Original Article

High Glucose, High Insulin, and Their Combination Rapidly Induce Laminin-β1 Synthesis by Regulation of mRNA Translation in Renal Epithelial Cells

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Laminin is a glycoprotein that contributes to renal extracellular matrix expansion in diabetes. We investigated regulation of laminin-β1 synthesis in murine renal proximal tubular epithelial cells by 30 mmol/l glucose (high glucose), 1 nmol/l insulin (high insulin), and their combination (high glucose+high insulin), simulating conditions observed during progression of type 2 diabetes. Compared with 5 mmol/l glucose and no insulin (control), high glucose alone, high insulin alone, or high glucose+high insulin together increased laminin-β1 chain protein synthesis within 5 min, lasting for up to 60 min with no change in laminin-β1 mRNA levels. Cycloheximide, but not actinomycin-D, abrogated increased laminin-β1 synthesis. High glucose, high insulin, and high glucose+high insulin stimulated phosphorylation of 4E-BP1, a repressor binding protein for eukaryotic initiation factor 4E (eIF4E), that was dependent on activation of phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin. High glucose, high insulin, and high glucose+high insulin also promoted release of eIF4E from 4E-BP1, phosphorylation of eIF4E, and increase in eIF4E association with eIF4G, critical events in the initiation phase of mRNA translation. High glucose, high insulin, and high glucose+high insulin increased Erk phosphorylation, which is an upstream regulator of eIF4E phosphorylation, and PD098059, which is a MEK inhibitor that blocks Erk activation, abolished laminin-β1 synthesis. This is the first demonstration of rapid increment in laminin-β1 synthesis by regulation of its mRNA translation by cells exposed to high glucose, high insulin, or high glucose+high insulin. *Diabetes* 56:476–485, 2007

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AMPK, AMP-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; eIF4E, eukaryotic initiation factor 4E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; MCT, mouse cortical tubule; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3 phosphate; TCA, trichloroacetic acid; VEGF, vascular endothelial growth factor.

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Insulin resistance and hyperinsulinemia are seen early in type 2 diabetes, followed by hyperglycemia, sometimes accompanied by continued elevation of plasma insulin, although insufficient to compensate for metabolic demands (1,2). Whereas the role of hyperglycemia in diabetes-related complications is established, the role of insulin remains unclear. Resistance to insulin actions in tissues in type 2 diabetes is not universal, with kidney and retina remaining sensitive in contrast to liver (3,4). Insulin may have a regulatory role in renal extracellular matrix (ECM) synthesis because renal cortical insulin receptor activation (3) coincides with the onset of accumulation of laminin-β1, an abundant constituent of the renal ECM (5).

Progressive accumulation of ECM proteins in the glomerular mesangium and tubulointerstitium in diabetes involves laminin in addition to type IV collagen and fibronectin (5–7). Laminin is a large heterotrimeric glycoprotein consisting of α-, β-, and γ-chains, with the subunit composition varying with compartment of the renal ECM. Thus, the glomerular basement membrane contains α5-, β2-, and γ1-chains, whereas the tubular basement membrane contains α5/1-, β1-, and γ1-chains (8). Laminin is involved in regulation of glomerular barrier function because mice lacking laminin-β2 develop severe proteinuria (9). Renal laminin-β1 content is increased in type 1 diabetes, correlating with increased abundance of mRNA (10,11). However, enhanced mRNA expression does not account for increase in renal laminin-β1 chain content in a murine model of type 2 diabetes at a time when the mice manifest hyperinsulinemia and hyperglycemia, suggesting nontranscriptional regulation (5). Accordingly, in the present study, we tested the hypothesis that high glucose, high insulin, and their combination augment mRNA translation to induce laminin-β1 chain synthesis in proximal tubular epithelial (mouse cortical tubule [MCT]) cells. These cells were chosen because they form the bulk of the kidney cortex on which observations on laminin-β1 chain were made in type 2 diabetes (5). The three experimental conditions were designed to simulate the three stages observed in the progression of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Cell culture. SV-40 immortalized murine proximal tubular epithelial (MCT) cells (provided by Dr. Eric Neilson, Vanderbilt University, Nashville, TN) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 7% fetal bovine serum, 5 mmol/l glucose, 100 units/ml penicillin, 100 μg/ml streptomy-

cin, and 2 mmol/l glutamine. MCT cells express in vivo properties of proximal tubular epithelial cells (12). Confluent cells were growth-arrested for 18 h in serum-free DMEM before experiments (13). Some experiments were conducted on glomerular epithelial cells grown as described previously (14).

Immunoblotting. Equal amounts of protein from cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with primary antibody for 3 h. All the primary antibodies were from Cell Signaling (Beverly, MA), if not otherwise mentioned. After washing, the membrane was incubated with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were visualized by chemiluminescence using the ECL reagent. Trichloroacetic acid (TCA)-precipitable proteins from medium fractions were run on SDS-PAGE and immunoblotted as described above. Images of the bands were scanned by reflectance scanning densitometry, and the intensity of the bands was quantified using NIH Image software (15). For immunoblot analysis of 4E-BP1 phosphorylation, cell lysates were heated at 100°C for 7 min and treated with 15% TCA. Immunoblotting was performed using an antibody specific for 4E-BP1 phosphorylated at Thr37/46 (16).

Measurement of protein synthesis. Quiescent MCT cells were preincubated with 10 μCi/ml [^{35}S]methionine for 2 h before incubation with high glucose (30 mmol/l), high insulin (1 nmol/l), or their combination. Cells were washed and lysed, and an equal amount of protein (300 μg) was immunoprecipitated using anti–laminin-β1 antibody. The protein coupled to protein A agarose beads were separated by boiling with sample buffer lacking bromophenol blue and centrifuged. The supernatants were spotted on 3MM filter paper (Whatman International, Maidstone, U.K.). Filters were washed three times by boiling for 1 min in 10% TCA containing 0.1 g/l methionine before determining radioactivity (15).

Phosphatidylinositol 3-kinase activity assay. Equal amounts of cell lysates (300 μg) were immunoprecipitated using an antibody against p85-α regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (Santa Cruz Biotechnology). The immuno beads were washed with radioimmunoprecipitation assay buffer, PBS, 0.5 mol/l LiCl, distilled water, and finally with the PI 3-kinase assay buffer and used for activity assay. The reaction product was subjected to thin-layer chromatography, and the phosphatidylinositol 3 phosphate (PI3P) spots were visualized by autoradiography (16).

Real-time RT-PCR. Total RNA was prepared from MCT cells using TRI reagent (Sigma, St. Louis, MO) per the manufacturer's specifications. Five micrograms total RNA from each sample was reverse transcribed with random hexamers using a commercially available kit (Invitrogen). PCR primer sequences for amplification of mouse laminin-β1 (Primer Bank ID no. 21595540a2) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Primer Bank ID no. 6679937a1) were obtained from Primer Bank, a public resource for PCR primers (17). Two microliters cDNA was amplified using SYBR Green PCR Master mix (Applied Biosystems) containing 100 nmol/l forward and reverse primers. PCR amplification was performed using 7900HT Sequence Detection System (Applied Biosystem) using the manufacturer's protocol. Dissociation curve analysis was performed after PCR amplification to confirm the specificity of the primers. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method.

Transfection studies. We used stable transfection of MCT cells with a plasmid carrying 4E-BP1 mutant (pACTAG-BP1 Thr^{37,46}→Ala^{37,46}) that carries a hemagluttinin tag. For control, we used a plasmid vector that does not contain a 4E-BP1 insert (13). MCT cells were transiently transfected with plasmids containing the dominant negative constructs for p85 regulatory subunit of PI 3-kinase or mTOR using lipofectamine-Plus reagent (Invitrogen) (18). SRα expression vector containing dominant negative p85 regulatory subunit of PI 3-kinase (SRα-Δp85) was provided by Dr. W. Ogawa (Kobe University, School of Medicine, Kobe, Japan). The mutant lacks a region (residues Met^{479} to Lys^{513}) in p85-α necessary for its association with the catalytic p110 subunit of PI 3-kinase (19). Plasmid containing kinase-dead mTOR (S2481A) was provided by Dr. S.L. Schreiber (Harvard University, Boston, MA) (20).

Statistical analysis. All values are expressed as means ± SE obtained from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) for comparison between multiple groups and post hoc analysis using Student-Newman-Keuls multiple comparison test; P values <0.05 were considered statistically significant.

RESULTS

High glucose, high insulin, and their combination (high glucose+high insulin) stimulate laminin-β1 synthesis and secretion. Immunoblotting of cell lysates showed that 30 mmol/l glucose (high glucose) alone, 1 nmol/l insulin (high insulin) alone, or high glucose and high insulin together increased laminin-β1 synthesis in a time-dependent manner, evident at 5 min and lasting for 30 min (Fig. 1*A–C*), accompanied by increase in its secretion into medium (Fig. 1*D–F*). Dose-response studies showed optimal effects on laminin-β1 synthesis at these concentrations (Supplemental Fig. S1A, which is detailed in the online appendix [available at http://dx.doi.org/10.2337/db05-1334]). Longer incubation with high glucose, high insulin, or high glucose+high insulin showed that laminin-β1 synthesis was again stimulated at 12 h and lasted for up to 48 h (Supplemental Fig. S1B). However, the three conditions did not induce synthesis of fibronectin and type IV collagen in MCT cells in the time frame of 5–60 min (Supplemental Fig. S1C). Although high glucose, high insulin, and high glucose+high insulin augmented de novo protein synthesis by 20–25% (data not shown), immunoprecipitation of total protein with laminin-β1 antibody still showed significant increment in labeled laminin-β1 synthesis under the three conditions (Supplemental Fig. S1D). Selective increment in laminin-β1 chain but not of type IV collagen and fibronectin suggested that matrix proteins were regulated individually and were not a nonspecific part of increment in general protein synthesis. Equimolar mannitol, used as an osmotic control for high glucose, did not affect laminin-β1 synthesis (Fig. 1*A* and *D*, last lane). Combined incubation of high glucose with high insulin did not have an additive or synergistic effect compared with high glucose alone or high insulin alone.

Augmented laminin-β1 synthesis is not due to increase in transcription. Real-time RT-PCR showed that laminin-β1 chain transcript levels were unaffected during 5- to 60-min incubation with the three conditions (Fig. 2*A*). Preincubation with cycloheximide, a translation inhibitor, but not actinomycin D, a transcription inhibitor, effectively blocked increase in laminin-β1 synthesis under the three conditions (Fig. 2*B* and *C*). The three conditions promoted synthesis of laminin-β1 chain within 5 min in rat glomerular epithelial cells (Supplemental Fig. S2A), which was inhibited by cycloheximide and not actinomycin D (Supplemental Fig. S2B), suggesting that rapid regulation of laminin-β1 chain synthesis was due to regulation of mRNA translation in this cell type as well. Cycloheximide stimulated phosphorylation of p38 mitogen-activated protein (MAP) kinase; however, it was not seen with high glucose or high insulin (Supplemental Fig. 3). These results show that the rapid increase in laminin-β1 content by high glucose, high insulin, and high glucose+high insulin involves mechanisms other than transcription, i.e., decreased degradation (21) and/or increased mRNA translation; the regulation via mRNA translation was further investigated.

High glucose, high insulin, and high glucose+high insulin induce 4E-BP1 phosphorylation. During the initiation phase of mRNA translation, binding of the 43S ribosomal preinitiation complex to the mRNA is facilitated by eukaryotic initiation factor 4E (eIF4E) in association with eIF4G and eIF4A (22). In the resting cell, eIF4E is held in an inactive complex by its binding protein 4E-BP1. Upon stimulation, 4E-BP1 is phosphorylated on Thr37/46 and other threonine and serine residues (23). This results in dissolution of the complex, freeing eIF4E to bind eIF4G and eIF4A and promote mRNA translation. Insulin is a powerful stimulus for 4E-BP1 phosphorylation in MCT cells (16,24). Immunoblot analysis showed that high glucose, high insulin, and high glucose+high insulin augmented phosphorylation of 4E-BP1 on Thr37/46 in a time

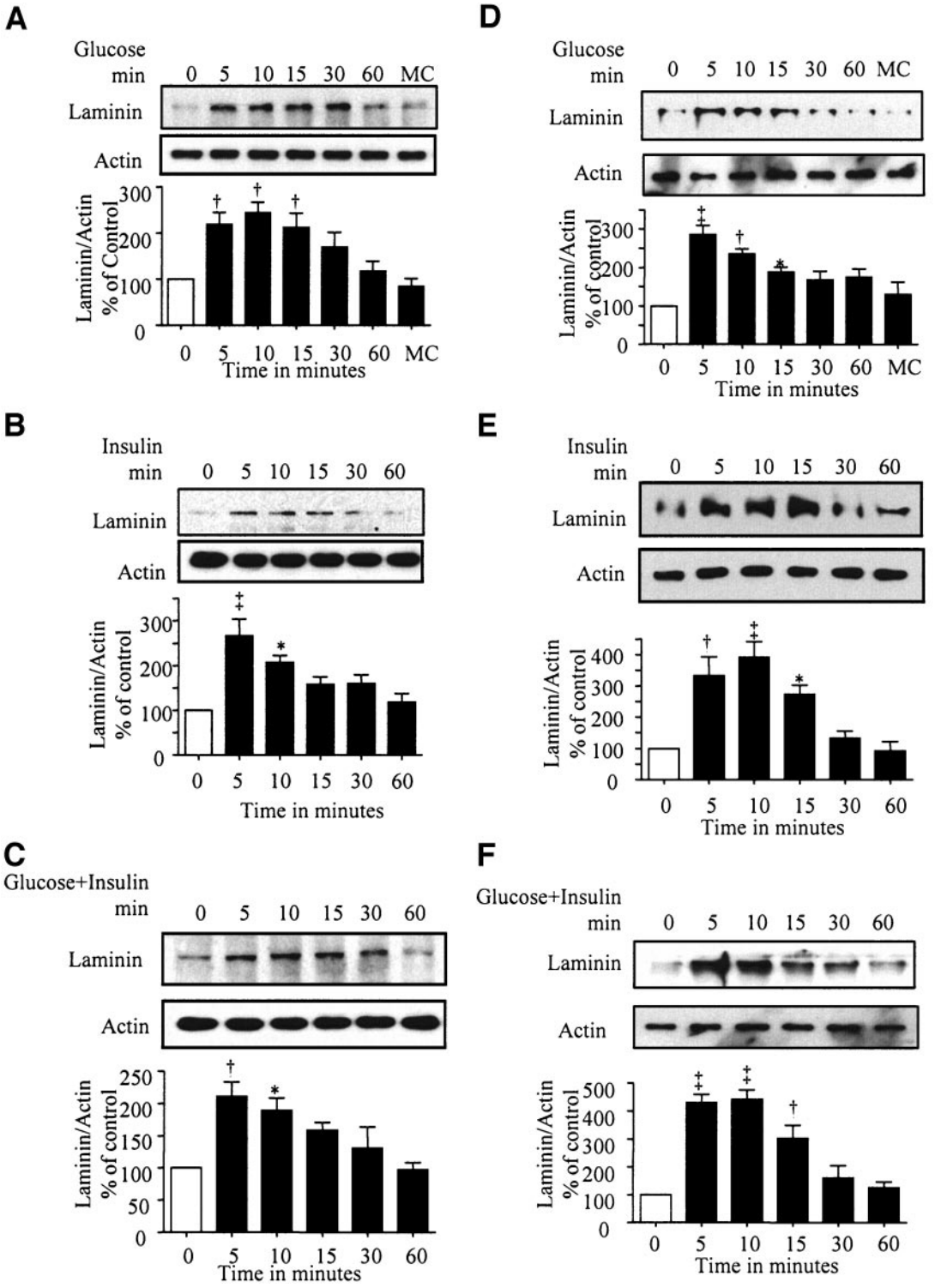

FIG. 1. Laminin-β1 synthesis and secretion are stimulated by 30 mmol/l glucose (high glucose), 1 nmol/l insulin (high insulin), and their combination. Western blotting shows an increase in laminin-β1 synthesis (*top*) in both cell (*A–C*) and medium (*D–F*) compartments. Mannitol (MC) was used as an osmotic control. *Bottom panels* show blots immunoblotting for actin done to assess loading. Representative blots from four to six experiments are shown, and composite data are given in histograms. *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$ vs. control by ANOVA.

course that corresponded to induction of laminin-β1 synthesis (Fig. 3*A*).

High glucose, high insulin, and high glucose+high insulin induce dissociation of 4E-BP1 from eIF4E and association of eIF4E with eIF4G. The eIF4E binding sites on 4E-BP1 and eIF4G are similar, and the two proteins compete with each other for eIF4E (25). eIF4G serves as a molecular bridge between mRNA, eIF4E, and components of the ribosomal preinitiation complex (26–28). Cell lysates were immunoprecipitated with a monoclonal anti-eIF4E antibody. The immunoprecipitates were immunoblotted with anti-4E-BP1 or anti-eIF4G antibody. The three incubation conditions promoted dissociation of 4E-BP1 from eIF4E in a time-dependent manner accompa-

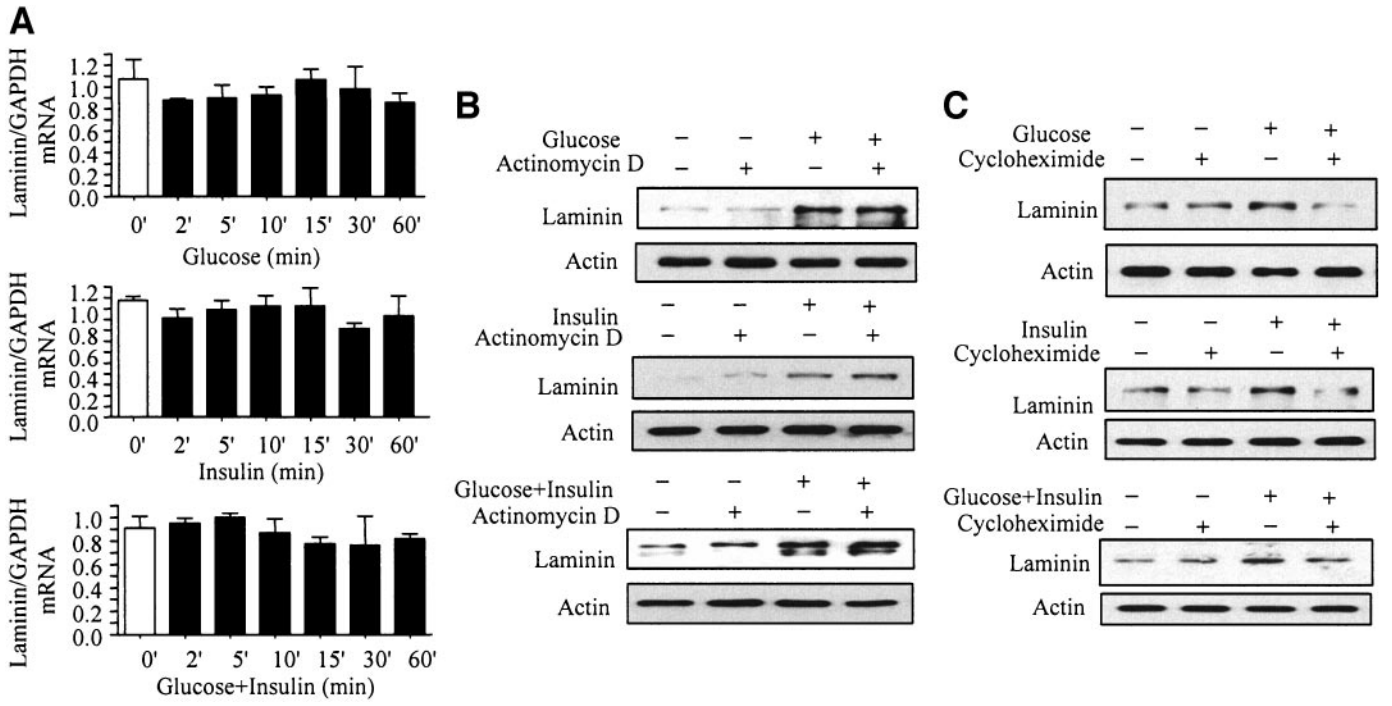

FIG. 2. ***A*****: High glucose, high insulin, and high glucose+high insulin do not change laminin-β1 mRNA levels. Real-time RT-PCR using SYBR Green method shows no significant change in the transcript level of laminin-β1 during incubation with high glucose, high insulin, or high glucose+high insulin. GAPDH served as an internal control. The laminin-β1–to–GAPDH mRNA ratio is shown.** ***B*** **and** ***C*****: Actinomycin D (50 μmol/l) did not inhibit laminin-β1 synthesis (*B*), but cycloheximide (10 μmol/l) did (*C*) in cells treated with high glucose, high insulin, and high glucose+high insulin. Loading was assessed by immunoblotting with actin antibody.**

nied by increased association of eIF4E with eIF4G (Fig. 3*B*).

4E-BP1 phosphorylation is required for high glucose–, high insulin–, and high glucose+high insulin–induced laminin-β1 synthesis. We examined the requirement of 4E-BP1 phosphorylation by using MCT cells stably expressing a mutant of 4E-BP1 (Thr37/46→Ala37/46). Because the mutant protein is tagged with hemagluttinin, efficiency of transfection was monitored by immunoblotting with a monoclonal anti-hemagluttinin antibody (Fig. 4*B*). Thr37/46 serve as priming sites for phosphorylation of other Ser and Thr residues on 4E-BP1

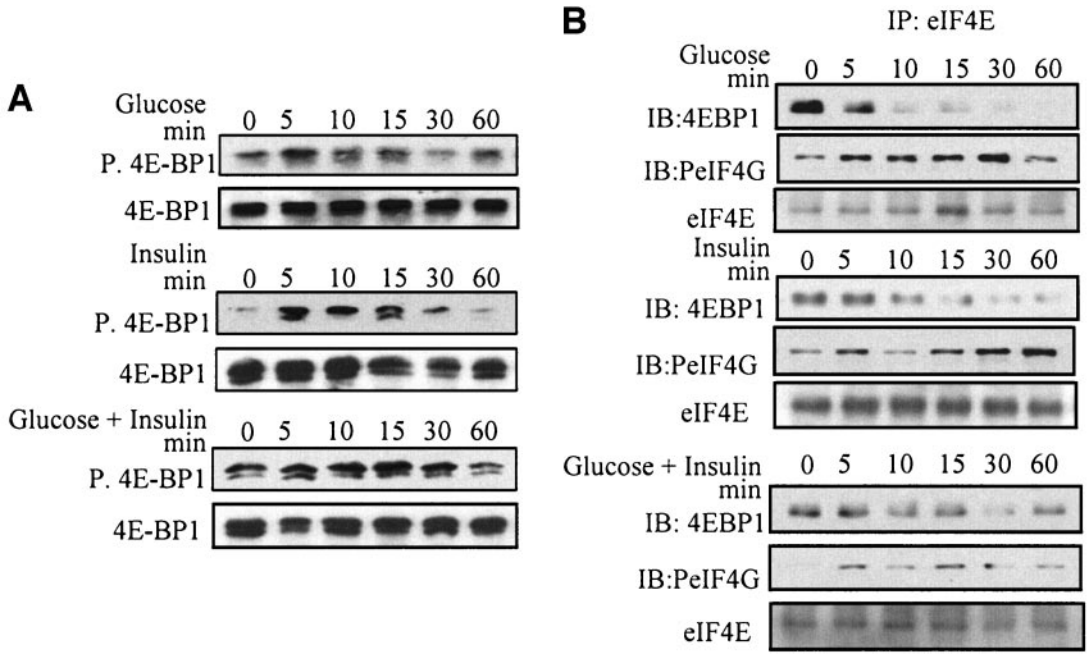

FIG. 3. ***A*****: High glucose, high insulin, and high glucose+high insulin induce phosphorylation of 4E-BP1. Cell homogenates were immunoblotted with an antibody for phospho-Thr37/46 4E-BP1 or 4E-BP1 antibody.** ***B*****: High glucose, high insulin, and high glucose+high insulin induce dissociation of 4E-BP1-eIF4E complex and association of eIF4E with eIF4G. Equal amounts of protein (500 μg) immunoprecipitated (IP) with a monoclonal eIF4E antibody were separated by SDS-PAGE and immunoblotted with an anti-4E-BP1 antibody or with an anti-phospho eIF4G antibody. Immunoblotting with eIF4E antibody was done to assess loading (*bottom*).**

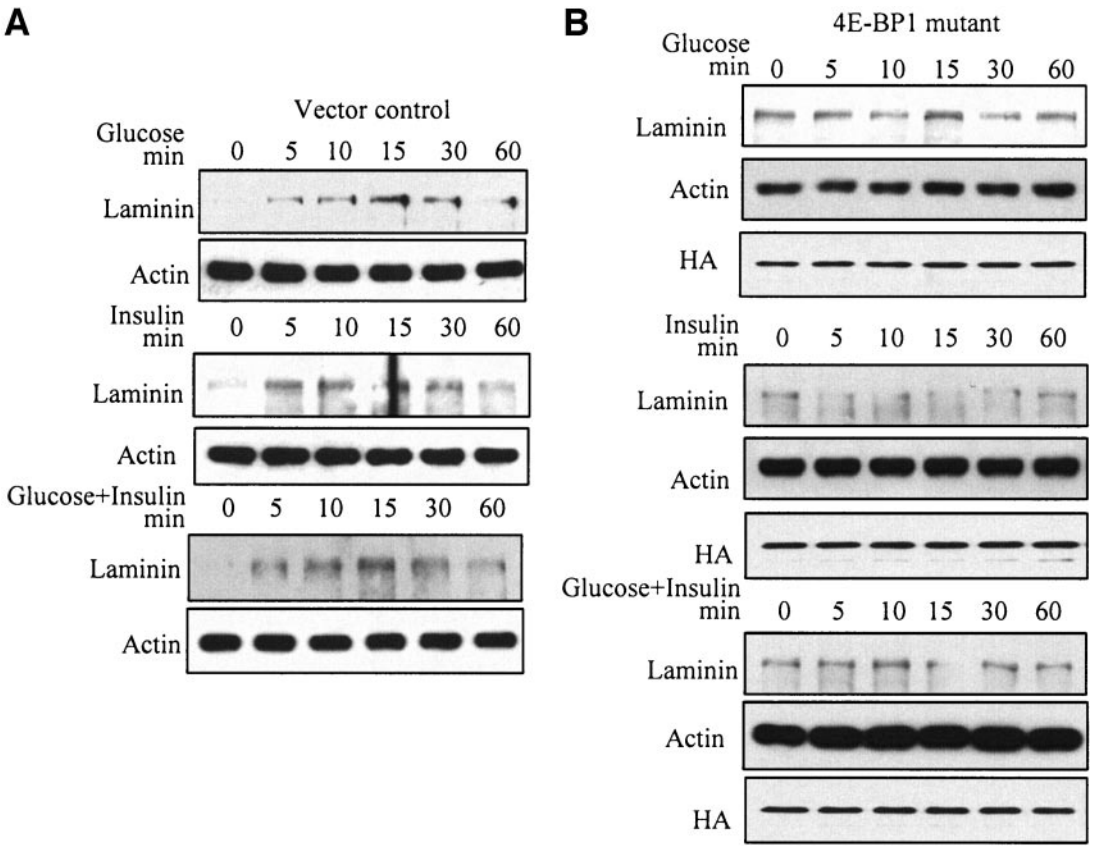

FIG. 4. Laminin-β1 mRNA translation requires phosphorylation of 4E-BP1. Immunoblotting of cell lysates shows increased laminin-β1 synthesis with the three treatments in vector-transfected MCT cells (*A*) but not in the hemagluttinin-tagged 4E-BP1 phosphorylation mutant transfected cells (*B*). *B*: Immunoblotting with actin antibody was done to assess loading. Immunoblotting for hemagluttinin showed successful transfection of the 4E-BP1 mutant.

(23). High glucose, high insulin, and high glucose+high insulin stimulated laminin-β1 synthesis in vector-transfected control MCT cells but not in cells expressing the 4E-BP1 mutant (Fig. 4*A* and *B*).

High glucose, high insulin, and high glucose+high insulin modulate upstream regulators of 4E-BP1 phosphorylation. 4E-BP1 phosphorylation is regulated by the canonical PI 3-kinase–Akt–mammalian target of rapamycin (mTOR) pathway (29–31). Although insulin, a growth factor, promotes PI 3-kinase activity leading to 4E-BP1 phosphorylation in MCT cells (16,24), regulation of PI 3-kinase activity by glucose has not been well studied. High glucose, high insulin, and high glucose+high insulin rapidly promoted activation of PI 3-kinase (Fig. 5*A*). Akt is activated downstream of PI 3- kinase and is required for Thr37/46 phosphorylation of 4E-BP1, although it is not the direct kinase (32). The three experimental conditions also induced phosphorylation of Akt within 2 min (Fig. 5*B*), occurring before the increase in 4E-BP1 phosphorylation. Akt indirectly leads to mTOR activation (33). mTOR is a central modulator of protein synthesis because its downstream targets control initiation and elongation phases of translation; it is a direct kinase for Thr37/46 of 4E-BP1 (34). High glucose, high insulin, and high glucose+high insulin stimulated mTOR phosphorylation within 2 min (Fig. 5*C*), preceding induction of 4E-BP1 phosphorylation.

Expression of dominant negative mutant of PI 3-kinase and kinase-dead mTOR abolished laminin-β1 synthesis induced by the three conditions (Fig. 6*A* and *B*). LY294002, a PI 3-kinase inhibitor, and rapamycin, an mTOR inhibitor, also exhibited similar effects (Supplemental Fig. S4). Success of mutant transfection was demonstrated functionally by showing that high glucose–, high insulin–, and high glucose+high insulin–induced increment in phosphorylation of Akt and p70S6 kinase, downstream targets of PI 3-kinase and mTOR, respectively, was blocked (Supplemental Fig. S5). These data show that activation of PI 3-kinase and mTOR is mandatory for induction of laminin-β1 synthesis by high glucose, high insulin, and high glucose+high insulin, perhaps by stimulating 4E-BP1 phosphorylation.

High glucose, high insulin, and high glucose+high insulin induce phosphorylation of eIF4E and eIF4G. Stimulation of mRNA translation is associated with increased phosphorylation of eIF4E and eIF4G (35–37). High glucose, high insulin, and high glucose+high insulin increased phosphorylation of Ser209 of eIF4E and Ser1108 of eIF4G in a time course that was similar to stimulation of laminin-β1 synthesis (Fig. 7*A*–*C*).

Erk activation is required for laminin-β1 synthesis in MCT cells. Erk1/2 MAP kinase (Erk) controls eIF4E phosphorylation, although it is not a direct kinase; it acts through its downstream target kinase Mnk1, which phosphorylates Ser209 on eIF4E (38). Immunoblotting showed a time-dependent increase in phosphorylation of Erk by high glucose, high insulin, and high glucose+high insulin, which was evident at 2 min preceding laminin-β1 chain synthesis (Fig. 8*A*). PD098059, a MEK inhibitor that results in suppression of Erk activation, blocked increased laminin-β1 synthesis stimulated by the three conditions (Fig.

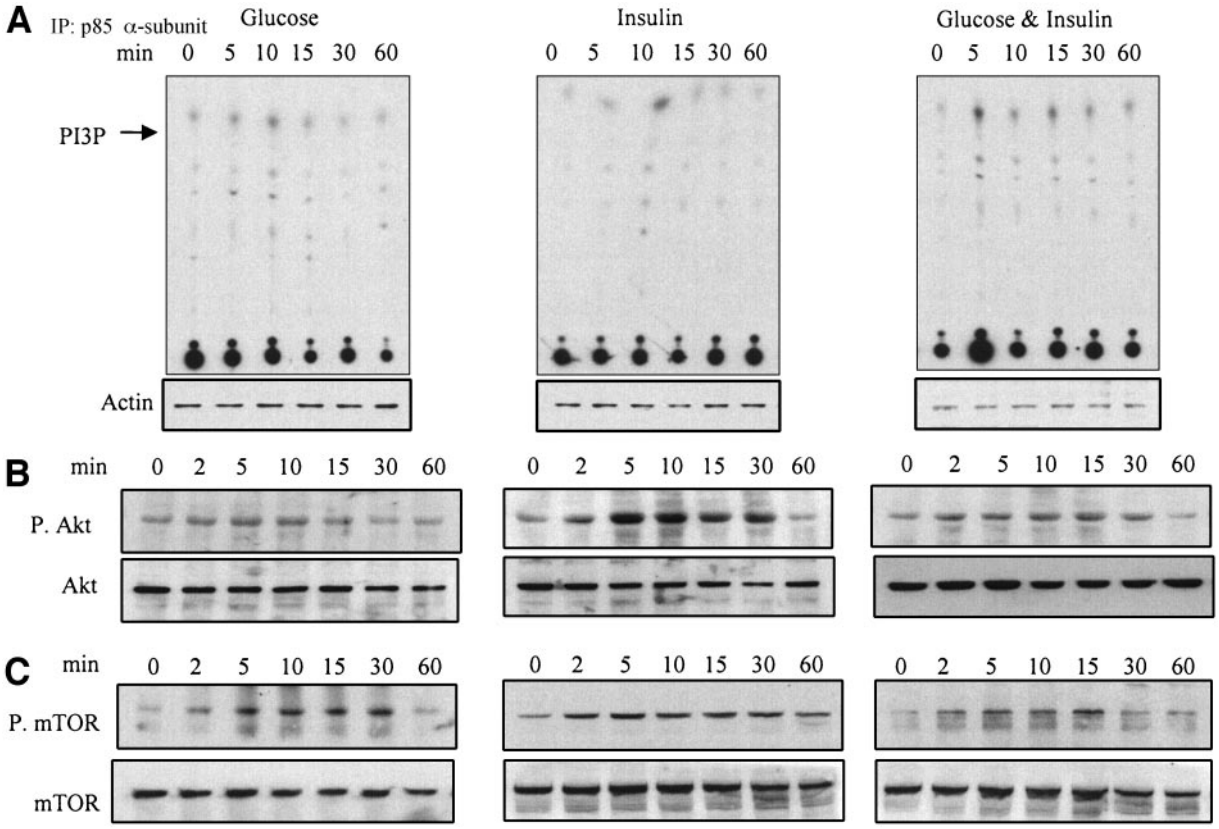

FIG. 5. ***A*****: High glucose, high insulin, and high glucose + high insulin stimulate PI 3-kinase activity and phosphorylation of Akt and mTOR. Equal amounts of protein from the lysates were immunoprecipitated with antibody against p85-α subunit of PI 3-kinase, and kinase activity was measured.** ***B*** **and** ***C*****: The three conditions increased phosphorylation of Akt and mTOR in a time-dependent manner (*** ***top*** **). Immunoblotting using antibody against Akt and mTOR was done to assess loading (*** ***bottom*** **).**

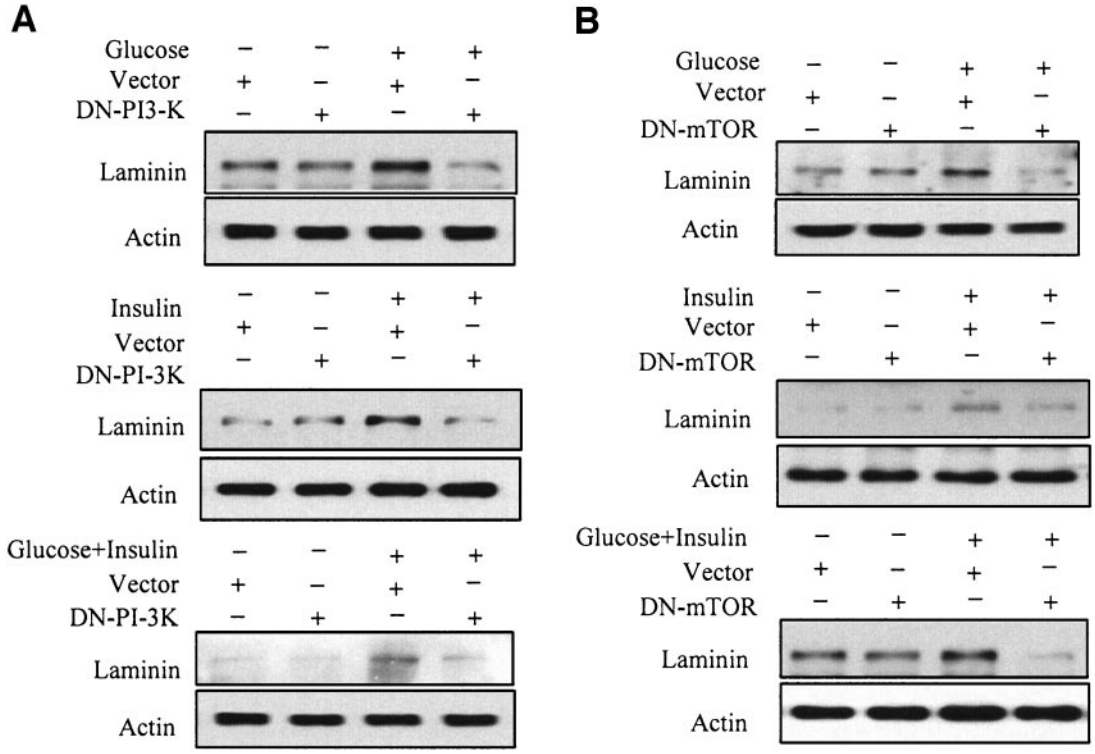

FIG. 6. High glucose–, high insulin–, and high glucose+high insulin–induced laminin-β1 synthesis requires PI 3-kinase and mTOR activation. High glucose, high insulin, or high glucose+high insulin stimulated laminin-β1 synthesis in vector transfected cells but not in cells expressing the dominant negative constructs of PI 3-kinase (* ***A*** **) or mTOR (*** ***B*** **). Immunoblotting with actin antibody was done to assess loading.**

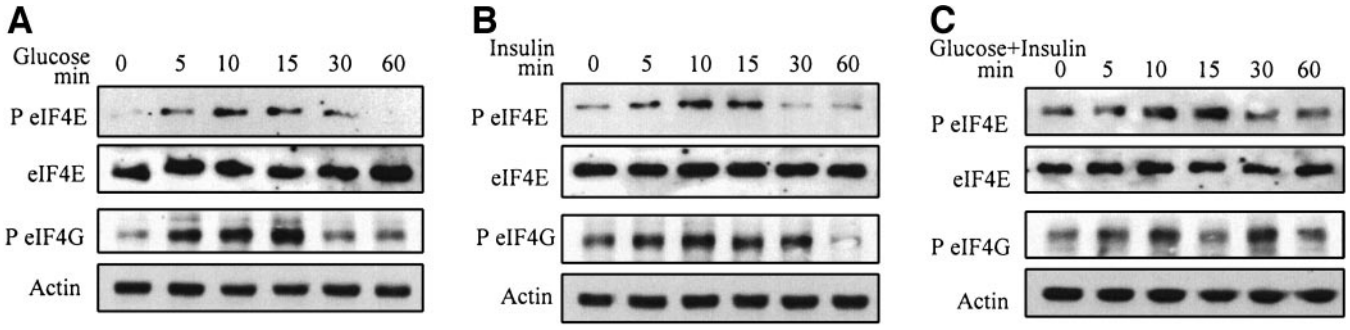

FIG. 7. High glucose (*A*), high insulin (*B*), and high glucose + high insulin (*C*) induce phosphorylation of eIF4E and eIF4G. Immunoblotting showed that phosphorylation of both eIF4E and eIF4G was augmented by high glucose, high insulin, and high glucose+high insulin. *Bottom panels* show immunoblots with eIF4E or actin antibodies done to assess loading.

8*B*), demonstrating the requirement for Erk activation. Figure 9 shows a scheme proposed for the regulation of laminin-β1 synthesis at the level of translation initiation by high glucose, high insulin, and both together in MCT cells.

DISCUSSION

Our results demonstrate that synthesis of laminin-β1 chain in MCT cells is increased within minutes of exposure to high glucose, high insulin, or their combination due to stimulation of its mRNA translation; this requires activation of PI 3-kinase–Akt–mTOR and Erk signaling pathways and 4E-BP1 phosphorylation. Increase in laminin-β1 chain synthesis was not associated with increase in its mRNA. These data demonstrate for the first time, to our knowledge, that mRNA translation can be an independent focus of control of rapid-phase synthesis of laminin-β1 chain in proximal tubular and glomerular epithelial cells under conditions encountered during progression of type 2 diabetes. Rapid-phase laminin-β1 chain appears to be selective in MCT cells under the conditions used because the contents of other matrix proteins, type IV collagen and fibronectin, were unchanged. Interestingly, increment in synthesis of laminin-β1 was not associated with progressive accumulation of the protein in the cell or media compartment; it is possible that proteinase activity prevents laminin-β1 accumulation in the short term. However, laminin-β1 degradation along with that of other matrix proteins is reduced in prolonged diabetes because of a combination of impaired matrix metalloproteinase activity and resistance of nonenzymatically glycated laminin-β1 to degradation.

Appearance of hyperglycemia in type 2 diabetes is preceded by a phase of hyperinsulinemia, which is able to overcome tissue resistance and transport glucose into cells. This is followed by a stage of hyperglycemia that may overlap with hyperinsulinemia for some time. Progressive injury to β-cells of the pancreas during long-term diabetes results in a decrease in insulin levels and persistence of overt hyperglycemia. Thus, the experimental conditions of high insulin, high glucose+high insulin, and high glucose were designed to recapitulate the successive phases of type 2 diabetes. Emerging data suggest that each of these phases may provoke tissue injury in diabetes (39). Even in late stages of type 2 diabetes, "hypoinsulinemia " may be relative and may apply only to glucose transport function. It is probable that prevailing insulin levels are adequate to stimulate other actions of insulin such as protein synthesis. Additionally, resistance to actions of insulin, defined by impaired activation of insulin receptor signaling pathway, is not universal among tissues in type 2

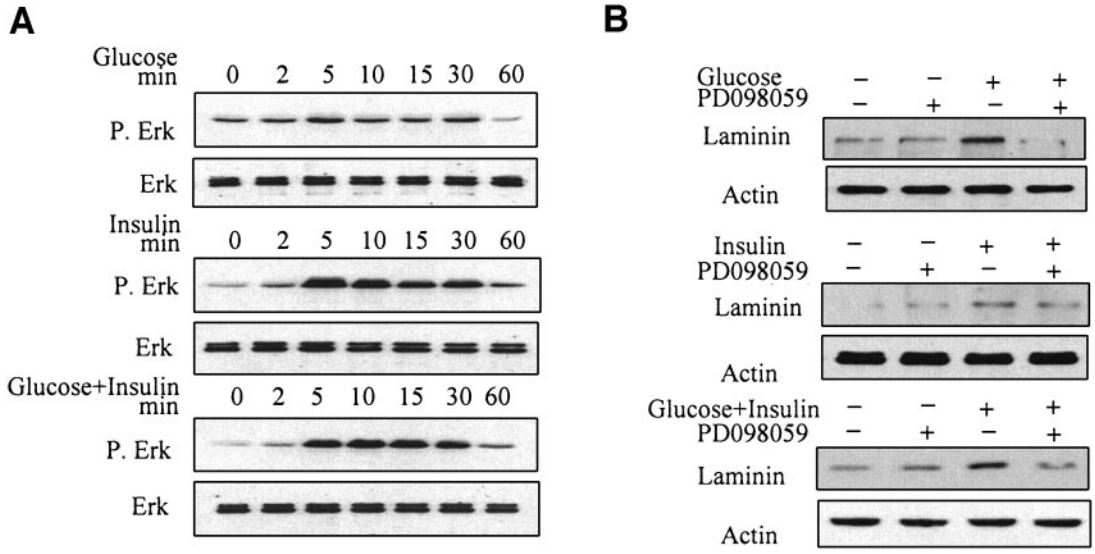

FIG. 8. *A*. Erk activation induced by high glucose, high insulin, and high glucose+high insulin is required for laminin-β1 synthesis. *A*: Immunoblotting showed that high glucose, high insulin, and high glucose+high insulin increased phosphorylation of Erk. Loading was assessed by immunoblotting with an antibody against Erk. *B*: MEK inhibitor, PD98059 (25 μmol/l), abrogated laminin-β1 synthesis stimulated by the three incubation conditions. Immunoblotting with an actin antibody was done to assess loading.

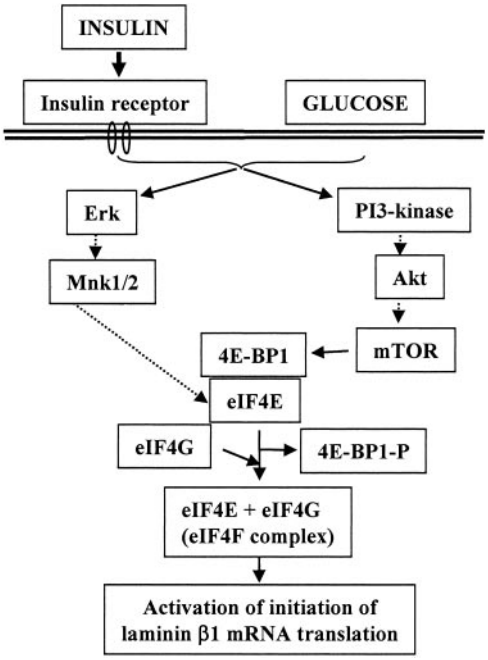

FIG. 9. Schematic for the regulation of laminin-β1 synthesis induced by high glucose, high insulin, and their combination in MCT cells.

diabetes. In contrast to liver, skeletal muscle, and adipose tissue, other tissues such as the vessel wall (40), retina (4), and the kidney (3) remain insulin sensitive and may be subject to injury by hyperinsulinemia. Our previous observation that insulin receptor signaling is increased in the renal cortex in mice with type 2 diabetes (3) prompted us to examine the role of insulin in events that are seen in the hyperinsulinemic phase of type 2 diabetes, i.e., increase in laminin-β1 synthesis.

Although increase in synthesis of most of renal matrix proteins, i.e., type IV collagen and fibronectin, is accounted for by elevated levels of their mRNA in type 2 diabetes (41,42), laminin-β1 accumulation was found to occur without increase in its mRNA in *db/db* mice with type 2 diabetes (5), suggesting augmented efficiency in translation of its mRNA or decreased rate of degradation as possible mechanisms. In the present study, we have focused on the former possibility. Our data show that mRNA translation can be stimulated rapidly after exposure to stimuli. This is possible because signaling reactions that control mRNA translation can be stimulated rapidly as shown by activation of Akt, mTOR, and Erk within 2 min in this study. Translation of mRNA is well suited to serve immediate synthetic needs of the cell because it can proceed at a rate of 20 amino acids per second (43).

Interestingly, all three conditions of incubation recruited the same regulatory signaling pathways in stimulation of laminin-β1 synthesis. The canonical PI 3-kinase–Akt–mTOR axis is used by growth factors such as insulin, IGF-I, and vascular endothelial growth factor (VEGF) in stimulation of protein synthesis (13,15,24). These growth factors are also known to stimulate Erk MAP kinase pathway in stimulation of mRNA translation (15,24). The pathways recruited by glucose, a metabolic fuel, have not been well studied, and we show that it activates the same pathway as insulin. High glucose may stimulate MCT cells to synthesize growth factors such as VEGF, which in turn activate PI 3-kinase and Erk pathways via their respective receptor tyrosine kinases (13,18). Additionally, high glucose may directly activate these pathways. Both Akt and mTOR are nutrient sensors, which may detect changes in energy status after exposure to high glucose. This pathway may involve another energy sensor, AMP-activated protein kinase (AMPK). Under basal conditions, AMPK phosphorylates tuberin (TSC-2), promoting its dimerization with TSC-1, resulting in inhibition of Rheb, an inducer of mTOR activity (44). Upon stimulation with high glucose, AMPK activity is reduced, which results in stimulation of mTOR with consequent increase in mRNA translation and protein synthesis (45). Our current study does not identify events involved in stimulation of PI 3-kinase activity by high glucose. Addition of high insulin to high glucose did not abolish high glucose effects. Such effects may be relevant to the phase of type 2 diabetes in which both hyperglycemia and hyperinsulinemia coexist. Evidently, our data with high glucose could be applicable to laminin-β1 regulation in both type 1 and type 2 diabetes; however, data from high insulin and high insulin+high glucose can reflect a mechanism of laminin-β1 regulation unique to type 2 diabetes. Additionally, in vivo data show that in type 1 diabetes, increment in laminin-β1 content of the kidney correlates with increase in its mRNA levels (10), whereas laminin-β1 accumulation in the kidney in type 2 diabetes occurs despite reduction in its mRNA (5). Taking in vivo and in vitro data together, we suggest that mechanisms regulating laminin-β1 chain may be different in the two types of diabetes.

Our observations may have clinical implications. Short-term postprandial elevation of plasma glucose is recognized as an independent risk factor for endothelial dysfunction (46) and cardiovascular complications in type 2 diabetes (47,48). Our data on pathologic effects of short-term exposure to high glucose find support in recent reports, which show that a 10-min exposure to high glucose stimulates proliferation followed by apoptosis in proximal tubular epithelial cells (49) and that cyclical short-term exposure to high glucose increases synthesis of matrix proteins by renal interstitial fibroblasts (50). A pathologic role for insulin is supported by the finding that pre-diabetic hyperinsulinemia contributes to glomerular hypertrophy (51). Elevated insulin levels that occur with intensive insulin therapy in type 1 or type 2 diabetes have been associated with transient worsening of diabetic retinopathy (52,53); similarly, short-term peaks in insulin may stimulate laminin-β1 mRNA translation in renal epithelial cells. Thus, recurrent episodes of high glucose and high insulin may stimulate synthesis of small amounts of laminin-β1, which over time may contribute to significant expansion of matrix in the kidney in type 2 diabetes. Our data provide additional rationale for maintaining tight control of plasma glucose in preventing renal complications in diabetes.

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