

A role for AMP-activated protein kinase in diabetes-induced renal hypertrophy

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Divisions of ¹Nephrology and ²Diabetes, Department of Medicine, O'Brien Kidney Research Center, University of Texas Health Science Center, and ⁵Geriatric Research, Education, and Clinical Center, South Texas Veterans Healthcare System, San Antonio, Texas; ³Institut Cochin, Departement Endocrinologie Metabolisme et Cancer, Inserm U567, Universite Paris 5, Paris, France; and ⁴University of Michigan School of Medicine, Ann Arbor, Michigan

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Lee M-J, Feliers D, Mariappan MM, Sataranatarajan K, Mahimainathan L, Musi N, Foretz M, Viollet B, Weinberg JM, Choudhury GG, Kasinath BS. A role for AMP-activated protein kinase in diabetes-induced renal hypertrophy. *Am J Physiol Renal Physiol* 292: F617–F627, 2007. First published October 3, 2006; doi:10.1152/ajprenal.00278.2006.—We tested the hypothesis that AMP-activated protein kinase (AMPK), an energy sensor, regulates diabetes-induced renal hypertrophy. In kidney glomerular epithelial cells, high glucose (30 mM), but not equimolar mannitol, stimulated de novo protein synthesis and induced hypertrophy in association with increased phosphorylation of eukaryotic initiation factor 4E binding protein 1 and decreased phosphorylation of eukaryotic elongation factor 2, regulatory events in mRNA translation. These high-glucose-induced changes in protein synthesis were phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin (mTOR) dependent and transforming growth factor- β independent. High glucose reduced AMPK α -subunit threonine (Thr) 172 phosphorylation, which required Akt activation. Changes in AMP and ATP content could not fully account for high-glucose-induced reductions in AMPK phosphorylation. Metformin and 5-aminoimidazole-4-carboxamide-1 β -ribose (AICAR) increased AMPK phosphorylation, inhibited high-glucose stimulation of protein synthesis, and prevented high-glucose-induced changes in phosphorylation of 4E binding protein 1 and eukaryotic elongation factor 2. Expression of kinase-inactive AMPK further increased high-glucose-induced protein synthesis. Renal hypertrophy in rats with Type 1 diabetes was associated with reduction in AMPK phosphorylation and increased mTOR activity. In diabetic rats, metformin and AICAR increased renal AMPK phosphorylation, reversed mTOR activation, and inhibited renal hypertrophy, without affecting hyperglycemia. AMPK is a newly identified regulator of renal hypertrophy in diabetes.

protein synthesis; mRNA translation

THE REPERTOIRE OF KIDNEY CELL response to injury includes cell division, cell hypertrophy, and apoptosis. Hyperplasia and hypertrophy contribute to enlargement of the kidney in physiological or pathological states, including diabetes. The first structural change in the kidney in diabetes is enlargement. Most of the kidney enlargement is accounted for by hypertrophy, i.e., increased cellular content of RNA and protein, rather than proliferation (27, 42). Renal hypertrophy occurs rapidly, with significant increases in kidney weight being evident within days of onset of Type 1 or Type

2 diabetes (48). The importance of hypertrophy lies in the possibility that it may predispose to long-term complications of diabetes (27, 51, 58), and, as such, its underlying mechanisms merit further investigation. Exploration of mechanism of diabetes-associated hypertrophy in renal cells has implicated transforming growth factor- β (TGF- β) and ANG II (21, 51, 57). These mediators are also involved in matrix accumulation in later stages of diabetes (7, 43, 52), suggesting a potential link between the early and late events in diabetic renal disease. Regulation of high-glucose-induced renal cell growth also involves cell cycle events (15, 50, 58). There could be additional regulatory pathways yet to be identified, including those regulated by energy status of the cell.

Glucose is a metabolic fuel that regulates energy status of the cell. States of energy depletion lead to the activation of AMP-activated protein kinase (AMPK), resulting in inhibition of energy-consuming pathways (55) and activation of ATP-generating processes (22). Cell hypertrophy entails an increased rate of protein synthesis, which accounts for a significant part of cell energy consumption (29). We hypothesized that AMPK, an energy sensor, regulates diabetes-induced renal hypertrophy. High-glucose-induced hypertrophy was studied in glomerular epithelial cells (GECs). Understanding the mechanisms of injury to GEC is important because, recently, attention has been drawn to decrease in their number and/or density as a determinant in proteinuria and progression of diabetic renal disease (10, 41, 53).

MATERIALS AND METHODS

Cell culture. Rat GECs, kindly provided by Dr. Jeffrey Kreisberg (Department of Surgery, University of Texas Health Science Center at San Antonio), were cultured as described previously (34). GECs display several of the characteristics of visceral GECs in vivo, including expression of Heymann nephritis antigen (52a), CD2AP, laminin, fibronectin, and basement membrane heparan sulfate proteoglycan (34) and sensitivity to puromycin aminonucleoside (34a). GECs were grown in DMEM containing 7% FBS, 5 mM glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine. Cells were used between passages 9 and 15. GECs were incubated with normal glucose (5 mM glucose), high glucose (30 mM glucose), or mannitol (5 mM glucose + 25 mM mannitol) for the indicated time points; medium was changed every 2 days.

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Protein synthesis measurement. Protein synthesis measurement was as previously described (49). Serum-starved cells were labeled with 10 $\mu\text{Ci/ml}$ of [^{35}S]methionine for the terminal 2 h of incubation. Cells were washed in PBS and lysed in radioimmunoprecipitation assay buffer. Cell protein content was measured with a Bio-Rad reagent (Bio-Rad, Hercules, CA). An equal amount of protein (20 μg) was spotted onto the 3-mm filter paper (Whatman, Maidstone, UK). Filters were washed three times by boiling for 1 min in 10% TCA containing 0.1 g/l methionine before radioactivity was determined.

Immunoblotting. Immunoblotting was as previously described (48). Equal amounts of cell lysate protein (15–30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was probed with primary antibody overnight at 4°C. After an extensive washing, the membrane was incubated with secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) or IRDye (Rockland Immunochemicals, Gibertsville, PA). Proteins were visualized by chemiluminescence using enhanced chemiluminescence reagent. Alternatively, Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to detect fluorochrome-coupled antibody. The membrane was stripped in 0.5 M NaOH and reprobed with indicated antibodies. Signal intensity was assessed by densitometric analysis.

Phosphatidylinositol 3-kinase assay. Equal amounts of cell lysate protein (500 μg) were immunoprecipitated with an antibody against p85 α -subunit of phosphatidylinositol 3-kinase (PI3-kinase) (14, 47, 48). Immunobeads were incubated with 10 μg of phosphatidylinositol for 10 min at 25°C in the PI3-kinase buffer. [γ - ^{32}P]ATP (5 μCi) was added and incubated for another 10 min at 25°C. Reaction was stopped by the addition of a mixture of chloroform-methanol. The reactants were extracted with 100 μl of chloroform, and the organic layer was washed with methanol and 1 N HCl. Reaction products were dried, resuspended in chloroform, separated by TLC, and developed with chloroform-methanol-28% ammonium hydroxide-water in the ratio of 129:114:15:21. The spots were visualized by autoradiography.

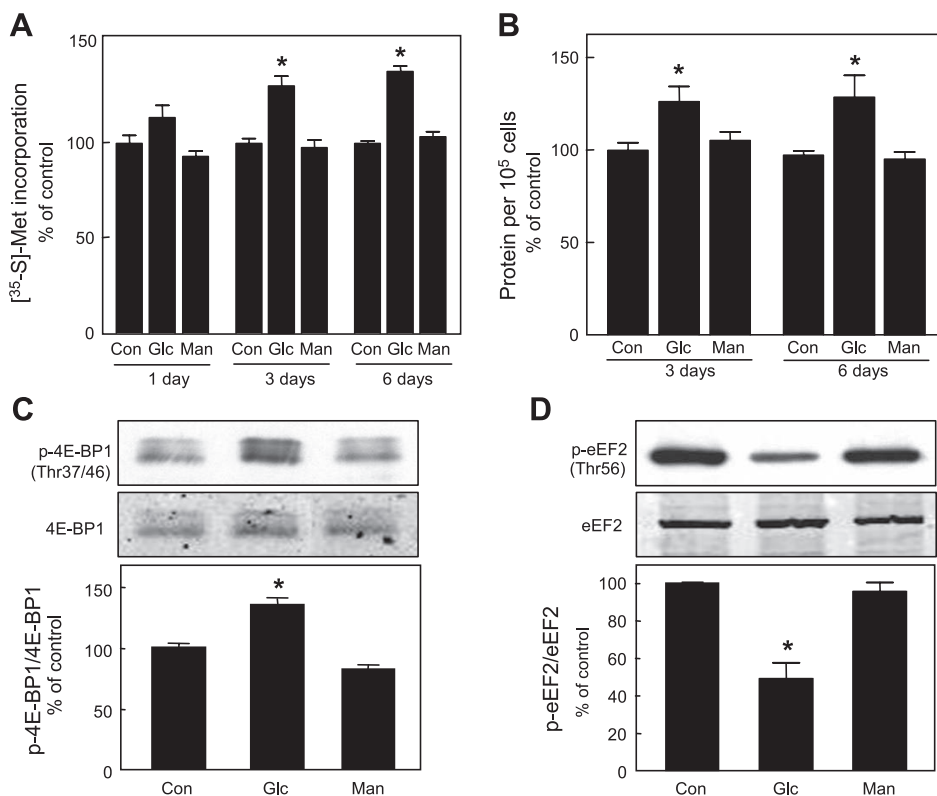
AMPK activity assay. Equal amounts of cell lysate protein (500 μg) were immunoprecipitated with an antibody against AMPK α (Cell Signaling, Danvers, MA) and incubated at 30°C with 100 μM SAMS peptide (Upstate, Lake Placid, NY) in reaction buffer (50 mM HEPES, pH 7.5, 10 mM MgCl_2 , 5% glycerol, 1 mM DTT, 0.05% Triton X-100) with 250 μM [γ - ^{32}P]ATP for 8 min; 25 μl of reaction mixture were spotted onto the phosphocellulose P81 paper (Upstate) and washed with phosphoric acid. Radioactivity on the filter paper was measured (16).

Transfection. The plasmid containing AMPK carrying K45R mutation of the α_1 -subunit (pCAGGS) (12) (kindly provided by Dr. N. Fujii, Joslin Clinic, Boston MA) was employed for transient transfection using lipofectamine and Lipo-plus reagent (Invitrogen) (38).

TGF- β bioassay. The TGF- β bioassay was as previously described (1). TGF- β was measured with a mink lung epithelial cell (MLEC) responder line, stably transfected with a construct containing the TGF- β -responsive element from the human plasminogen activator inhibitor-1 promoter fused to a luciferase reporter gene. MLECs were treated for 24 h with conditioned medium from control or high-glucose-treated GECs. Recombinant TGF- β (R&D Systems, Minneapolis, MN) was used as a standard. The cell extracts were prepared and assayed for luciferase activity (Promega, Madison, WI).

Animal study. Animal protocols were approved by the Institutional Animal Care and Use Committee. To induce Type 1 diabetes, male Sprague-Dawley rats weighing 200–220 g (Harlan, Indianapolis, IN) received a single injection of 60 mg/kg streptozotocin in citrate buffer (pH 4.5) into the tail vein. The rats became diabetic the next day as indicated by elevation in blood glucose concentration, which was measured by a glucometer (Ascensia Elite XL, Mishawaka, IN). On day 4 of hyperglycemia, rats were killed. One of the kidneys was immediately frozen in liquid nitrogen-chilled 2-methylbutane and stored at -70°C ; the other kidney was weighed and used for glomerular isolation. In additional experiments, metformin was administered by gavage starting on the first day of diabetes for 4 days at 300

Fig. 1. Regulation of protein synthesis and phosphorylation of 4E binding protein 1 (4E-BP1) and eukaryotic elongation factor 2 (eEF2) by high glucose (Glc). **A:** glomerular epithelial cells (GECs) were incubated with 5 mM Glc [control (Con)], 30 mM Glc, or 5 mM Glc + 25 mM mannitol (Man) for 1, 3, or 6 days. Protein synthesis was measured by incorporation of [^{35}S]methionine (Met) into TCA-precipitable protein. Composite data from 3 experiments are shown (* $P < 0.001$ high Glc vs. control by ANOVA). **B:** cells were treated as described in A. At end of incubation for 3 or 6 days, protein content was measured and expressed per 10^5 cells. Composite data from 3 experiments are shown (* $P < 0.05$ high Glc vs. control by ANOVA). **C and D:** after 3-day incubation with 5 mM Glc (control), 30 mM Glc, or 5 mM Glc + 25 mM Man, equal amounts of cell lysate protein were separated by SDS-PAGE and immunoblotted with an antibody specific for 4E-BP1 phosphorylated on Thr37/46 (C) or for eEF2 phosphorylated on Thr56 (D). The blot was stripped and probed with an antibody against 4E-BP1 or eEF2 to assess loading. Representative blots from 3 experiments are shown. **C and D, bottom:** composite densitometric data from 3 experiments (* $P < 0.001$ high Glc vs. control by ANOVA).



mg·kg⁻¹·day⁻¹ (35). 5-Aminoimidazole-4-carboxamide-1 β -riboside (AICAR; 750 mg·kg⁻¹·day⁻¹) was given by intraperitoneal route (37) for the same duration. Body weight and blood glucose concentration were measured daily. C57BL6/KsJ *db/db* mice, a model of Type 2 diabetes, were maintained on regular laboratory chow. Blood glucose concentration was monitored for emergence of diabetes. The mice were killed at 2 wk of diabetes at which time the kidneys are hypertrophic (48). Renal cortex was dissected out for further analysis.

Measurement of AMP and ATP levels. Samples of GEC suspension were immediately deproteinized in TCA, neutralized with triethylamine-CFC 113, and stored at -20°C. Purine nucleotides and their metabolites in 20- μ l aliquots of the neutralized extracts were separated and quantified by the reverse-phase ion-pairing gradient HPLC method as previously described (13).

Statistics. Data were obtained from at least three independent experiments and are expressed as means \pm SE. Statistical comparisons between multiple groups were performed by ANOVA, and Bonferroni's method was applied to control for multiple testing. Statistical comparisons between two groups were performed by the Student's *t*-test with a minimum value of *P* < 0.05 considered to represent statistical significance.

RESULTS

High glucose induces hypertrophy of GEC. Cells were incubated with 5 mM glucose (control), 30 mM glucose (high glucose), or 5 mM glucose + 25 mM mannitol (osmotic control) for 1, 3, or 6 days; blood glucose levels are in the 30 mM range in rodents with diabetes (20). High glucose increased protein synthesis by 30 and 40% on *days 3* and *6*, respectively; the increment on *day 1* was not statistically significant (Fig. 1A; *P* < 0.001); osmotic control had no effect. High glucose also induced cell hypertrophy, defined as protein content of the GEC per unit cell number, by nearly 20% compared with 5 mM glucose-treated cells (Fig. 1B; *P* < 0.05, for *days 3* and *6*). We employed 3-day incubation for further studies.

High glucose stimulates 4E binding protein 1 phosphorylation. The initiation phase of mRNA translation during which the ribosome binds to mRNA is the rate-limiting step in protein

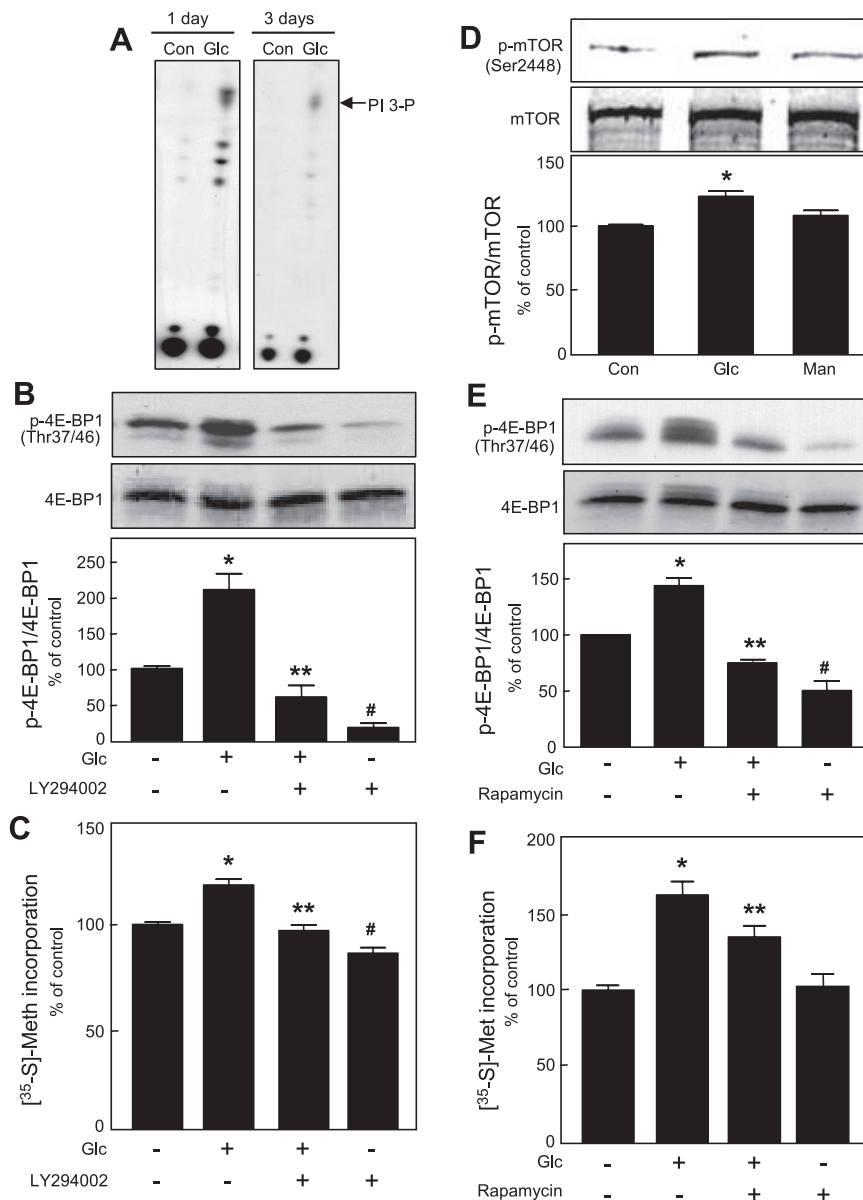


Fig. 2. High-Glc-induced protein synthesis is phosphatidylinositol 3-kinase (PI3-kinase) and mammalian target of rapamycin (mTOR) dependent. **A:** GECs were incubated with 30 mM Glc for different durations and processed for PI3-kinase assay. Representative blots from 3 experiments are shown. PI3-P refers to phosphatidylinositol 3-phosphate, a product of PI3-kinase. **B:** GECs were incubated with 30 mM Glc for 3 days with or without addition of 25 μ M LY-294002 for the terminal 2 h of incubation. *Top:* immunoblotting for 4E-BP1 was done as described in Fig. 1. *Bottom:* composite data from 3 experiments (**P* < 0.001 for high Glc vs. control, ***P* < 0.001 for high Glc vs. high Glc + LY-294002, #*P* < 0.001 for control + LY-294002 vs. control, by ANOVA). **C:** measurement of protein synthesis was done as described in Fig. 1 except for preincubation with or without 25 μ M LY-294002 for 2 h before addition of the label. Graph shows composite data from 3 experiments (**P* < 0.001 for high Glc vs. control, ***P* < 0.001 for high Glc vs. high Glc + LY-294002, #*P* < 0.001 for control + LY-294002 vs. control, by ANOVA). **D:** cells were treated as described in Fig. 1C, and immunoblotting (*top*) was done with an antibody against phosphorylated Ser2448 on mTOR. *Bottom:* composite data from 3 experiments (**P* < 0.001 for high Glc vs. control by ANOVA). **E:** GEC were incubated with 30 mM Glc for 3 days, with or without addition of 22 nM rapamycin for the terminal 2 h of incubation. *Top:* immunoblotting for 4E-BP1 was done as described in Fig. 1. *Bottom:* composite data from 3 experiments (**P* < 0.01 for high Glc vs. control, ***P* < 0.001 for high Glc vs. high Glc + rapamycin, #*P* < 0.001 control vs. control + rapamycin, ANOVA). **F:** measurement of protein synthesis was done as described in Fig. 1 except for preincubation with or without 22 nM rapamycin for 2 h before addition of the label. Graph shows composite data from 3 experiments (**P* < 0.001 for high Glc vs. control, ***P* < 0.05 for high Glc vs. high Glc + rapamycin, by ANOVA).

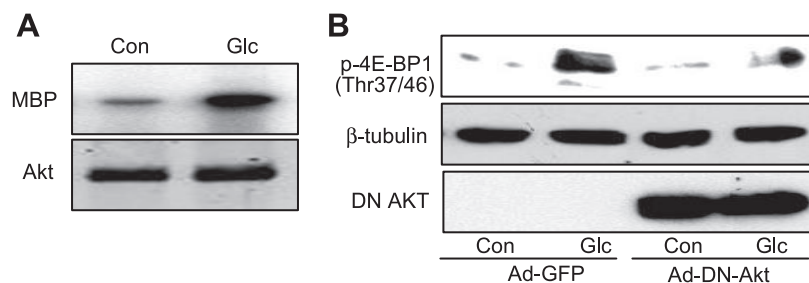
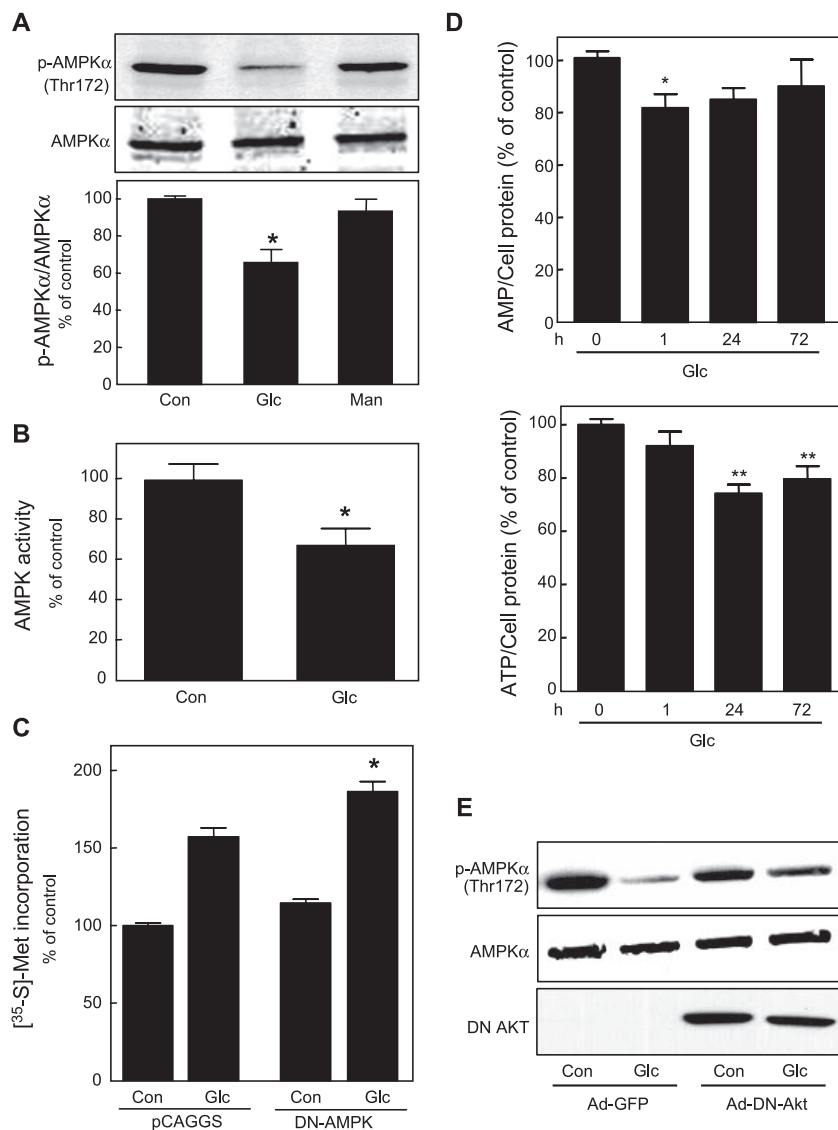


Fig. 3. High Glc regulates Akt activity. *A*: GECs were incubated with 30 mM Glc for 3 days and processed for Akt immunokinase assay with myelin basic protein (MBP) substrate. An immunoblot of cell lysates using anti-Akt antibody was performed to assess loading. A representative blot from 3 experiments is shown. *B*: GECs were quiesced in serum-free medium for 24 h and infected at room temperature for 1 h with a multiplicity of infection of 70 of replication-defective adenovirus vector carrying mouse Akt mutated at its phosphorylation sites (T308A, S473A) with an HA tag (Ad-DN-Akt). Control cells were infected with the same multiplicity of infection of adenovirus carrying green fluorescent protein (Ad-GFP). After a 3-day exposure to 5 mM Glc (control) or 30 mM Glc, immunoblotting was done with the indicated antibodies. A representative blot from 3 experiments is shown.

synthesis (40, 54). This process is controlled by several eukaryotic initiation factors (eIFs), including the mRNA cap binding protein eIF4E. In the basal state, eIF4E is held inactive by its binding protein, 4E binding protein 1 (4E-BP1). Phos-

phorylation of 4E-BP1 dissolves the complex and releases eIF4E, which, in association with eIF4G and eIF4A, promotes the initiation phase of translation. In renal epithelial cells, phosphorylation of 4E-BP1 is essential for protein synthesis

Fig. 4. High Glc reduces AMP-activated protein kinase (AMPK) phosphorylation and activity. *A*: cells were treated as described in Fig. 1C, and immunoblotting was done with an antibody against phosphorylated Thr172 on AMPK α -subunit. *Top*: representative blot from 3 experiments. *Bottom*: composite densitometric data from 3 experiments ($*P < 0.001$, high Glc vs. control by ANOVA). *B*: GECs were incubated with 30 mM Glc for 3 days and processed for AMPK activity using SAMS substrate. Composite data from 6 experiments are shown ($*P < 0.01$, high Glc vs. control, by *t*-test). *C*: GECs were transfected with either control plasmid or pCAGGS containing a K45R mutation in the α -catalytic subunit of AMPK. After a 3-day exposure to 5 mM Glc (control) or 30 mM Glc, de novo protein synthesis was measured as described in Fig. 1. Composite data from 3 experiments are presented ($*P < 0.01$, high Glc vs. high Glc + AMPK mutant, by ANOVA). *D*: GECs were incubated with 30 mM Glc for different durations, and AMP (*top*) and ATP (*bottom*) contents were measured by HPLC and corrected for protein content. Composite data ($n = 3$ at each time point) are shown with control as 100% ($*P < 0.05$, $**P < 0.001$, by ANOVA). *E*: cells were treated as described in Fig. 3B. After a 3-day exposure to 5 mM Glc (control) or 30 mM Glc, immunoblotting was done with the indicated antibodies. A representative blot from 5 experiments is shown.



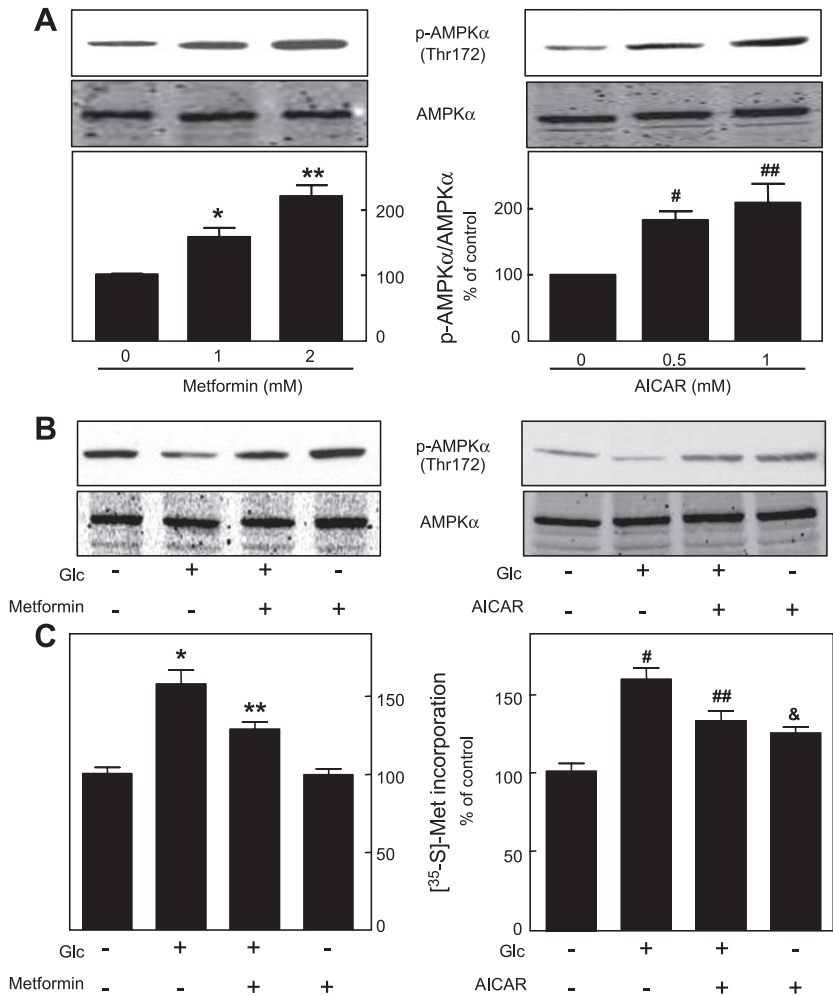


Fig. 5. Metformin and 5-aminoimidazole-4-carboxamide-1 β -riboside (AICAR) reverse high-Glc effects on AMPK phosphorylation and protein synthesis. *A*: GECs were incubated with different doses of metformin (*left*) or AICAR (*right*) for 24 h. Equal doses of cell lysate protein were separated by SDS-PAGE and immunoblotted with AMPK antibodies as described in Fig. 4. *Top*: representative blot from 3 experiments. *Bottom*: composite densitometric data from 3 experiments (* $P < 0.05$, ** $P < 0.001$ for metformin vs. control and # $P < 0.05$, ## $P < 0.01$ for AICAR vs. control, by ANOVA). *B*: after 3-day incubation with 30 mM Glc, metformin (1 mM) or AICAR (0.5 mM) and equal amounts of cell lysate protein were separated by SDS-PAGE and immunoblotted with AMPK antibodies as described in Fig. 4. A representative blot from 3 experiments is shown. *C*: after 3-day exposure to 30 mM Glc, metformin, or AICAR, de novo protein synthesis was measured as described in Fig. 1. Composite data from 3 experiments are shown (* $P < 0.001$ for high Glc vs. control, ** $P < 0.001$ for high Glc vs. high Glc + metformin, # $P < 0.001$ for high Glc vs. control, ## $P < 0.05$ for high Glc vs. high Glc + AICAR, & $P < 0.05$ for AICAR vs. control, by ANOVA).

induced by insulin, ANG II, IGF-I, and VEGF (2, 47–49). In contrast to the growth factors, glucose regulation of 4E-BP1 phosphorylation has not been well studied. We examined phosphorylation of Thr37/46 because it is a priming event that

leads to phosphorylation of other sites in 4E-BP1 (18, 25). High glucose increased 4E-BP1 phosphorylation at early time points of incubation (30 and 60 min, data not shown) and at 3 days (Fig. 1C; $P < 0.001$).

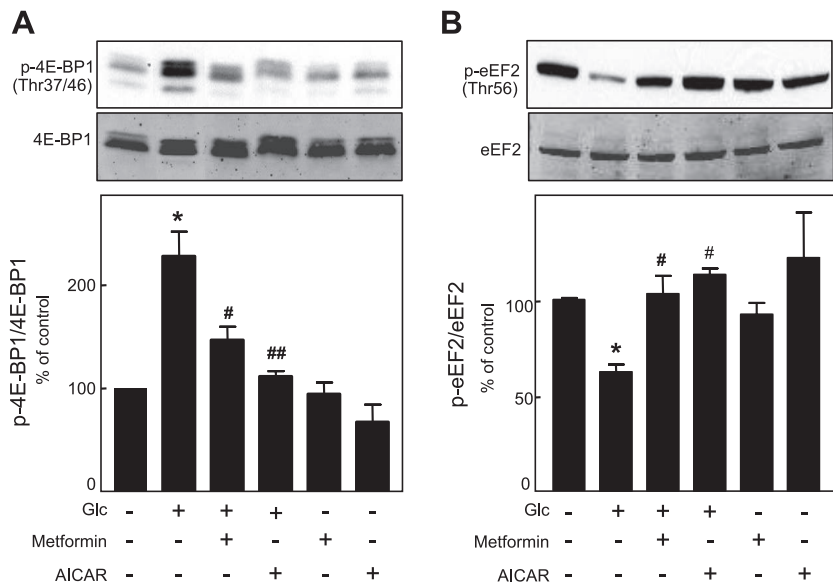


Fig. 6. AMPK regulation of initiation and elongation phases of mRNA translation. *A*: immunoblotting for 4E-BP1 was done as described in Fig. 1. *Top*: representative blot from 3 experiments. *Bottom*: composite densitometric data from 3 experiments (* $P < 0.001$ for high Glc vs. control, # $P < 0.05$ for high Glc vs. high Glc + metformin, ## $P < 0.01$ for high Glc vs. high Glc + AICAR, by ANOVA). *B*: immunoblotting for eEF-2 was done as described in Fig. 1. *Top*: representative blot from 3 experiments. *Bottom*: composite densitometric data from 3 experiments (* $P < 0.01$ high Glc vs. control, # $P < 0.01$ high Glc vs. high Glc + metformin or high Glc + AICAR, by ANOVA).

High glucose regulates eukaryotic elongation factor 2 phosphorylation. During the elongation phase of mRNA translation, amino acids are sequentially added to the nascent peptide chain by the participation of aminoacyl tRNA (6, 54). Movement of the ribosome in the 5' to 3' direction along the mRNA, resulting in translocation of aminoacyl tRNA from the A site to the P site on the ribosome, is regulated by eukaryotic elongation factor 2 (eEF2). Activation of eEF2 occurs following dephosphorylation at Thr56 (45). High glucose reduced eEF2 Thr56 phosphorylation at early time points of incubation (30 and 60 min, data not shown) and at 3 days (Fig. 1D; $P < 0.001$). These data show that high glucose regulates important steps in the initiation and elongation phases of mRNA translation.

High-glucose-induced protein synthesis is PI3-kinase and mammalian target of rapamycin dependent. We studied PI3-kinase-Akt-mammalian target of rapamycin (mTOR) pathways because these regulate 4E-BP1 phosphorylation (2). High glucose increased PI3-kinase activity in anti-p85 α immunoprecipitates at early time points (30 and 60 min, data not shown) and at 1 and 3 days of incubation, showing a rapid onset and sustained stimulation of the kinase activity (Fig. 2A). Because we used p85 α antibody in immunoprecipitation, these data show that high glucose induces activation of class IA PI3-kinase. LY-294002, a selective PI3-kinase inhibitor, inhibited both basal and high-glucose-induced increases in 4E-BP1 phosphorylation (Fig. 2B; $P < 0.001$) and protein synthesis (Fig. 2C; $P < 0.001$). These data suggest that constitutive and high-glucose-induced protein synthesis in the GEC requires PI3-kinase activity.

We next examined whether high glucose stimulated mTOR activity because mTOR is a direct kinase for 4E-BP1 (25). High glucose significantly augmented mTOR Ser2448 phosphorylation at both early and late points of incubation, suggesting activation (Fig. 2D; $P < 0.001$). Requirement of mTOR in high-glucose-induced protein synthesis was examined with rapamycin, a selective inhibitor of mTOR (25). Rapamycin inhibited both basal and high-glucose-induced increases in 4E-BP1 phosphorylation (Fig. 2E; $P < 0.001$). Rapamycin partially but significantly inhibited protein synthesis induced by high glucose (Fig. 2F).

Role of Akt in mTOR regulation was explored. High glucose augmented Akt activity at both early and late time points of incubation (Fig. 3A). In GECs infected with a recombinant adenovirus carrying dominant-negative Akt, but not control green fluorescent protein, high glucose failed to stimulate 4E-BP1 phosphorylation (Fig. 3B); increment in 4E-BP1 phosphorylation is a readout of mTOR activation (26). Thus high glucose induction of mTOR activity is Akt dependent.

High glucose reduces AMPK phosphorylation. AMPK regulates activity of tuberous sclerosis complex (TSC)-2 (tuberin), which, in the basal state, forms a heterodimer with TSC-1 (hamartin) and tonically inhibits mTOR activity, the key controller of 4E-BP1 phosphorylation (19). Activity of AMPK depends on phosphorylation of Thr172 on its α -catalytic subunit (23). AMPK phosphorylation on Thr172 was significantly reduced by high glucose but not by mannitol (Fig. 4A; $P < 0.001$). Immunokinase assay showed that high glucose reduced the activity of AMPK by nearly 40% relative to control (Fig. 4B; $P < 0.01$), confirming that changes in AMPK phosphorylation reflected changes in AMPK activity.

To further explore the role of AMPK in protein synthesis, GECs were transfected with a pCAGGS plasmid containing dominant-negative AMPK carrying a K45R mutation. High glucose stimulated protein synthesis in cells expressing the control vector; expression of dominant-negative AMPK resulted in further increment in high-glucose-induced protein synthesis (Fig. 4C; $P < 0.01$). Basal protein synthesis increased in cells expressing the mutant but did not reach statistical significance.

Upstream factors that regulate AMPK phosphorylation were examined. Reduced AMP content and increased ATP content are known to decrease AMPK phosphorylation (8). Contents of AMP and ATP were measured after high-glucose exposure. High glucose significantly reduced AMP content at 1 h but not at 24 and 72 h (Fig. 4D). Reduction in AMPK phosphorylation was seen at all time points of incubation with high glucose, but correlation with reduced AMP content was seen only at the 1 h time point. ATP content of the GEC was unchanged at 1 h but was reduced

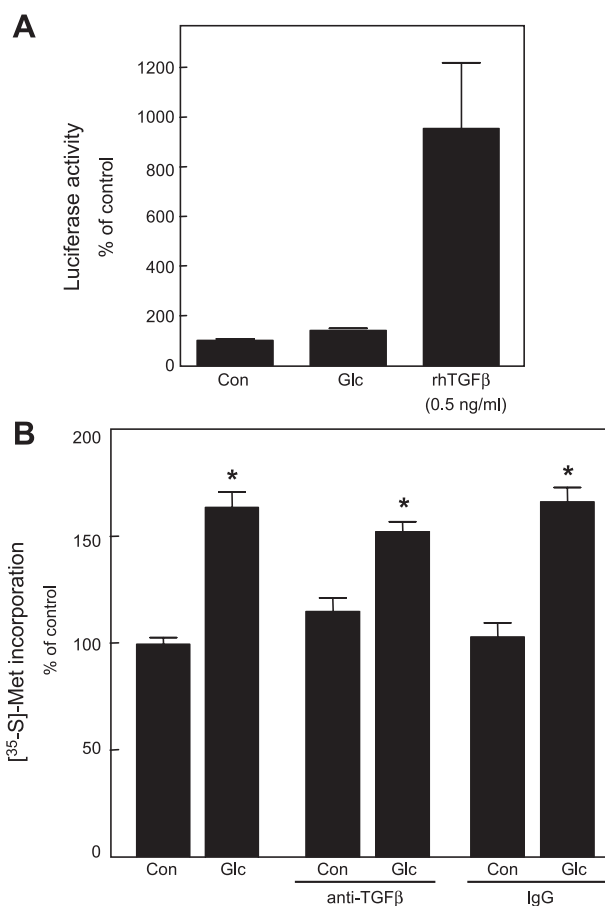


Fig. 7. Transforming growth factor- β (TGF- β) is not involved in high-glucose-induced hypertrophy in the GEC. *A*: mink lung epithelial cells, stably transfected with the human plasminogen activator inhibitor-1 promoter containing TGF- β responsive element fused to a luciferase reporter gene, were treated with conditioned medium from control or high-Glc-treated GEC and human TGF- β standard (rh) for 24 h. TGF- β activity was assessed by luciferase activity. Composite data from 3 experiments are presented. *B*: after 3-day exposure to 5 mM Glc (control) or 30 mM Glc with or without addition of 30 μ g/ml neutralizing anti-TGF- β antibody or irrelevant IgG, de novo protein synthesis was measured as described in Fig. 1. Composite data from 3 experiments are presented (* $P < 0.001$, high Glc vs. control, by ANOVA).

at 24 and 72 h. However, because high glucose decreased AMPK phosphorylation at these time points and because reduced ATP should increase AMPK phosphorylation, ATP content did not correlate with high-glucose-induced changes in AMPK phosphorylation. Role of Akt was examined because it is reported to modulate AMPK and TSC-2 (9, 28). Compared with reduction in AMPK phosphorylation in adenovirus-green fluorescent protein-infected control cells, high glucose barely decreased AMPK phosphorylation in adenovirus-DN-Akt-infected cells (Fig. 4E), identifying a possible role for Akt as one of the upstream regulators of AMPK.

Metformin and AICAR reverse high-glucose effects on AMPK phosphorylation and protein synthesis. We next investigated whether AMPK activators metformin (61) and AICAR could affect high-glucose stimulation of protein synthesis. Metformin and AICAR dose dependently increased Thr172 phosphorylation (Fig. 5A) and prevented high-glucose-induced reduction in AMPK phosphorylation (Fig. 5B). Both metformin and AICAR caused a significant but partial inhibition of high-glucose-induced protein synthesis (Fig. 5C; $P < 0.001$ and $P < 0.05$). Metformin and AICAR reversed high-glucose-induced changes in phosphorylation of 4E-BP1 and eEF2 (Fig. 6, A and B). These data show that stimulation of AMPK inhibits important events in high-glucose regulation of initiation and elongation phases of mRNA translation.

TGF- β is not involved in high-glucose-induced protein synthesis. We next examined the potential role of TGF- β because high-glucose-induced hypertrophy is TGF- β dependent in some renal cells (51, 60). High glucose did not increase TGF- β

expression as measured by luciferase activity linked to TGF- β -sensitive plasminogen activator inhibitor-1 promoter in MLECs (Fig. 7A). Addition of neutralizing TGF- β antibody did not affect high-glucose-induced protein synthesis (Fig. 7B), showing high-glucose induction of protein synthesis in GECs is independent of TGF- β .

Renal AMPK phosphorylation is reduced in Type 1 and Type 2 diabetes. We explored whether diabetes-induced renal hypertrophy was associated with changes in AMPK phosphorylation. On *day 4* of streptozotocin-induced insulin-deficient diabetes, a model of Type 1 diabetes, kidney weight corrected to body weight was significantly increased by 31% (Fig. 8A; $P < 0.0001$). In rats with Type 1 diabetes, AMPK phosphorylation was reduced by nearly 60% ($P < 0.001$) and 80% ($P < 0.01$) in renal cortex (Fig. 8B) and glomeruli (Fig. 8C), respectively. AMPK phosphorylation was also reduced by nearly 70% in renal cortex of *db/db* mice, a model of Type 2 diabetes, at ~ 2 wk of hyperglycemia, at which time the kidneys are hypertrophic (48) (Fig. 8D; $P < 0.01$). These data suggest an association between reduced AMPK phosphorylation and renal hypertrophy in rodents with Type 1 or Type 2 diabetes.

Metformin and AICAR reverse Type 1 diabetes-induced renal hypertrophy. Preliminary studies were performed to determine the doses of metformin and AICAR that increase AMPK phosphorylation in the renal cortex without affecting plasma glucose (data not shown). Metformin and AICAR did not affect elevation in blood glucose or reduction in body weight in diabetic rats (Table 1). The gain in kidney-to-body weight ratio seen in diabetic rats on *day 4* of diabetes was partially but significantly inhibited by metformin or AICAR

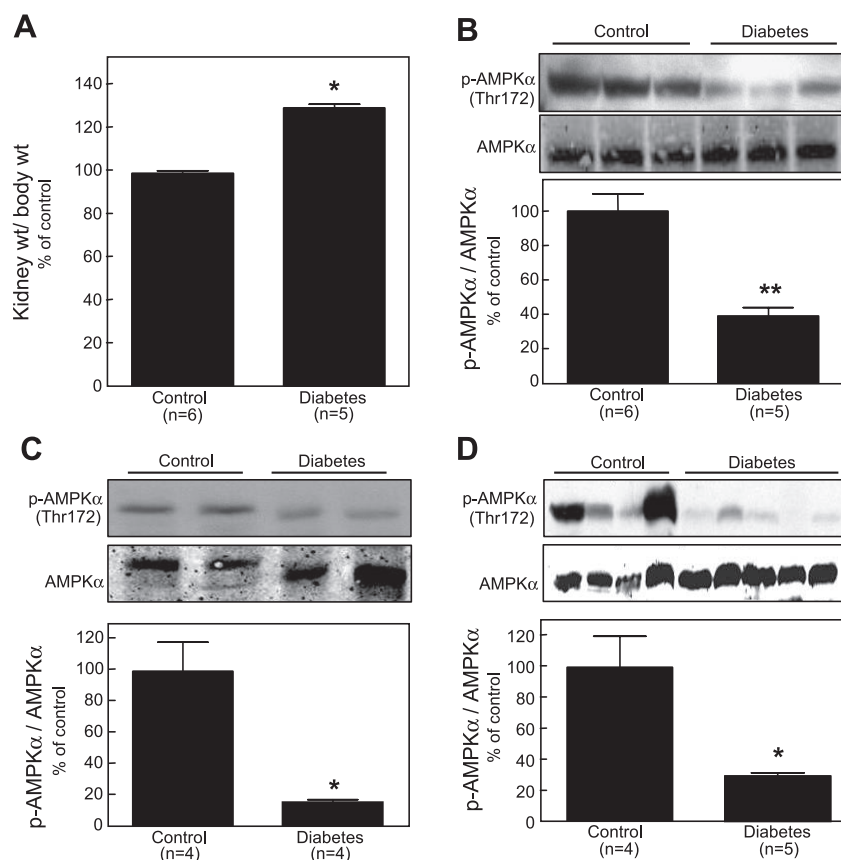


Fig. 8. AMPK phosphorylation is reduced in the kidney in Type 1 and Type 2 diabetes. A, B, and C: Type 1 diabetes was induced in Sprague-Dawley rats by a single injection of 60 mg/kg streptozotocin in citrate buffer (pH 4.5) into the tail vein. On *day 4* of hyperglycemia, rats were killed. A: kidney weight-to-body weight ratio ($*P < 0.0001$, diabetes vs. control, by Student's *t*-test). Equal amounts of renal cortical (B) and glomerular (C) homogenates from rats were separated by SDS-PAGE and immunoblotted with AMPK antibodies as described in Fig. 4 ($*P < 0.01$, $**P < 0.001$, diabetes vs. control, by Student's *t*-test). D: C57BL6/KsJ *db/db* mice were killed at ~ 2 wk of diabetes. Equal amounts of renal cortical homogenates from control and diabetic mice were separated by SDS-PAGE and immunoblotted with AMPK antibodies as described in Fig. 4 ($*P < 0.01$, diabetes vs. control, by Student's *t*-test).

Table 1. Physical and metabolic parameters in control and diabetic rats

	Control (n = 6)	Diabetes (n = 5)	Diabetes + Metformin (n = 5)	Diabetes + AICAR (n = 4)
Body weight, g	243.67 ± 5.55	210.40 ± 3.35*	205.00 ± 5.27*	210.50 ± 4.66*
Blood glucose, mg/dl	92.50 ± 2.19	440.80 ± 28.96*	459.80 ± 12.56*	472.25 ± 23.42*

Values are means ± SE; n = no. of animals. On *day 1* of streptozotocin-induced hyperglycemia, rats were treated with metformin or AICAR for a total of 4 days. AICAR, 5-aminoimidazole-4-carboxamide-1 β -riboside. * $P < 0.001$ vs. control by ANOVA.

(Fig. 9A). Metformin and AICAR prevented the reduction in AMPK phosphorylation in renal cortex in diabetic rats compared with untreated diabetic animals (Fig. 9B). These data demonstrate that reduction in AMPK activity contributes to diabetes-induced renal hypertrophy in rats.

Signaling pathways that regulate protein synthesis were investigated in the renal cortex of rats with Type 1 diabetes. At 4 days of diabetes, Akt phosphorylation was increased in the renal cortex (Fig. 9C). Activation of mTOR was reflected in increased phosphorylation of its direct substrates, p70S6 kinase and 4E-BP1 (Fig. 9D). Treatment with metformin and AICAR prevented increases in phosphorylation of 4E-BP1 and p70S6 kinase in diabetic rats (Fig. 9D), although the diabetes-induced

increase in phosphorylation of Akt was unaffected (Fig. 9C). These data show that, in the kidney, AMPK regulates mTOR activity, without any effect on Akt activation.

DISCUSSION

Protein synthesis consumes a significant amount of cellular energy (29). Induction of protein synthesis by glucose, a source of energy, is likely to involve regulation of energy sensors such as AMPK. Accordingly, in association with high-glucose-induced protein synthesis, we found inhibition of AMPK phosphorylation and activity. AMPK is a heterotrimeric protein consisting of a combination of $\alpha_{1/2}$ -, $\beta_{1/2}$ -, and $\gamma_{1/2/3}$ -

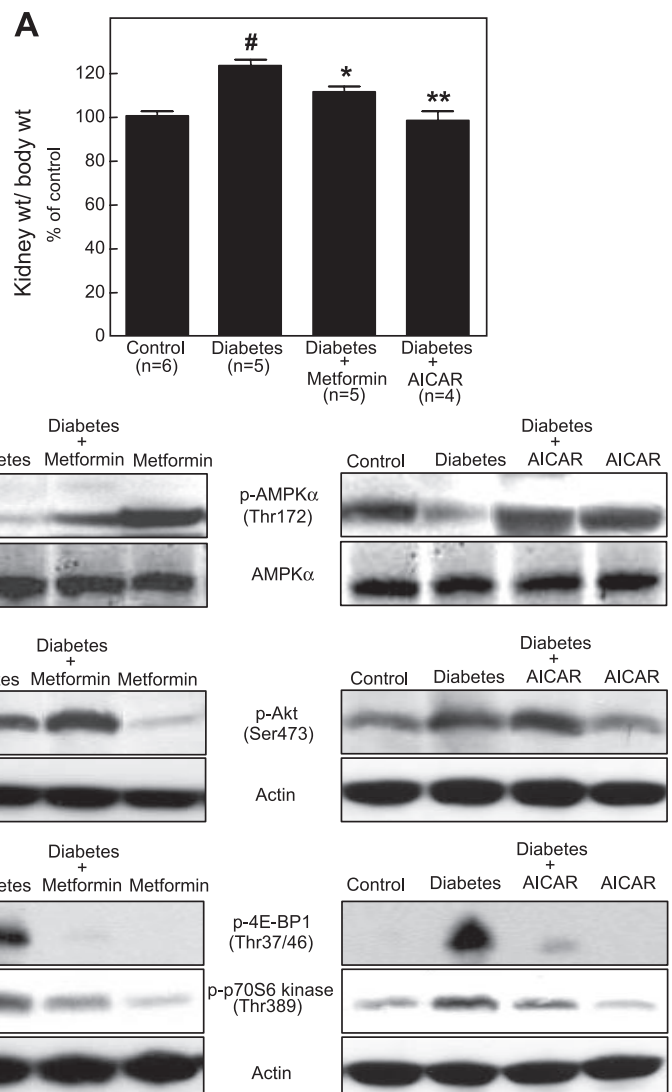


Fig. 9. Metformin and AICAR inhibit diabetes-induced renal hypertrophy. *A*: on *day 1* of streptozotocin-induced hyperglycemia, rats were treated with metformin or AICAR for a total of 4 days, as described in MATERIALS AND METHODS. *A*: kidney weight-to-body weight ($\#P < 0.001$ for diabetes vs. control, $*P < 0.05$ for diabetes vs. diabetes + metformin, $**P < 0.001$ for diabetes vs. diabetes + AICAR, by ANOVA). *B*, *C*, and *D*: equal amounts of renal cortical homogenates from rats were separated by SDS-PAGE and immunoblotted with the indicated antibodies (*left*: metformin; *right*: AICAR).

subunits (8), with the α_1 - rather than α_2 -subunit expressed in the kidney (16). The enzymatic activity of AMPK is dependent on phosphorylation of Thr172 of the α -subunit (23). The COOH terminus of β -subunit binds to α - and γ -subunits (30). The γ -subunit contains the cystathionine beta synthase-like domain to which AMP binds allosterically. Increased AMP content stimulates AMPK activity, whereas increased ATP content results in its suppression (8). On activation, AMPK inhibits energy-consuming reactions, such as synthesis of fatty acids and sterols (55), and activates ATP-generating processes, such as fatty acid oxidation (39).

The signal in diabetic kidney that alters activity of AMPK is not known but may include changes in levels of AMP and ATP. An increase in proximal tubular ATP content and mitochondrial enlargement has been observed in diabetes (32). However, infusion of high glucose into an isolated kidney has been reported to reduce ATP content (33). Careful measurement of ATP and AMP content in the GEC at various time points of incubation with high glucose revealed a single time point of 1-h exposure to high glucose when reduced AMP content correlated with decreased AMPK phosphorylation. Reduced AMPK phosphorylation did not correlate with AMP content at other time points or with ATP content at any time point. These data suggest that, in addition to adenine nucleotides, other factors may be involved in high-glucose regulation of AMPK in GECs. High osmolality, known to augment AMPK activity (17), is unlikely to be involved in GECs because mannitol did not affect AMPK phosphorylation. Ca^{2+} /calmodulin-dependent kinase kinase- β also stimulates AMPK activity (24). Because Ca^{2+} /calmodulin-dependent kinase kinase- β is expressed mostly in the central nervous system, other factors yet undiscovered may be involved (3). High-glucose-induced reduction in AMPK activity could increase fatty acid synthesis, which, in turn, could affect mRNA translation. Arachidonic acid derivatives $\text{PGF}_{2\alpha}$ and HETE have been reported to stimulate the initiation phase of mRNA translation (44, 59). In addition to modulation of fatty acid synthesis, recent investigations have implicated AMPK in protein synthesis (46), which is regulated at the level of mRNA translation.

Important events in mRNA translation, e.g., phosphorylation of 4E-BP1 and eEF2, are partly under the control of the PI3-kinase-Akt-mTOR signaling pathway. Reversal of high-glucose-induced changes in 4E-BP1 and eEF2 phosphorylation by stimulation of AMPK activity suggests that AMPK interacts with this pathway. High-glucose-induced increases in 4E-BP1 phosphorylation and protein synthesis in the GECs were found to be dependent on PI3-kinase and mTOR activation. Inhibition of high-glucose-induced Akt activation by expression of dominant-negative Akt construct partly restored AMPK phosphorylation, suggesting AMPK is downstream of Akt. Because stimulation of AMPK also inhibited high-glucose-induced mTOR activity, as shown by inhibition of phosphorylation of 4E-BP1 and p70S6 kinase, AMPK appears to be interposed between Akt and mTOR (Fig. 10). Inhibition of AMPK by Akt has also been observed in cardiac myocytes (36). In addition, Akt induces phosphorylation of TSC-2 and inhibits the activity of the TSC-1/TSC-2 dimer (28), which would contribute to increase in mTOR activity. Akt could also stimulate the activity of a phosphatase or inhibit upstream kinase for AMPK such as LKB-1, which could result in reduction in AMPK phosphor-

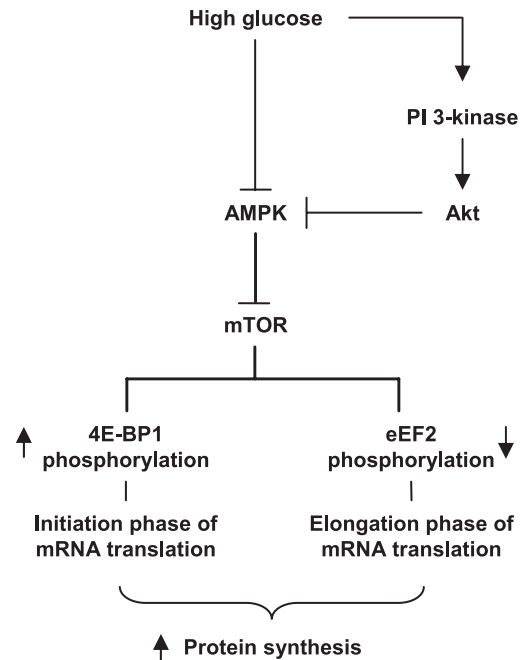


Fig. 10. Schematic of AMPK regulation in diabetes-induced renal hypertrophy.

ylation. There could be additional mechanisms by which high glucose may suppress AMPK activity in the GEC.

mTOR plays a central role in regulation of initiation and elongation phases of mRNA translation. 4E-BP1, an important controller of eIF4E activity in the initiation phase, is a direct substrate of mTOR. Increase in activity of p70S6 kinase, another important mTOR substrate, promotes phosphorylation of eEF2 kinase on Ser366 (56), resulting in inhibition of its activity. eEF2 kinase directly phosphorylates eEF2 on Thr56, leading to its inactivation (45). Thus phosphorylation and inactivation of eEF2 kinase contribute to decreased eEF2 phosphorylation. Recently, AMPK has been reported to regulate eEF2 kinase by phosphorylation of Ser398, which increases activity of eEF2 kinase, resulting in inhibition of the elongation phase (5). Thus high-glucose-induced reduction in AMPK activity in the GEC appears to reduce activity of eEF2 kinase, resulting in decreased phosphorylation of eEF2 and facilitation of the elongation phase of protein synthesis.

Observations with dominant-negative AMPK suggest that AMPK normally inhibits protein synthesis and that its suppression appears to mediate high-glucose-induced protein synthesis. That inhibition of AMPK is not a parallel event but is actually required for high-glucose-induced protein synthesis is suggested by abrogation of the high-glucose effect with metformin and AICAR, two distinct activators of AMPK. Studies in cardiomyocytes have shown a similar reduction in AMPK phosphorylation in hypertrophy induced by phenylephrine and its reversal by metformin and AICAR (9). Furthermore, mutations in the γ_2 -subunit of AMPK are associated with cardiac hypertrophy (4). Administration of metformin and AICAR partly or fully prevented renal hypertrophy seen in early stages of insulin-deficient diabetes in association with increased AMPK phosphorylation in the renal cortex without affecting the degree of hyperglycemia. These observations support a definitive role for AMPK in control of renal protein synthesis in diabetes.

Findings in this report extend previous observations in which AMPK was found to be involved in high-glucose regulation of L-type pyruvate kinase gene expression in islet cells and in glucose regulation of its own uptake in skeletal muscle (11, 31). Identification of AMPK as a regulating factor in diabetes-induced renal hypertrophy has implications for management. Hypertrophy of GEC and the rest of the kidney cells may be linked to subsequent events that result in demise of kidney function (27, 58). Preliminary data indicate that AMPK may be also involved in abnormal extracellular matrix protein synthesis induced by high glucose in the GEC (Lee and Kasinath, unpublished observations). Accumulation of matrix proteins has been directly linked to loss of kidney function in diabetes. Ready availability of agents such as metformin and thiazolidinediones, which increase AMPK activity, make AMPK an attractive therapeutic target for reversing diabetes-induced renal injury.

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