Hydrophobic motif site-phosphorylated protein kinase CβII between mTORC2 and Akt regulates high glucose-induced mesangial cell hypertrophy

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1Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas; 2Veterans Affairs Research, South Texas Veterans Health Care System, San Antonio, Texas; 3Geriatric Research, Education and Clinical Research, South Texas Veterans Health Care System, San Antonio, Texas; and 4Departments of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

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Das F, Ghosh-Choudhury N, Mariappan MM, Kasinath BS, Choudhury GG. Hydrophobic motif site-phosphorylated protein kinase CβII between mTORC2 and Akt regulates high glucose-induced mesangial cell hypertrophy. Am J Physiol Cell Physiol 310: C583–C596, 2016. First published January 6, 2016; doi:10.1152/ajpcell.00266.2015.—PKCβII controls the pathologic features of diabetic nephropathy, including glomerular mesangial cell hypertrophy. PKCβII contains the COOH-terminal hydrophobic motif site Ser-660. Whether this hydrophobic motif phosphorylation contributes to high glucose-induced mesangial cell hypertrophy has not been determined. Here we show that, in mesangial cells, high glucose increased phosphorylation of PKCβII at Ser-660 in a phosphatidylinositol 3-kinase (PI3-kinase)-dependent manner. Using siRNAs to downregulate PKCβII, dominant negative PKCβII, and PKCβII hydrophobic motif phosphorylation-deficient mutant, we found that PKCβII regulates activation of mechanistic target of rapamycin complex 1 (mTORC1) and mesangial cell hypertrophy by high glucose. PKCβII via its phosphorylation at Ser-660 regulated phosphorylation of Akt at both catalytic loop and hydrophobic motif sites, resulting in phosphorylation and inactivation of its substrate PRAS40. Specific inhibition of mTORC2 increased mTORC1 activity and induced mesangial cell hypertrophy. In contrast, inhibition of mTORC2 decreased the phosphorylation of PKCβII and Akt, leading to inhibition of PRAS40 phosphorylation and mTORC1 activity and prevented mesangial cell hypertrophy in response to high glucose; expression of constitutively active Akt or mTORC1 restored mesangial cell hypertrophy. Moreover, constitutively active PKCβII reversed the inhibition of high glucose-stimulated Akt phosphorylation and mesangial cell hypertrophy induced by suppression of mTORC2. Finally, using renal cortexes from type 1 diabetic mice, we found that increased phosphorylation of PKCβII at Ser-660 was associated with enhanced Akt phosphorylation and mTORC1 activation. Collectively, our findings identify a signaling route connecting PI3-kinase to mTORC2 to phosphorylate PKCβII at the hydrophobic motif site necessary for Akt phosphorylation and mTORC1 activation, leading to mesangial cell hypertrophy.

diabetic nephropathy; signal transduction; mTOR complex; protein kinase C

EARLY CHANGES IN DIABETIC nephropathy comprise whole kidney hypertrophy including glomerular hypertrophy and altered hemodynamics (41, 79). Pathologic manifestations that follow these early changes include hyperfiltration, microalbuminuria, effacement and detachment of podocytes, tubulointerstitial fibrosis, and glomerulosclerosis, which lead to loss of renal function (25, 66, 86). Although podocytes contribute significantly to development and progression of albuminuria in diabetic nephropathy, abnormal urinary albumin loss regresses in nearly 50% of patients. However, enhanced mesangial matrix expansion correlates well with development of diabetic nephropathy (5, 59). These results suggest a role of mesangial cells in the loss of renal function in this disease. In fact increase in expression of many hormones and growth factors including angiotensin II, TGFβ, VEGF, and IGF-1 in response to hyperglycemia contributes to mesangial cell hypertrophy and amassing of matrix proteins. Control of hyperglycemia represents the best way to prevent metabolic derangements of diabetic nephropathy; however, current therapies for glycemic control are fraught with complications and difficulty in compliance. High glucose can contribute to diabetic nephropathy by producing sorbitol, advanced glycation end products, and diacylglycerol (DAG) (1, 4, 7, 31, 69, 81). Among these factors, DAG contributes to the activation of protein kinase C (PKC).

The PKC family of serine/threonine kinases belongs to the AGC superfamily and consists of three groups of enzymes: classical, novel, and atypical. The classical (α, β, βII, and γ) and novel (δ, ε, η, and θ) isoforms are activated by DAG, while the former also requires Ca2+. The atypical PKCs (ζ and λ) are Ca2+ and DAG independent (44, 50, 54). Among all the isoforms, PKCβII is activated in hyperglycemia and has been linked to various vascular complications of diabetes including diabetic nephropathy (28, 76). Using a PKC inhibitor, which predominantly suppresses both PKCβ1 and βII isoforms, Ishi et al. (28) showed amelioration of changes in glomerular filtration rate and albumin excretion in a streptozotocin-induced rat model of type 1 diabetes. Mice with homozygous deletion of PKCβ showed protection towards complications of diabetic nephropathy. These included reduction in glomerular and mesangial hypertrophy, expression of NADPH oxidases, and urinary excretion of isoprostane and 8-hydroxy-dG (56). Furthermore, expression of markers of renal fibrosis such as TGFβ and connective tissue growth factor (CTGF) was diminished in diabetic PKCβ null mice although no effect was observed on renal albumin excretion (49, 56). A significant drawback of these studies is that both PKCβ1 and βII were deleted in this mouse model (42). Since PKCβII has been predominantly implicated in complications of diabetes (76), in employing PKCβ null mouse in the aforementioned studies, the specific contribution of PKC βII isotype to the renal outcome of diabetic nephropathy could not be determined.
In rat mesangial cells high glucose increased the activation of various classical (α and β), novel (δ and ε), and atypical (ζ) PKC isotypes (32, 35, 37, 78). There are conflicting data on whether adult rat mesangial cells express PKCβII (32, 67). More recently, PKCβII has been characterized in adult mouse and human mesangial cells and reported to be activated in lupus nephritis (82). In the present study, using human mesangial cells, we show that high glucose increases the phosphorylation of hydrophobic motif site (Ser-660) of PKCβII in a phoshatidylinositol 3-kinase (PI3-kinase) and mechanistic target of rapamycin complex 2 (mTORC2)-dependent manner. We demonstrate that both PKCβII and mTORC2 contribute to high glucose-induced phosphorylation of Akt at Thr-308 and Ser-473. High glucose-stimulated mTORC2 and PKCβII regulate inactivation and activation of PRAS40 and mTORC1, respectively, resulting in protein synthesis and hypertrophy of mesangial cells.

MATERIALS AND METHODS

Reagents. d-glucose, d-mannitol, Nonidet P-40, PMSF, Na3VO4, protease inhibitor cocktail, and actin antibody were purchased from Sigma. Phospho-PKCβII (Ser-660), phospho-Akt (Ser-473), phospho-Act (Thr-308), Akt, phospho-4EBP-1 (Thr-37/46), 4EBP-1, phospho-S6 kinase (Thr-389), S6 kinase, phospho-PRA540 (Thr-246), PRAS40, mTOR, and rictor antibodies were purchased from Cell Signaling Technology. HA antibody was purchased from Covance. PKCβII, p85 regulatory subunit of PI3-kinase, phosphatase and tensin homolog deleted from chromosome 10 (PTEN), and GFP antibodies and pooled siRNAs against PKCβII were purchased from Santa Cruz Biotechnology. shRNA expression vectors against PKCβII and PTEN were described previously (2, 10, 11, 45). GFP-tagged PKCβII expression vector was a gift from Dr. Y. Hannnun, Medical University of South Carolina. Dominant negative PKCβII K371R and constitutively active PKCβII CAT plasmids were obtained from Addgene and described previously (10). GFP-tagged PKCβII expression vector was a gift from Dr. Y. Hannnun, Medical University of South Carolina. Dominant negative PKCβII K371R and constitutively active PKCβII CAT plasmids were obtained from Addgene and described previously (10). GFP-tagged PKCβII expression vector was a gift from Dr. Y. Hannnun. PKCβII and PTEN were described previously (2, 10, 11, 45).

Cell culture and adenovirus infection. Previously, we and Abboud and coworkers (9, 68) have described the preparation of normal human mesangial cells from renal cortex of human kidneys unsuitable for transplantation. These cells have been extensively used by us and were cultured in DMEM in the presence of 10% fetal bovine serum as described previously (6, 9). Cells were incubated in serum free medium for 24 h before incubation with 25 mM glucose (high glucose). As osmotic control 5 mM glucose plus 20 mM mannitol (low glucose) was used. Where necessary, mesangial cells were infected with adenovirus vectors in serum free medium at a multiplicity of infection of 50 for 24 h as described previously (11). As control, infection with an adenovirus vector expressing green fluorescence protein (Ad GFP) was used.

Animals. OVE26 mice expressing pancreatic β-cell-specific calmodulin transgene and their control littermate FVB mice were purchased from The Jackson Laboratories. OVE26 mice are hyperglycemic at 3 days of age and develop significant renal hypertrophy, glomerular hypertrophy, and albuminuria at 2 mo of age (15, 84). The mice were kept in the University of Texas Health Science Center animal facility. They had free access to food and water. After the control FVB and OVE26 mice were euthanized at 3 mo of age, both kidneys were removed. Renal cortical tissues from each mouse were isolated and frozen as described previously (13). The University of Texas Health Science Center Institutional Animal Care and Use Committee approved the animal protocol.

Cell lysis, immunoprecipitation, and immunoblotting. After high-glucose treatment, the cells were washed with PBS. The monolayer was harvested in radiommune precipitation buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1% NP-40, and 0.1% protease inhibitor cocktail). Similarly, renal cortices from control and diabetic mice were harvested in the same RIPA buffer. The cells and renal cortices were lysed for 30 min at 4°C. The cell extracts were centrifuged at 12,000 g for 30 min. The supernatant was collected and the protein concentration was determined. Equal amounts of proteins were separated by SDS-PAGE. Separated proteins were transferred to membrane. Immunoblotting was performed using indicated antibodies. The protein bands were developed with horseradish peroxidase-conjugated secondary antibodies (10, 12). For immunoprecipitation, equal amounts of protein were incubated with PKCβII antibody and immunoprecipitated using protein G agarose beads. The immunobeads were suspended in sample buffer followed by electrophoresis and immunoblotting using phospho-PKCβII (Ser-660) antibody as described above.

mTORC2 immunocomplex kinase assay. Renal cortical lysates were immunoprecipitated with rictor antibody. The immunoprecipitates were washed three times with the RIPA buffer at 4°C. Then, the immunoprecipitates were washed twice with the mTORC2 immunocomplex kinase assay buffer. One-hundred and twenty-five nanograms of recombinant PKCβII and 500 μM ATP were added. The reaction mixture was incubated at 37°C for 30 min and was terminated by adding 4× SDS sample buffer. The protein was analyzed by immunoblotting with phospho-PKCβII (Ser-660) antibody. For control, 25 ng of recombinant PKCβII were immunoblotted separately with PKCβII antibody.

Protein synthesis. After incubation, the mesangial cells were labeled with 35S-methionine. Protein synthesis was determined as 35S-methionine incorporation as described previously (11, 14).

Measurement of cellular hypertrophy. The cells were trypsinized and counted in a hemocytometer. Cells were then centrifuged at 4,000 g at 4°C. The cell pellet was washed with PBS and lysed in RIPA buffer as described above. The protein content in the total number of cells was determined. Hypertrophy was determined as a ratio of total protein to number of cell, as described previously (14).

Statistics. The data were analyzed by ANOVA followed by Student-Newman-Keuls analysis or by paired Student’s t-test. A P < 0.05 was considered significant (10, 11).

RESULTS

High glucose-induced increase in hydrophobic motif site phosphorylation of PKCβII is PI3-kinase dependent and regulates mesangial cell hypertrophy. Glomeruli from diabetic rodents and high glucose-treated glomerular mesangial cells in culture show increased activity of different PKC isoforms (28). However, activation of PKCβII is involved in diabetic complications of kidney. Similar to other PKC isotypes, PKCβII requires phosphorylation for activation. All members of the AGC kinase family including PKCβII contain a hydrophobic motif site, Ser-660, which undergoes phosphorylation. Al-
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with other isotypes of PKC when they are phosphorylated. Therefore, PKCβII immunoprecipitates were used from lysates of mesangial cells incubated with high glucose. High glucose increased phosphorylation of PKCβII at Ser-660 in a time-dependent and sustained manner for up to 48 h (Fig. 1, A and B). Many PKC isotypes have been shown to be activated by PI3-kinase-dependent PDK1-mediated activation loop phosphorylation (40). However, the role of PI3-kinase in phosphorylation of PKCβII hydrophobic motif site has not been characterized. The PI3-kinase inhibitor Ly294002 significantly blocked high glucose-stimulated Ser-660 phosphorylation of PKCβII (Fig. 1C). To confirm this observation, we used a deletion mutant of the p85 regulatory subunit of PI3-kinase, which confers dominant negative function to this enzyme. Expression of dominant negative PI3-kinase inhibited high glucose-induced PKCβII phosphorylation (Fig. 1D). Similarly, expression of the tumor suppressor protein PTEN, which is an endogenous inhibitor of PI3-kinase signaling, abolished phosphorylation of PKCβII induced by high glucose (Fig. 1E).

We have shown previously that high glucose-induced PI3-kinase activity contributes to mesangial cell hypertrophy (14, 45). Our results above show that PKCβII is a downstream target of PI3-kinase signaling. Also, PKCβ knockout mouse showed protection from diabetic renal, especially glomerular hyper trophy (49). Since this model does not discriminate between the roles of PKCβ1 and PKCβII, and PKCβII activity is increased in diabetic renal glomeruli, we tested the specific role of PKCβII in mesangial cell hypertrophy. We used a pool of siRNAs specifically targeting PKCβII. The results showed that downregulation of PKCβII significantly inhibited high glucose-induced protein synthesis (Fig. 1F). Consequently, mesangial cell hypertrophy as determined by the ratio of total protein to cell number in response to high glucose was also inhibited (Fig. 1G). To determine the role of PKCβII kinase activity in mesangial cell hypertrophy, we employed a kinase dead mutant with a mutation at the Lys-371 to Arg (70). Expression of this mutant markedly inhibited the high glucose-stimulated protein synthesis and hypertrophy of mesangial cells (Fig. 1, H and J). The hydrophobic motif site phosphorylation is associated with the activities of various PKC isotypes. However, its role in a specific biological activity such as protein synthesis is poorly understood. Expression of PKCβII nonphosphorylatable mutant significantly prevented both protein synthesis and hypertrophy of mesangial cells in response to high glucose (Fig. 1, J and K). These results suggest that the hydrophobic motif site phosphorylation of PKCβII is essential for high glucose-induced mesangial cell hypertrophy.

*PKCβII increases mTORC1 signaling.* We and others have shown that mTORC1 activation is necessary for renal hypertrophy in rodents (61, 65). Also, we showed the requirement of mTORC1 in high glucose-induced mesangial cell hypertrophy (14). To examine the role of PKCβII in mTORC1 activation, we determined the phosphorylation of its two endogenous substrates, S6 kinase and 4EBP-1. As expected, high glucose increased phosphorylation of these two proteins (Fig. 2). siRNAs targeting PKCβII blocked high glucose-stimulated phosphorylation of S6 kinase and 4EBP-1 (Fig. 2, A and B). Expression of dominant negative PKCβII inhibited high glucose-stimulated phosphorylation of S6 kinase and 4EBP-1 (Fig. 2, C and D). Similarly, nonphosphorylatable PKCβII S660A abrogated the phosphorylation of both mTORC1 substrates (Fig. 2, E and F).

*Ser-660 phosphorylation of PKCβII is necessary for Akt activation.* Our previous studies demonstrated that high glucose-induced mesangial cell hypertrophy is dependent on phosphorylation/inactivation of PRAS40, an Akt substrate and a negative regulator of mTORC1 activity (14). We determined the role of PKCβII in phosphorylation of PRAS40. Both siRNAs against PKCβII and dominant negative PKCβII inhibited high glucose-induced phosphorylation of PRAS40 at Thr-246 (Fig. 3, A and B). Similarly, the PKCβII Ser-660A phosphorylation-deficient mutant blocked the phosphorylation of PRAS40 in response to high glucose (Fig. 3C). We and others previously reported that Akt kinase activation is associated with renal hypertrophy in rodents (33, 45). It is known that activated Akt kinase phosphorylates PRAS40 at Thr-246 (62). Thus inhibition of its phosphorylation by PKCβII mutant indicates a role of this kinase in activation of Akt. Indeed high glucose increased the phosphorylation of Akt at both the hydrophobic motif (Ser-473) and catalytic loop (Thr-308) sites in a time-dependent manner similar to that observed with PKCβII Ser-660 phosphorylation (Figs. 3, D and E, and 1, A and B). Note that the hydrophobic motif site phosphorylation of Akt at Ser-473 is mediated by mTORC2 (64). However, downregulation of PKCβII by siRNAs or dominant negative PKCβII expression abrogated high glucose-induced phosphorylation of Akt at both sites (Fig. 3, F and G). Moreover, the expression of PKCβII Ser-S660A inhibited the phosphorylation of Akt at these sites (Fig. 3H). These results indicate that Ser-660 phosphorylated PKCβII regulates high glucose-stimulated Akt activation necessary for the phosphorylation of PRAS40, which results in activation of mTORC1 leading to mesangial cell hypertrophy.

*mTORC2 regulates high glucose-induced PKCβII hydrophobic motif phosphorylation and mTORC1 activation.* It was shown previously that kinase activity of mTORC2 phosphorylates the hydrophobic motif site of conventional PKCα iso type (63). We tested whether mTORC2 could phosphorylate the hydrophobic motif site of PKCβII. A classical way of studying the role of mTORC2 is to downregulate one of its exclusive components rictor (10, 63). With the use of two independent siRNAs to downregulate endogenous rictor, which is necessary for mTORC2 activity (63), our results showed inhibition of high glucose-induced phosphorylation of PKCβII at Ser-660 (Fig. 4A). Importantly, high glucose-induced phosphorylation of PKCβII at this site was inhibited by the PI3-kinase inhibitor Ly294002 (Fig. 1C). To directly determine the involvement of PI3-kinase in mTORC2-mediated phosphorylation of PKCβII, we immunoprecipitated rictor, the specific and required component of mTORC2, from lysates of mesangial cells treated with Ly294002 before incubation with high glucose. The immunoprecipitates were used in an immunocomplex kinase assay to determine mTORC2 activity using recombinant PKCβII protein as substrate in the presence of ATP. The reaction product was immunoblotted with phospho-PKCβII (Ser-660). High glucose significantly increased the mTORC2 activity, which was inhibited by Ly 294002 (Fig. 4B). These results indicate that high glucose activates mTORC2 in a PI3-kinase-sensitive manner.

We have shown above that hydrophobic motif site phosphorylated PKCβII regulates mTORC1 activation by high glucose.
Fig. 1. Phosphatidylinositol 3-kinase (PI3-kinase)-dependent phosphorylation of PKCβII at Ser-660 regulates high glucose-induced mesangial cell hypertrophy. A and B: Mesangial cells were incubated with 25 mM glucose or 5 mM glucose plus 20 mM mannitol for indicated periods of time. The cell lysates were immunoprecipitated (IP) with PKCβII antibody followed by immunoblotting (IB) with phospho-PKCβII (Ser-660) and PKCβII antibodies. C–E: Mesangial cells were treated with 25 μM Ly294002 for 1 h (C) or infected with adenovirus vector expressing dominant negative PI3-kinase (D) or phosphatase and tensin homolog deleted from chromosome 10 (PTEN; E) for 24 h before incubation with HG as described in MATERIALS AND METHODS. The PKCβII immunoprecipitates were immunoblotted with phospho-PKCβII (Ser-660) and PKCβII antibodies. Representative blots from 3 independent experiments are shown. F–K: mesangial cells were transfected with pooled siRNAs against PKCβII (F and G) or dominant negative PKCβII K371R (H and I) or PKCβII S660A (J and K). The cells were then incubated with high glucose (HG; 25 mM glucose) or low glucose (LG; 5 mM glucose plus 20 mM mannitol); F, H, and J, protein synthesis was determined as a measure of 35S-methionine incorporation as described in the MATERIALS AND METHODS. G, I, and K: mesangial cell hypertrophy was determined as the ratio of total protein content to cell number. F–K, bottom: expression of proteins from parallel experiments. For F–K, mean ± SE of triplicate measurements is shown. For F, *P < 0.01 vs. control; **P < 0.01 vs. HG stimulated. For G, *P < 0.01 vs. control; **P < 0.05 vs. HG stimulated. For H, *P < 0.001 vs. control; **P < 0.01 vs. HG stimulated. For I, *P < 0.001 vs. control; **P < 0.01 vs. HG stimulated. For J, *P < 0.01 vs. control; **P < 0.01 vs. HG stimulated. For K, *P < 0.001 vs. control; **P < 0.01 vs. HG stimulated.
glucose (Fig. 2, E and F). Since mTORC2 regulates phosphorylation of PKCβII, we systematically examined the involvement of mTORC2 in mTORC1 activation in high glucose-treated mesangial cells. Since phosphorylation of PRAS40 is necessary for mTORC1 activation by high glucose, we first tested the effect of inhibition of mTORC2 activity on its phosphorylation. Inhibition of mTORC2 by downregulation of rictor with two independent shRNAs showed suppression of PRAS40 phosphorylation in response to high glucose (Fig. 4C). Also, downregulation of rictor resulted in the inhibition of phosphorylation of Akt at both sites (Fig. 4D), which is necessary for its activation and PRAS40 phosphorylation (62, 64). Therefore, we tested the role of mTORC2 in high glucose-stimulated mTORC1 activity. Downregulation of rictor by two individual shRNAs inhibited high glucose-increased mTORC1 activity as judged by phosphorylation of S6 kinase (Fig. 4E). To confirm mTORC2 regulation of mTORC1, we examined phosphorylation of 4EBP-1, another direct substrate of mTORC1. Inhibition of expression of rictor abrogated high glucose-stimulated 4EBP-1 phosphorylation (Fig. 4F). Note that inhibition of mTORC2 by shRNA against rictor increased basal phosphorylation of both S6 kinase and 4EBP-1 (Fig. 4, E and F; see DISCUSSION). These results conclusively show that mTORC2 contributes to the activation of mTORC1 in response to high glucose in mesangial cells; in contrast, mTORC2 appears to constitutively inhibit mTORC1 in the basal state.

mTORC2 regulates mesangial cell hypertrophy via Akt-mTORC1. We have previously shown that both Akt and mTORC1 regulate mesangial cell hypertrophy (14, 45). Since we have shown above that mTORC2 contributes to the activation of mTORC1 in response to high glucose (Fig. 4), we directly examined directly the role of mTORC2 in mesangial cell hypertrophy. Specific inhibition of rictor by shRNAs to block mTORC2 activity significantly abrogated the protein synthesis in mesangial cells induced by high glucose (Fig. 5A). Similarly, shRNAs against rictor markedly inhibited high glucose-induced mesangial cell hypertrophy (Fig. 5B). These results suggest an involvement of mTORC2 in mesangial cell hypertrophy. Interestingly, inhibition of mTORC2 under basal condition increased both protein synthesis and hypertrophy of mesangial cells (Fig. 5, A and B).

However, we have shown above that mTORC2 is upstream of mTORC1, which in turn is regulated by Akt kinase (Fig. 4). Therefore, to directly test the involvement of Akt and mTORC1 downstream of mTORC2, we overexpressed constitutively active mutant of Akt (Myr Akt) along with shRNA against rictor. As expected, shRictor significantly inhibited the high glucose-induced protein synthesis and mesangial cell hypertrophy (Fig. 5, C and D). Expression of constitutively active Akt kinase significantly reversed the inhibition of protein synthesis and hypertrophy by shRictor in the presence of high glucose (Fig. 5, C and D). Similarly, expression of constitutively active mTORC1 (CA mTOR) prevented the shRictor-induced suppression of high glucose-induced protein synthesis and hypertrophy (Fig. 5, C and D). These results conclusively indicate that both Akt and mTORC1 act downstream of mTORC2 to regulate mesangial cell hypertrophy in response to high glucose.

mTORC2-regulated PKCβII controls Akt Ser-473 phosphorylation for the induction of mesangial cell hypertrophy. It is known that mTORC2 phosphorylates Akt Ser-473 for its full activation (64). We showed above that mTORC2 phosphory-
ylates PKCβII at its hydrophobic motif site Ser-660 (Fig. 4A). Also, our results above conclusively demonstrate that active PKCβII is necessary for Akt Ser-473 phosphorylation in response to high glucose in mesangial cells (Fig. 3, F, G, and H). Therefore, we investigated the connection between mTORC2 and PKCβII for Akt Ser-473 phosphorylation. As expected, when mTORC2 was blocked by downregulation of rictor, phosphorylation of Akt at Ser-473 by high glucose was inhibited (Fig. 6A). Interestingly, coexpression of constitutively active PKCβII reversed the shRictor-induced inhibition of high glucose-stimulated phosphorylation of Akt (Fig. 6A, compare lane 8 with lane 4). Importantly, expression of constitutively active PKCβII alone under low glucose condition was sufficient to induce Akt Ser-473 phosphorylation (Fig. 6A, compare lane 5 with lane 1). Moreover, under basal mTORC2-inhibited condition in the presence of shRictor, PKCβII increased phosphorylation of Akt at Ser-473 (Fig. 6A, compare lane 7 with lane 1). These results suggest that downstream of mTORC2, PKCβII is the kinase for phosphorylation of Akt hydrophobic motif site Ser-473 in the presence of high glucose.

We have shown that mTORC2 and PKCβII independently regulate high glucose-induced mesangial cell hypertrophy (Figs. 1, F–K, and 5). To examine the direct contribution of PKCβII downstream of mTORC2, we coexpressed CA PKCβII along with shRNAs against rictor. As expected down-regulation of rictor to inhibit mTORC2 activity blocked high glucose-induced protein synthesis and hypertrophy in mesangial cells (Fig. 6, B and C). Coexpression of CA PKCβII with shRNAs against Rictor reversed the shRictor-mediated inhibition of high glucose-induced protein synthesis and hypertrophy (Fig. 6, B and C; compare bars 8 with bar 4). Furthermore, CA PKCβII alone or along with shRNAs against Rictor significantly induced the protein synthesis and hypertrophy of mesangial cells (Fig. 6, B and C; compare bars 5 and 7 with bar 1). Together our results indicate that not only mTORC2 but PKCβII downstream of mTORC2 contributes to the high glucose-induced mesangial cell hypertrophy.

Phosphorylation of PKCβII Ser-660 in kidneys of OVE26 type 1 diabetic mice. Activation of PKCβII in diabetic rat kidney has been reported (28). Our results above show that hydrophobic motif site phosphorylation of PKCβII is nec-
PKCβII regulates mTORC1 and mTORC2. For PKCβII blot, 25 ng of recombinant PKCβII were run in parallel. For C–F, the cell lysates were immunoblotted with phospho-PRAS40 (Thr-246; C), phospho-Akt (Ser-473) and phospho-Akt (Thr-308; D), phospho-S6 kinase (Thr-389; E), and phospho-4EBP-1 (Thr-37/46; F) and with antibodies for indicated proteins. Representative blots from 3 independent experiments are shown for A and C–F. Representative blot from 4 independent experiments is shown for B.

Fig. 4. mTORC2 regulates HG-induced phosphorylation of PKCβII at Ser-660 and mTORC1 activation. Mesangial cells were transfected with 2 independent shRNA against rictor (shRictor 1 and shRictor 2) or vector (A, C, D, E, and F). The cells were incubated with HG as described in Fig. 3 legend. For B, the cells were treated with 25 μM Ly for 1 h before incubation with HG. For A, the cell lysates were immunoprecipitated with PKCβII followed by immunoblotting with phospho-PKCβII (Ser-660) and PKCβII antibodies. The cell lysates were immunoblotted with rictor and actin antibodies. For F, the cell lysates were immunoprecipitated with PKCβII and PKCβII antibody was used to immunoprecipitate mTORC2 followed by immunocomplex kinase assay with the 120 ng of recombinant PKCβII as described in the MATERIALS AND METHODS. For PKCβII blot, 25 ng of recombinant PKCβII were run in parallel. For C–F, the cell lysates were immunoblotted with phospho-PRAS40 (Thr-246; C), phospho-Akt (Ser-473) and phospho-Akt (Thr-308; D), phospho-S6 kinase (Thr-389; E), and phospho-4EBP-1 (Thr-37/46; F) and with antibodies for indicated proteins. Representative blots from 3 independent experiments are shown for A and C–F. Representative blot from 4 independent experiments is shown for B.

necessary for mesangial cell hypertrophy (Fig. 1, J and K). To examine the relevance of our observations in vivo, we used OVE26 mouse model, which displays type 1 diabetes within 3 days after birth (15). The transgenic OVE26 mouse exhibits pathologic features of diabetic nephropathy, including renal hypertrophy, augmented mesangial volume, and matrix expansion (84). We examined phosphorylation of PKCβII at Ser-660 in the renal cortexes of diabetic OVE26 mice. The results show a significant increase in phosphorylation of PKCβII at Ser-660 (Fig. 7, A and B). These results indicate that activation of mTORC2 may phosphorylate PKCβII.

We directly examined the activation of mTORC2 in the renal cortical lysates of the diabetic mice. Rictor antibody was used to immunoprecipitate mTORC2 followed by an immunocomplex kinase assay using recombinant PKCβII protein as substrate in vitro in the presence of ATP. The reaction product was analyzed by immunoblotting with PKCβII phospho-Ser-660 antibody. The results show significantly increased phosphorylation of PKCβII at Ser-660 in the diabetic mice compared with the mTORC2 immunoprecipitates from control mice (Fig. 7, C and D). These results indicate that mTORC2 is activated in the diabetic kidney.

We have shown above that PKCβII promoted phosphorylation of Akt in mesangial cells (Fig. 3, F–H); we tested this phenomenon. Increase in PKCβII phosphorylation was associated with enhanced phosphorylation of Akt at both hydrophobic motif and catalytic loop sites in diabetic renal cortexes (Fig. 7, E and F). Since activation of Akt results in phosphorylation of its substrate PRAS40, we determined its phosphorylation in the diabetic renal cortex. As evident in Fig. 7, G and H, PRAS40 phosphorylation was significantly increased in the diabetic animals. PRAS40 phosphorylation and hence inactivation results in activation of mTORC1, which was associated with mesangial cell hypertrophy (14). Therefore, we determined its activation by measuring phosphorylation of two of its substrates S6 kinase and 4EBP-1 in vivo. Phosphorylation of both S6 kinase and 4EBP-1 was significantly enhanced in the
renal cortex of diabetic animals compared with those in controls (Fig. 7, I–L). These results indicate a possible role of PKCβII hydrophobic motif site phosphorylation in pathology of diabetic nephropathy.

**DISCUSSION**

We conclude that high glucose concentration increases PKCβII hydrophobic motif phosphorylation. Furthermore, our data establish PKCβII as the downstream target of mTORC2 for Akt hydrophobic motif site phosphorylation/activation and induction of mesangial cell hypertrophy. We show a causal effect of mTORC2 and PKCβII on inactivation of PRAS40 and activation of mTORC1 necessary for mesangial cell hypertrophy. Finally, using renal samples from OVE26 type 1 diabetic mice, we provide the first evidence for a direct correlation between PKCβII hydrophobic motif phosphorylation and phosphorylation of Akt, PRAS40, S6 kinase, and 4EBP-1, which may contribute to hyperglycemia-induced renal hypertrophy (Fig. 8).

PKCβII undergoes three phosphorylations, one at the catalytic loop (Thr-500) and two in the COOH-terminal domain at turn motif (Thr-641) and hydrophobic motif (Ser-660) (53, 55). In fact, the catalytic loop site is phosphorylated by PDK-1, which phosphorylates Akt kinase at Thr-308 (40, 71, 74). In the initial studies, the COOH-terminal sites were shown to be autophosphorylated. However, more recent studies show that the turn motif is phosphorylated by mTORC2 in a growth factor-independent manner (16, 27). Similarly, hydrophobic motif phosphorylation of conventional PKCα was shown to be stimulus independent (24). Interestingly, we found that high glucose concentration increased phosphorylation of the PKCβII at the hydrophobic motif site Ser-660 (Fig. 1, A and B).

A previous report demonstrated that in the endothelial cells expression of PKCβ inhibited insulin-induced PI3-kinase activity (38). On the other hand, PI3-kinase increased the expression of PKCβII (58). Moreover, PI3-kinase regulates phosphorylation of the catalytic loop site of many conventional and novel PKC isotypes (40). It occurs via PKD1, which is activated by direct binding of the PI3-kinase product phosphatidylinositol 3,4,5-trisphosphate to its pleckstrin homology domain (74). Although autophosphorylation of hydrophobic motif site of PKCβII was initially reported, we demonstrate that this phosphorylation is PI3-kinase dependent (Figs. 1, C–E, and 4B). We previously demonstrated that PI3-kinase is essential for high glucose-induced hypertrophy of mesangial cells (45). Furthermore, a role of PKCβ has been reported in renal hypertrophy. With our present results using dominant negative
PKCβII as well as siRNAs against the PKCβII isotype, we conclusively demonstrate a specific contribution of this isotype in regulating high glucose-induced protein synthesis and mesangial cell hypertrophy (Fig. 1, F–I). In fact, we for the first time demonstrate that the hydrophobic motif site phosphorylation of PKCβII is necessary for this pathological action of high glucose (Fig. 1, J and K).

We and others have shown that high glucose increases mTOR activity in mesangial cells (14, 43). In cardiomyocytes, activation of mTORC1 is sensitive to inhibition of novel PKC isotypes (52). A more recent report described mTORC1 activation in glioblastoma by PKCα (17). Interestingly, this activation of mTORC1 was shown to be Akt independent. In contrast to these results, in mesangial cells we showed that high glucose-stimulated mTORC1 activity in mesangial cells (14, 43). In cardiomyocytes, activation of mTORC1 is Akt kinase dependent (14). Furthermore, a recent study demonstrated that global PKC inhibition including PKCβII did not have any effect on mTORC1 activity (23). However, in the present study, specific inhibition of PKCβII abundance, expression of dominant negative PKCβII, as well as the nonphosphorylatable mutant PKCβII S660A inhibited high glucose-stimulated mTORC1 activity (Fig. 2). These results demonstrate a role of PKCβII, specifically its hydrophobic motif site phosphorylation, in mTORC1 activation.

PRAS40 is a negative regulatory exclusive component of mTORC1 (62). Initially it was identified as a substrate of Akt kinase in insulin-stimulated cells (20, 36, 83). Recently, it was shown that Akt-mediated phosphorylation of PRAS40 relieves its inhibitory action on mTORC1, resulting in increase in its kinase activity (62). We have recently shown that high glucose-stimulated Akt kinase phosphorylates PRAS40 and activates mTORC1 in mesangial cells (14). However, Akt-independent phosphorylation of PRAS40 has been reported (83). In the context of present study, one possibility is PKC-mediated phosphorylation of PRAS40. In fact a recent report showed activation of mTORC1 by phorbol ester, indicating an apparent role of PKC (20). However, no PRAS40 phosphorylation was detected by phorbol ester (20). In the present study we show that dominant negative PKCβII as well as the nonphosphorylatable mutant PKCβII S660A inhibited high glucose-stimulated PRAS40 phosphorylation (Fig. 3, A–C), consequently suppressing mTORC1 activity (Fig. 2).
Phosphorylation at both catalytic loop site Thr-308 and hydrophobic motif site Ser-473 of Akt is required for its full activation (47). Although PDK1 is the only enzyme, which phosphorylates the catalytic loop site Thr-308, many kinases including Ilk, ATM, MAPKAPK-2, DNA-PK, PAK1, PKC, and mTORC2 phosphorylate Akt Ser-473 in a context-dependent manner (3, 18, 48, 60, 64, 75, 77). Also, autophosphorylation of Akt at Ser-473 was reported (73). In the present study, expression of dominant negative PKCβII or siRNAs targeting PKCβII inhibited high glucose-stimulated phosphorylation of Akt at Ser-473 (Fig. 3, A and C). In fact, our results demonstrate a specific requirement of the hydrophobic motif site phosphorylation of PKCβII for increased phosphorylation of Akt at both catalytic loop (Thr-308) and hydrophobic (Ser-473) sites (Fig. 3H).

Unlike mTORC1, the three proteins rictor, Sin1, and protor contribute to the specificity of mTORC2 kinase activity (80, 87). Using rictor-downregulated HeLa cells, Sarbassov et al. (63) first showed that mTORC2 is responsible for phosphorylation of the hydrophobic motif site of PKCα. Also, murine embryonic fibroblasts (MEFs) isolated from rictor-deficient mouse demonstrated mTORC2 as the principal kinase for PKCα (16, 27). Interestingly, in these cells the endogenous and exogenously expressed PKCα and other conventional PKCs including PKCβII and novel PKC isozymes were degraded (16, 27). Therefore, it is difficult to draw any conclusion about the role of mTORC2 in hydrophobic motif site phosphorylation of PKCβII from these studies. In contrast, in mesangial cells using rictor downregulation, we for the first time show that mTORC2 phosphorylates PKCβII hydrophobic motif Ser-660 (Fig. 4A). Furthermore, in contrast to the previous reports (16, 27), our data demonstrate that reduction of rictor does not have any effect on PKCβII levels in mesangial cells (Fig. 4A). Interestingly, inhibition of PI3-kinase signaling by three independent means significantly blocked this phosphorylation (Fig. 1, C-E). Furthermore, high glucose-induced mTORC2 activity was inhibited by Ly294002 (Fig. 4B). These data are consistent with the recent reports showing that activation of PI3-kinase is necessary for mTORC2 activity (8, 22, 85).

mTORC2 has been considered as the predominant kinase for Akt Ser-473 (64). Our results confirm that high glucose-induced phosphorylation of Akt Ser-473 is mediated by mTORC2 (Fig. 4D). However, in the present study our results conclusively demonstrate that in mesangial cells mTORC2-dependent PKCβII contributes to the phosphorylation of Akt at Ser-473 (Figs. 3, F-H, and Fig. 4, A and D). In rictor null MEFs, which showed mTORC2 inhibition, the inducible phosphorylation of Akt at the catalytic loop site Thr-308 was not
affected (24). In contrast to these results, we found that the Thr-308 phosphorylation of Akt was also dependent on mTORC2 and PKCβII (Figs. 3, F–H, and 4D), suggesting that inhibition of mTORC2 blocks the PDK-1 activity towards Akt Thr-308. To test this, we investigated the activating phosphorylation of PDK-1 under the rictor-downregulated condition. High glucose increased phosphorylation of PDK-1. Downregulation of rictor did not have any effect on PDK-1 phosphorylation (data not shown). Therefore, the inhibition of Akt Thr-308 phosphorylation we observed was not due to inhibition of PDK-1 activity by rictor downregulation. Alternatively, the hydrophobic motif site of AGC kinases, which includes Akt, has been shown to serve as a docking site for PDK-1, which lacks its own hydrophobic motif and catalyzes the phosphorylation of the catalytic loop site such as Thr-308 of Akt kinase (29). Therefore, our results demonstrating lack of Thr-308 phosphorylation under both mTORC2- and PKCβII-inhibited conditions suggest that lack of phosphorylation of Akt Ser-473 may hinder PDK-1 binding and hence inhibits phosphorylation at Thr-308 (Figs. 3, F–H, and 4D).

A role of mTORC1 is established in protein synthesis and hypertrophy of mesangial cells. One mechanism of mTORC1 activation depends on Akt kinase. Differential phosphorylation of Akt substrates has been reported in MEFs lacking rictor. In fact these cells display a significant defect in mTORC2-mediated phosphorylation of Akt at the hydrophobic motif site Ser-473. Insulin-stimulated phosphorylation of GSK3β by Akt was retained while phosphorylation of FoxO3α, another substrate of Akt, was defective (24). PRAS40 exclusively present in the mTORC1 is a direct substrate of Akt. In conjunction with the Akt-mediated phosphorylation defect of FoxO3α, we found defective phosphorylation of PRAS40 in rictor-downregulated mesangial cells (Fig. 4C). As a result, mTORC1 activity was inhibited as judged by the phosphorylations of S6 kinase and 4EBP-1 (Fig. 4, E and F). As mTORC2 regulates phosphorylation of Akt at both Thr-308 and Ser-473, we conclude that full activation of Akt is necessary for PRAS40 phosphorylation by this kinase and mTORC1 activation. Interestingly, we found increased phosphorylation of S6 kinase and 4EBP-1 in rictor-downregulated cells (Fig. 4, E and F, compare lane 3 with lane 1). One hypothesis is that downregulation of rictor results in loss of mTORC2 formation, thus leaving more mTOR kinase to be incorporated in mTORC1, leading to increase in its kinase activity. Therefore, downregulation of rictor increases the basal mTORC1 activity observed in the absence of enhanced phosphorylation of PRAS40 as the latter is upstream of mTORC1 (Fig. 4, C, E, and F). Since mTORC1 regulates protein synthesis and hence hypertrophy, downregulation of rictor blocked high glucose-induced protein synthesis and hypertrophy (Fig. 5, A–D) due to inhibition of mTORC1 activity (Fig. 4, E and F). However, in support of the above hypothesis, due to increased formation of mTORC1 in the presence of shRictor under basal condition, protein synthesis was increased resulting in hypertrophy of mesangial cells (Fig. 5, A and B, compare bar 3 with bar 1). This mode of activation of specific mTOR complex by subunit availability has been postulated recently (46). Although our results conclusively demonstrate the role of Akt and mTORC1 downstream of mTORC2 in high glucose-induced mesangial cell hypertrophy (Fig. 5, C and D), we provide evidence that mTORC2-mediated hydrophobic motif-phosphorylated PKCβII and its kinase activity are necessary for Akt Ser-473 phosphorylation and mesangial cell hypertrophy (Figs. 3, 4, and 6).

In summary, we show a specific role of mTORC2 in high glucose-induced mesangial cell hypertrophy. Our results manifest a specific role of PKCβII hydrophobic motif phosphorylation in activation of Akt and mTORC1, which in turn contributes to mesangial cell hypertrophy. Furthermore, phosphorylation of PKCβII, Akt, and PRAS40 necessary for mTORC1 activation and hypertrophy is confirmed in the renal tissues of type 1 diabetic OVE26 mice (Fig. 7). Chronic use of rapamycin, which blocks both mTORC1 and mTORC2, produces glucose intolerance, insulin resistance, and new-onset diabetes in both rodents and humans (21, 26, 30, 51, 72). A more recent study in mice showed that the effect of rapamycin on glucose intolerance is due to its effect on mTORC2 (39). Our results in the present study posit a significant role of mTORC2 upstream of PKCβII, which regulates Akt-dependent mTORC1 activation for the induction of mesangial cell hypertrophy, a significant complication of diabetic kidney disease. Thus instead of the use of rapamycin, development and use of specific inhibitor of mTORC1 may be beneficial for renal complications of diabetes without having any adverse effects as it would not have any effect on mTORC2.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.D. and M.M.M. performed experiments; F.D., N.G.-C., M.M.M., B.S.K., and G.G.C. interpreted results of experiments; G.G.C. conceived and designed the research; G.G.C. analyzed data; G.G.C. prepared figures; G.G.C. drafted manuscript; G.G.C. edited and revised manuscript.

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