could also promote dephosphorylation of eEF2. Since these steps in elongation phase and high glucose and high insulin-induced laminin beta 1 chain synthesis in the proximal tubular epithelial cells could

be blocked by rapamycin, effect of that agent on laminin accumulation in db/db mice was examined. Rapamycin administration for 2 weeks did not affect plasma glucose levels in either the control or diabetic mice. Rapamycin inhibited diabetes associated renal hypertrophy in the diabetic mice. In addition, rapamycin significantly inhibited laminin accumulation in both glomeruli and tubules. This ameliorative effect was associated with reversal of diabetes-induced reduction in Thr56 phosphorylation of eEF2, increase in Ser366 phosphorylation of eEF2 kinase and activation of mTOR. Investigators have previously reported that rapamycin improves renal disease in rodents with type 1 diabetes or type 2 diabetes [78, 79]; however, underlying mechanism of rapamycin effects was not studied in depth. Studies of Sataranatarajan et al. provide a mechanistic basis for these observations. Taken together, these studies suggest mTOR as a potential target for inhibition in diabetic nephropathy.

Signaling pathways could yield other potential sites for intervention in diabetic nephropathy. Similar to AMPK reviewed above, one can strive to identify kinases that may inhibit translation and help ameliorate diabetic nephropathy. In this context, we have examined the role of glycogen synthase kinase 3 beta (GSK 3 beta). GSK 3 beta regulates numerous pathways involved in glycogen metabolism [80], cytoskeletal regulation [81], cell cycle progression [82] and cell survival. GSK 3 beta can influence protein synthesis by regulating the activity of eIF2B. As described earlier, during initiation phase of translation activation of eIF2 alpha, a constituent of preinitiation complex, requires exchanging the associated GDP for GTP, a step catalyzed by eIF2B, a guanine nucleotide exchange factor consisting of five subunits [12]. In the resting state of the cell, GSK 3 beta phosphorylates eIF2B and keeps it in an inactive state, thus inhibiting protein synthesis [83]. The role of GSK 3 beta in diabetes-induced laminin beta 1 synthesis was examined [84]. As discussed above, purely translational regulation of laminin beta1 was found with short term incubation with high glucose and high insulin in proximal tubular epithelial cells; however, prolonging the duration of exposure to 24 h caused increase in laminin beta 1 that was accompanied by increase in mRNA suggesting that at longer duration of incubation laminin beta1 was regulated at the transcriptional level by high glucose and high insulin. Stimulation of laminin beta 1 synthesis was associated with increase in Ser9 phosphorylation of GSK 3 beta which results in its inactivation; this was confirmed by reduction in phosphorylation of Ser539 on eIF2B epsilon, a direct substrate of GSK 3 beta. These data suggested reduced phosphorylation of eIF2B would now allow it to function as a guanine nucleotide exchange factor and promote initiation phase of mRNA translation. Accordingly, these changes in GSK 3 beta and eIF2B epsilon were associated with increase in phosphorylation of 4E-BP1 and reduction in phosphorylation of eEF2, important regulatory steps in initiation and elongation phases of translation, respectively. Examination of signaling pathways with dominant negative constructs and selective chemical inhibitors showed that Ser9 phosphorylation of GSK 3 beta was under the control of PI 3 kinase-Akt-mTOR and Erk pathways. The relevance of changes in GSK 3 beta and eIF2B phosphorylation induced by high glucose and high insulin was evaluated in the renal cortex of db/db mice with type 2 diabetes. During the phase of renal hypertrophy and at a time when laminin beta 1 synthesis is evident, there was a reduction in Ser9 phosphorylation of GSK 3 beta and in Ser539 phosphorylation of eIF2B epsilon, suggesting a role for GSK 3 beta in renal hypertrophy and

matrix accumulation in the kidney in type 2 diabetes. Since Akt activity is increased in renal cortex at this time [69], it is likely that Akt may promote Ser9 phosphorylation of GSK3 beta, as reported in non-renal cells. These data extend the role of GSK 3 beta to protein synthesis and suggest GSK 3 beta as a potential therapeutic target in diabetic renal disease. However, it must be noted that generalized stimulation of GSK 3 beta is not desirable in type 2 diabetes as it would further inhibit glycogen synthesis and promote more resistance to insulin; thus, techniques that selectively stimulate GSK 3 beta in the kidney will have to be developed. Nevertheless, these data show how examination of translation can yield novel therapeutic targets in diabetic nephropathy.

2 Conclusions

As enhanced synthesis of protein is an important contributing factor in the pathogenesis of diabetic nephropathy, control of that process is a legitimate therapeutic target. Protein levels can be reduced by interfering with either the process of transcription or of mRNA translation, or by augmenting degradation. Although inhibition of transcription could lead to reduction in protein synthesis, it should be noted that this entails a lag period during which the existent mRNA residues can still be translated. On the other hand, inhibiting translation can result in nearly immediate cessation of synthesis of new copies of the peptide [22]. This can be achieved by RNA silencing via siRNA or microRNA, by targeting individual steps in initiation or elongation stage of translation, or, by modulating the activity of signaling kinases and phosphatases. Whereas the use of siRNA or microRNA can lend specificity in terms of protein target, other steps may inhibit general protein synthesis in the kidney and may be useful in blocking general renal hypertrophy. Thus, translation can be an attractive additional site of therapeutic intervention in diabetic nephropathy.

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Fig. 1.
Scanning model of translation initiation

Fig. 2.
Signaling pathways involved in regulation of initiation phase of translation in kidney in diabetes. Adapted from [5] with permission from the American Society of Nephrology

Fig. 3.

Signaling pathways involved in regulation of elongation phase of translation in kidney in diabetes. Adapted from [5] with permission from the American Society of Nephrology