

Dysregulated intracellular signaling impairs CTGF-stimulated responses in human mesangial cells exposed to high extracellular glucose

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Submitted 30 August 2006; accepted in final form 22 February 2007

Furlong F, Crean J, Thornton L, O'Leary R, Murphy M, Martin F. Dysregulated intracellular signaling impairs CTGF-stimulated responses in human mesangial cells exposed to high extracellular glucose. *Am J Physiol Renal Physiol* 292: F1691–F1700, 2007. First published February 27, 2007; doi:10.1152/ajprenal.00342.2006.—High ambient glucose activates intracellular signaling pathways to induce the expression of extracellular matrix and cytokines such as connective tissue growth factor (CTGF). Cell responses to CTGF in already glucose-stressed cells may act to transform the mesangial cell phenotype leading to the development of glomerulosclerosis. We analyzed cell signaling downstream of CTGF in high glucose-stressed mesangial cells to model signaling in the diabetic milieu. The addition of CTGF to primary human mesangial cells activates cell migration which is associated with a PKC- ζ -GSK3 β signaling axis. In high ambient glucose basal PKC- ζ and GSK3 β phosphorylation levels are selectively increased and CTGF-stimulated PKC- ζ and GSK3 β phosphorylation was impaired. These effects were not induced by osmotic changes. CTGF-driven profibrotic cell signaling as determined by p42/44 MAPK and Akt phosphorylation was unaffected by high glucose. Nonresponsiveness of the PKC- ζ -GSK3 β signaling axis suppressed effective remodeling of the microtubule network necessary to support cell migration. However, interestingly the cells remain plastic: modulation of glucose-induced PKC- β activity in human mesangial cells reversed some of the pathological effects of glucose damage in these cells. We show that inhibition of PKC- β with LY379196 and PKC- β siRNA reduced basal PKC- ζ and GSK3 β phosphorylation in human mesangial cells exposed to high glucose. CTGF stimulation under these conditions again resulted in PKC- ζ phosphorylation and human mesangial cell migration. Regulation of PKC- ζ by PKC- β in this instance may establish PKC- ζ as a target for constraining the progression of mesangial cell dysfunction in the pathogenesis of diabetic nephropathy.

GSK3 β ; PKC- ζ ; migration; diabetic nephropathy

DIABETIC NEPHROPATHY (DN) occurs in 25–40% of diabetic patients and is the leading cause of end-stage renal disease. The pathological hallmark of the disease is glomerulosclerosis arising from increased extracellular matrix (ECM) deposition in the mesangium (28). Hyperglycemia is a major stimulus for ECM protein overproduction by mesangial cells in DN (3). Through multiple effector mechanisms involving intermediate glucose metabolism, high glucose modifies target cell signaling and gene expression. Transcription of several cytokines and growth factors is upregulated as a consequence of hyperglycemia in DN, among them, transforming growth factor- β (TGF- β) and connective tissue growth factor (CTGF) which are important mediators of disease progression (32, 38).

CTGF expression becomes upregulated in early DN when mesangial expansion is mild (34). CTGF is a 38-kDa cysteine-rich heparin-binding protein and is a prototypic member of the CCN family of proteins (7). CTGF exhibits diverse biological actions in vitro depending on the cell type, including proliferation and matrix production (8, 10, 35). CTGF promotes cell adhesion and migration in a wide variety of cell types (5, 11, 12). The addition of CTGF to primary mesangial cells induced fibronectin production, cell migration, and cytoskeletal rearrangement (12, 32).

High glucose-induced activation of PKC is a major contributor of mesangial cell dysfunction and contributor to the propagation of diabetic glomerulosclerosis (2, 37). PKC- α , - β , - δ , - ϵ , and - ζ are expressed in the glomeruli of normal and streptozotocin (STZ)-induced diabetic rats (4). Similarly, the expression of these PKC isozymes has been identified in cultured mesangial cells (4). The importance of individual PKC isozyme activation in the diabetic milieu was first identified when the specific PKC- β inhibitor, LY333531, prevented an elevated glomerular filtration rate, increased albumin excretion rate and the overexpression of mRNA for glomerular TGF- β 1 when administered to STZ-diabetic rats (22, 27). High ambient glucose-heightened ERK and PKC δ activity was shown to enhance cellular responsiveness to TGF- β 1 and exacerbated the production of ECM proteins by mesangial cells (20). In addition, glucose-driven PKC- ζ activity was shown to mediate F-actin disassembly and alter mesangial cell contractile responses to endothelin-1 (14). It has become evident that the aberrant activation of the PKC isozymes and other signaling mediators by glucose can alter the response of the mesangial cell to external stimuli. Such potential aberrations in cell signaling by glucose stress may have an important role in the progression of DN (14, 20). In our experimental model, we assessed mesangial cell responses to CTGF in high glucose-stressed cells to study changes in signaling in the diabetic milieu.

METHODS

Cell culture. Primary human mesangial cells (HMCs; Cambrex Bio Science Wokingham) were routinely cultured and maintained as described previously (12, 32). Cells (passage 7–9) were proliferated in MCDB-131 medium supplemented with 10% FCS until the cells had reached 80% confluency. FCS was then removed and the cells were cultured in serum-free medium containing either 5 or 30 mM D-glucose (Sigma) for 48 h or serum-starved for 24 h before glucose treatment. Twenty-five millimolar mannitol was used as an osmotic control.

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Scratch wound assay. The scratch wound assay was performed as described in Ref. 6 on HMCs that were maintained in serum-free medium containing 5 or 30 mM glucose for 48 h. A scratch was applied to the monolayer of cells which were then stimulated with recombinant human CTGF (Lot no. CS 950–29, a gift from Fibrogen, San Francisco, CA; 25 ng/ml) for 24 h. The selective PKC- β inhibitor LY379196 (30 nM; a gift from Eli Lilly and Company) was added 14 h before cell stimulation with CTGF.

Cell migration “chemotaxis” assay. The assay was carried out as described in Ref. 13 on HMCs that were maintained in serum-free medium containing 5 or 30 mM glucose for 48 h.

cdc42 Activity assay. Cells grown to 80% confluency on 100-mm plates were serum-starved and maintained for 48 h in 5 or 30 mM glucose and stimulated with CTGF (25 ng/ml) for 30 min. Cells were pelleted and lysates were prepared using RIPA [50 mM Tris·HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM PMSF, phosphatase inhibitor cocktail II (Sigma) and protease inhibitor cocktail (Sigma) buffer]. Samples were normalized for total protein. cdc42 Activity was determined using the Pierce EZ-detect activation kit (Pierce Biotechnology). The kit uses a GST-fusion protein containing the p21-binding domain (PBD) of human Pak1 to specifically pull-down active cdc42. The pulled-down active cdc42 is detected by Western blot analysis with anti-cdc42 antibody. One milligram of cell protein per sample was used.

Fluorescence microscopy. HMCs were plated subconfluently onto chamber glass slides and allowed to attach in full medium for 24 h. Cells were serum-starved and maintained in 5 or 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 4 h. Cells were fixed in 3.7% paraformaldehyde for 18 min. The cells were permeabilized in 0.1% Triton X-100. Cells were washed twice (10 min) in PBS. Slides were blocked with 5% normal goat serum (NGS; Sigma) for 1 h at room temperature and incubated with primary antibodies, anti-vinculin (1/400) and anti-tubulin (1/300; Sigma) diluted in 5% NGS overnight at 4°C. Slides were incubated with secondary antibody conjugated to FITC (1/400, Molecular Probes) for 20 min at room temperature. Cells were mounted in SlowFade (Molecular Probes). Experiments in the absence of primary antibody (no antibody controls) were performed. Stained cells were visualized with a Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and AxioVision 3.0.6 software (Carl Zeiss).

SDS-PAGE and Western blotting. Whole cell lysates were prepared using RIPA buffer. Samples were normalized for total protein, resuspended in reducing sample buffer, and separated by SDS-PAGE (30). Proteins were then electrophoretically transferred to nitrocellulose membrane (200 V for 90 min) and analyzed with antibodies raised against phospho-GSK3 β (Ser9; 1/1,000), phospho-PKC- ζ (Thr410/403; 1/1,000), p42/44 MAPK (1/1,000), phospho-p42/44 MAPK (Thr202/Tyr204; 1/5,000), phospho-Akt (Ser473; 1/1,000; Cell Signaling Technology), PKC- ζ (1/1,000; Santa Cruz Biotechnology), β -actin (1/10,000), and PKC- β_1 (1/2,500; Sigma). Horseradish peroxidase-conjugated secondary antibodies (DakoCytomation) were used in conjunction with ECL Dura chemiluminescence detection system (Pierce). Densitometry was performed using Scion Image (<http://www.scioncorp.com>) and data are presented as mean fold change \pm SE, relative to unstimulated control values.

PKC- ζ kinase assay. PKC- ζ was immunoprecipitated from fresh RIPA whole cell lysates with 2 μ g of anti-PKC- ζ antibody (Santa Cruz Biotechnology). Immune complexes were captured with protein A sephrose, washed with PBS three times. PKC- ζ kinase assay was performed as outlined in the PKC assay kit (Upstate). To each sample, a specific PKC- ζ peptide substrate (ERMRRPRKRQGSVRRRV), PKA/CaMK inhibitor cocktail, reaction buffer, and lipid activator were added. The reaction was started by adding an Mg²⁺/ATP cocktail containing [γ -³²ATP] (50 μ Ci, \sim 6,000 Ci/mmol) to the samples and were incubated at 30°C for 15 min. The reaction was terminated by removing 25 μ l of the reaction mixture and transferring the aliquot onto the center of a P81 phosphocellulose filter. After the

filters were washed in phosphoric acid, the bound radioactivity on the filters was estimated in a scintillation counter. Background radioactivity was determined by performing a no enzyme control.

siRNA knock-down of PKC- β . Transfection of siRNAs targeted against PKC- β (Dharmacon, SMARTpool reagent) was achieved using lipofectamine 2000 according to the manufacturer’s instructions. Forty nanomolar of each siRNA was incubated with lipofectamine 2000 for 20 min at room temperature and transfected into HMCs in suspension at 37°C for 20 min. Cells were then plated onto chamber slides or six-well plates and maintained in the presence of transfection mix for a further 2 h before changing the medium. Fluorescent siRNA (siGlo, Dharmacon) was cotransfected to allow identification of transfected cells. In the image fields studied, most of the cells fluoresced red which clearly showed that the majority of the cells were transfected with siRNA.

Statistical analyses. All data are presented as means \pm SE for at least three independent experiments. Data analysis was performed using GraphPad Prism software. Statistical significance was evaluated by using a Student’s *t*-test.

RESULTS

HMCs cultured in high extracellular glucose fail to migrate in response to CTGF. To characterize how exposure to high glucose alters cell responsiveness reflective of HMC dysfunction, we measured the motility of these cells in response to CTGF by scratch wound assay (6) and in a cell migration chemotaxis assay. In the scratch wound assay, migration was impaired in HMCs cultured in 30 mM glucose compared with cells cultured for 48 h in 5 mM glucose and subsequently stimulated with CTGF (25 ng/ml) for 24 h (Fig. 1A, *a* and *b*). The number of migrating cells found in the wound decreased by up to 50% in three independent experiments (Fig. 1A, bar chart). This was not due to a change in osmolarity as cells cultured in normal glucose supplemented with 25 mM mannitol migrated in response to CTGF (The online version of this article contains supplemental data; Fig. 1*a*). Platelet-derived growth factor (PDGF) and FCS stimulated HMC migration in high glucose-exposed cells (supplemental data, Fig. 2) and show that the effect of glucose stress on the cells is CTGF stimulus specific. We verified this effect on cell motility using a chemotaxis-type assay. HMCs maintained in either 5 or 30 mM glucose for 48 h were transferred to collagen I-coated Transwell filters and the ability of the cells to migrate through the collagen matrix in response to CTGF was determined. The migration of high glucose-stressed HMCs compared with control cells was assayed 4 h post-CTGF stimulation (Fig. 1B). Results clearly show that the number of migrating cells in cultures exposed to 30 mM glucose was significantly reduced compared with those maintained in 5 mM glucose. Immunocytochemical analysis of the microtubules and focal adhesions in HMCs cultured in either 5 or 30 mM glucose revealed that the microtubule network in HMCs exposed to high extracellular glucose failed to orientate in the direction of movement: the microtubule network in unstimulated cells in 5 mM glucose radiates out from the nucleus and is distributed throughout the cell cytoplasm (Fig. 2A). In 5 mM glucose, stimulation with CTGF caused the microtubules to redistribute in a polarized fashion (Fig. 2B), typical of that associated with migration (17). HMCs exposed to 30 mM glucose for 48 h and treated with CTGF (Fig. 2C) failed to reorganize their microtubules. We conclude that the polarization of the microtubule network in response to CTGF is impaired in HMCs exposed to high

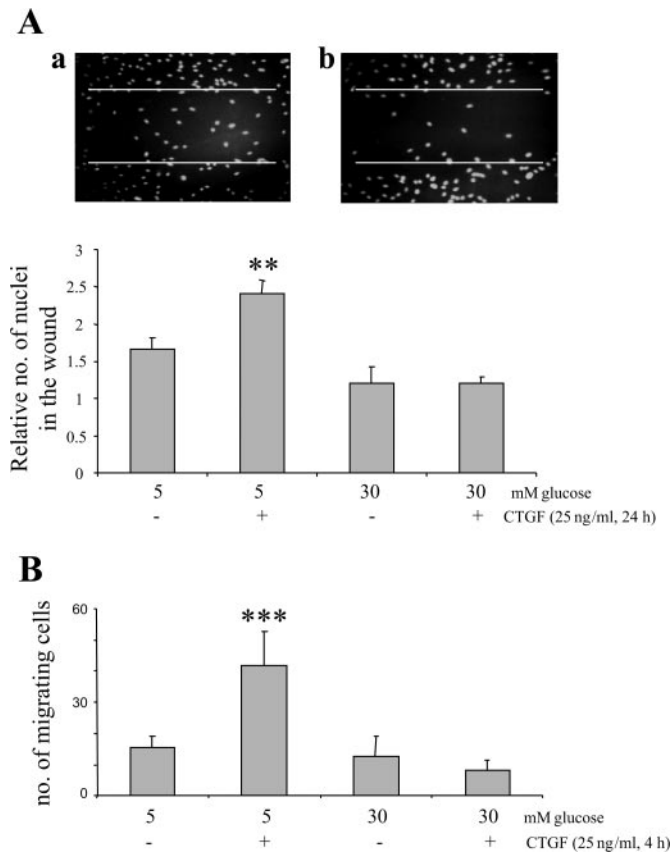


Fig. 1. Mesangial cells exposed to high extracellular glucose fail to migrate or polarize in response to connective tissue growth factor (CTGF). *A*: human mesangial cell (HMC) migration in serum-free medium containing either 5 (*a*) or 30 (*b*) mM glucose for 48 h before treatment and stimulated with CTGF (25 ng/ml) for 24 h under the same conditions of culture. Fixed cells stained with Hoechst 33343 were photographed at $\times 10$ magnification and 10 fields of each sample were photographed. Representative images are shown. The cells in the wound were counted and the relative number of cells that had migrated into the wound was estimated. In the accompanying bar chart, the relative fold increase (means \pm SE) in cells migrating into the wound for cultures exposed to 5 or 30 mM glucose and treated with CTGF is shown ($n = 3$ independent experiments, $**P = 0.003$, bar 2 vs. bar 1). *B*: HMCs cultured in serum-free medium containing either 5 or 30 mM glucose for 48 h were transferred to a Transwell filter and were stimulated with CTGF (25 ng/ml) for 4 h to carry out a modified Boyden chamber assay. The cells that had migrated through the filter were stained with crystal violet and counted. Results were graphed as number of migrating cells (means \pm SE, $n = 3$, $***P = 0.001$, bar 2 vs. bar 1).

extracellular glucose. Similarly, the focal adhesions of migrating cells distribute to the leading edge: vinculin staining of focal adhesions in HMCs cultured in 5 mM glucose and stimulated with CTGF was concentrated at the leading edge of the cell, whereas in unstimulated cells it was distributed across the entire cell surface (Fig. 2, compare *D* and *E*). CTGF failed to elicit this response in HMCs preexposed for 48 h to 30 mM glucose (Fig. 2*F*).

PKC- ζ and GSK3 β phosphorylation in mesangial cells exposed to high glucose. The temporal- and spatial-specific phosphorylation and activation of PKC- ζ with subsequent downstream GSK3 β phosphorylation are required to induce cell polarization that is necessary to support cell migration in HMCs (13, 15). We next sought to investigate the activational status of components of this signaling axis in glucose-stressed mesangial cells. Stimulation of serum-starved HMCs main-

tained in 5 mM glucose with CTGF induced PKC- ζ phosphorylation (on Thr410/403; Fig. 3*A*, lanes 1–4) and associated activation of the kinase (1.75 ± 0.14 -fold increase, $P = 0.01$; Fig. 3*B*). In cells preexposed to high glucose basal levels of phospho-PKC- ζ were elevated, compared with cells cultured in 5 mM glucose and CTGF stimulation did not further increase the level of phosphorylation (Fig. 3*A*, lanes 5–8). This was not due to a change in osmolarity as cells cultured in normal glucose supplemented with 25 mM mannitol demonstrated increased PKC- ζ phosphorylation over basal levels in response to CTGF (supplemental data, Fig. 1*b*). The associated PKC- ζ kinase activity in the HMCs exposed to high extracellular glucose was higher than basal (1.36 ± 0.1 , $P = 0.029$) but unaffected by CTGF (Fig. 3*B*). Similarly, CTGF induced the phosphorylation of GSK3 β on serine 9 under control conditions (5 mM glucose; Fig. 3*C*, lanes 1–4). Again, basal levels of phospho-GSK3 β were elevated in cells preexposed to high glucose (Fig. 3*C*, lanes 5–8) and GSK3 β phosphorylation levels were then unaffected by CTGF treatment. This too was not due to osmolarity changes as cells cultured in normal glucose supplemented with 25 mM mannitol demonstrated increased GSK3 β phosphorylation over basal levels in response to CTGF (supplemental data, Fig. 1*b*). Total PKC- ζ and GSK3 β protein levels were not affected by glucose treatment (supplemental data, Fig. 3). Treatment of HMCs maintained in 5 mM glucose with CTGF resulted in a clear increase in cellular β -catenin (Fig. 3*D*). In contrast, basal levels of β -catenin were elevated in cells preexposed to high glucose and these levels were unchanged by CTGF stimulation (Fig. 3*D*). In migrating cells inactivation of GSK3 β kinase activity by phosphorylation on serine 9 leads to stabilization of β -catenin making it available to support necessary morphological changes (15). Thus preexposure to high ambient glucose induced the sustained phosphorylation of both GSK3 β and PKC- ζ and stimulation of these cells with CTGF failed to further increase the phosphorylation of either protein or induce any of the migration-associated intracellular events, e.g., increases in cellular β -catenin.

The dysregulation of cell migration in cells exposed to high ambient glucose may occur due to a global loss of cellular responsiveness to CTGF. However, CTGF-treated HMCs also demonstrate a rapid and transient phosphorylation of p42/44 MAPK and Akt in mediating cellular responses (12). Phosphorylation of these proteins was not affected by preexposure to high glucose (Fig. 3*E*). Total p42/44 MAPK and Akt protein levels were also unchanged by glucose (supplemental data, Fig. 3). This suggests that the high glucose-exposed cells still respond to CTGF. The loss of the migration response is most likely caused by selective glucose-induced imbalance in the phosphorylation/activity of the PKC- ζ -GSK3 β axis and a loss in the ability of CTGF to activate this signaling pathway. Phosphorylation of p42/44 MAPK and Akt downstream of CTGF is profibrotic and leads to fibronectin production in HMCs (12). This would therefore suggest that activation of the profibrotic response to CTGF persists in HMCs exposed to high glucose.

CTGF-mediated activation of *cdc42* in high extracellular glucose. *cdc42*-dependent PKC- ζ -catalyzed phosphorylation of GSK3 β on serine 9 occurs specifically at the leading edge of migrating cells (15). We measured the activity of *cdc42* by a pull-down assay in cells precultured in 5 and 30 mM glucose

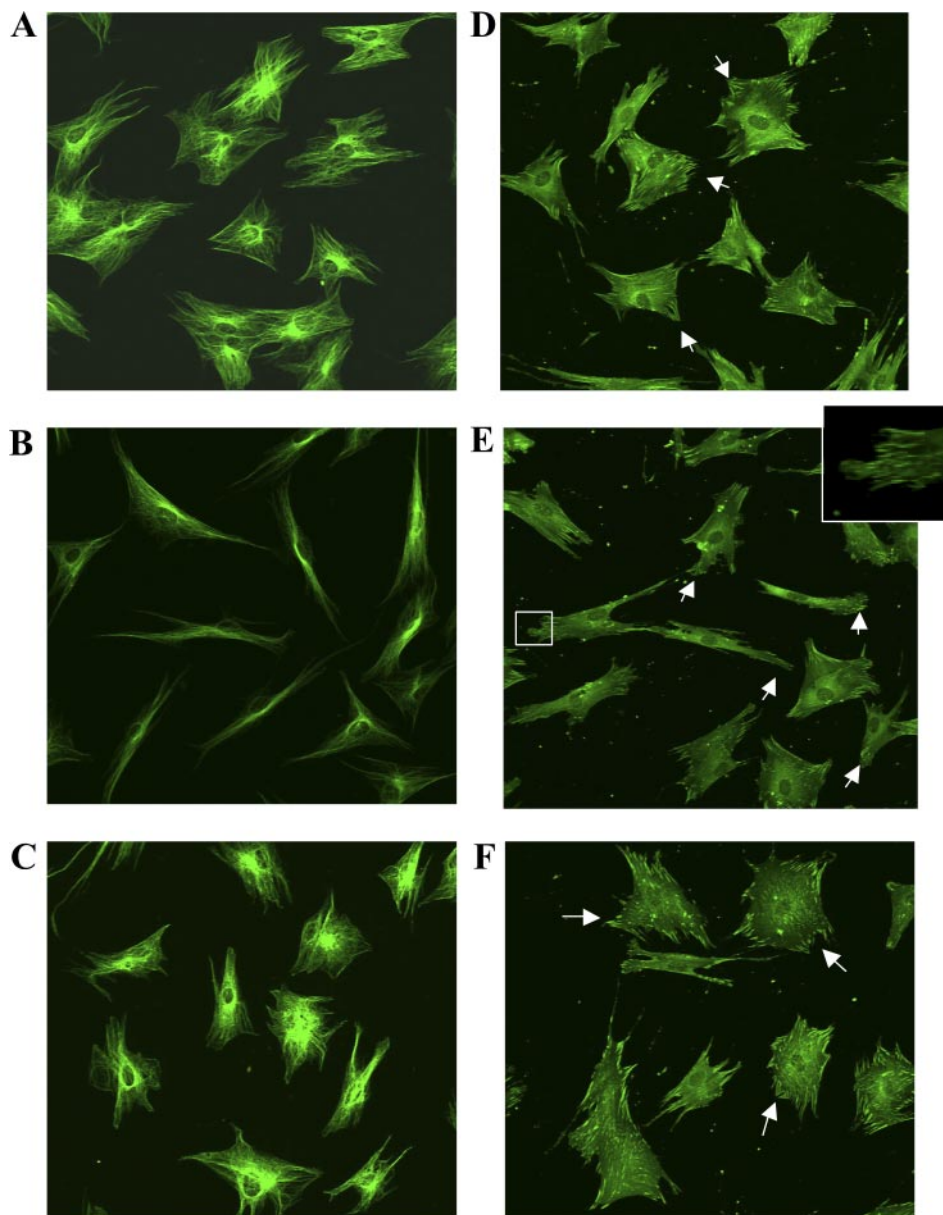


Fig. 2. Mesangial cells exposed to high extracellular glucose fail to adopt a motile-like morphology. HMCs cultured subconfluently in serum-free medium containing 5 or 30 mM glucose for 48 h and then exposed to CTGF (25 ng/ml) for 4 h were fixed and stained with anti-tubulin (A–C) and anti-vinculin (D–F) antibodies and appropriate FITC-conjugated secondary antibodies. Stained cells were visualized with an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and AxioVision 3.0.6 software (Carl Zeiss, Thornwood, NY); $\times 20$ magnification. A and D: 5 mM glucose-untreated cells. B and E: 5 mM glucose/CTGF-treated cells. C and F: 30 mM glucose/CTGF-treated cells. A and C: cells with an unpolarized microtubule morphology. B: cells with a polarized microtubule morphology. White arrows indicate long adhesive focal adhesions (D and F) and de novo focal adhesion formation (E). Images presented are a representative of images from 5 independent experiments.

and treated with CTGF. Exposure of control cells (5 mM glucose) to CTGF for 30 min induced activity above basal levels (Fig. 4, lanes 1–2). However, we found that preexposure to high glucose-impaired CTGF induced increases in cdc42 activation (Fig. 4, lanes 3–4). Total cdc42 protein levels were unchanged by exposure to glucose (supplemental data, Fig. 3).

Acute exposure to high extracellular glucose can elicit sustained phosphorylation of GSK3 β and PKC- ζ . We established that exposure to 30 mM glucose for 48 h drives the sustained phosphorylation of PKC- ζ and GSK3 β . Chronic activation of these signaling moieties renders the cells unresponsive to CTGF-induced cell polarization and migration. We proceeded to investigate how elevated glucose levels stimulate the phosphorylation of GSK3 β and PKC- ζ . In a time course of exposure to 30 mM glucose, we show by Western blot that glucose rapidly activates and sustains GSK3 β and PKC- ζ phosphorylation in HMCs (Fig. 5A). We continued to use this experimental strategy [acute exposure (6 h) of HMCs to high

glucose] to identify upstream regulators of the PKC- ζ -GSK3 β axis whose inhibition might relieve their sustained phosphorylation in HMCs exposed to high glucose for the prolonged period (48 h). Phosphorylation of GSK3 β by PKC- ζ was demonstrated in other model systems (33) and we also show (Fig. 5B) that HMCs exposed to 30 mM glucose (6 h) resulted in clear elevation of phospho-[ser9]-GSK3 β and that this increase is blocked with a specific myristoylated PKC- ζ pseudosubstrate inhibitor. However, we previously showed that inhibition of PKC- ζ inhibited CTGF-induced HMC migration (13). This would limit PKC- ζ inhibition as a strategy for reversing the phenotype.

PKC- β has been implicated as the key PKC isozyme responsible for high glucose-induced cell damage (2, 37). Inhibition of PKC- β with Ruboxistaurin (LY333531) can reverse the effects of glucose and resolve fibrosis in a number of cell models of hyperglycemia and animal models of DN (1, 27). We were therefore interested in investigating the possibility

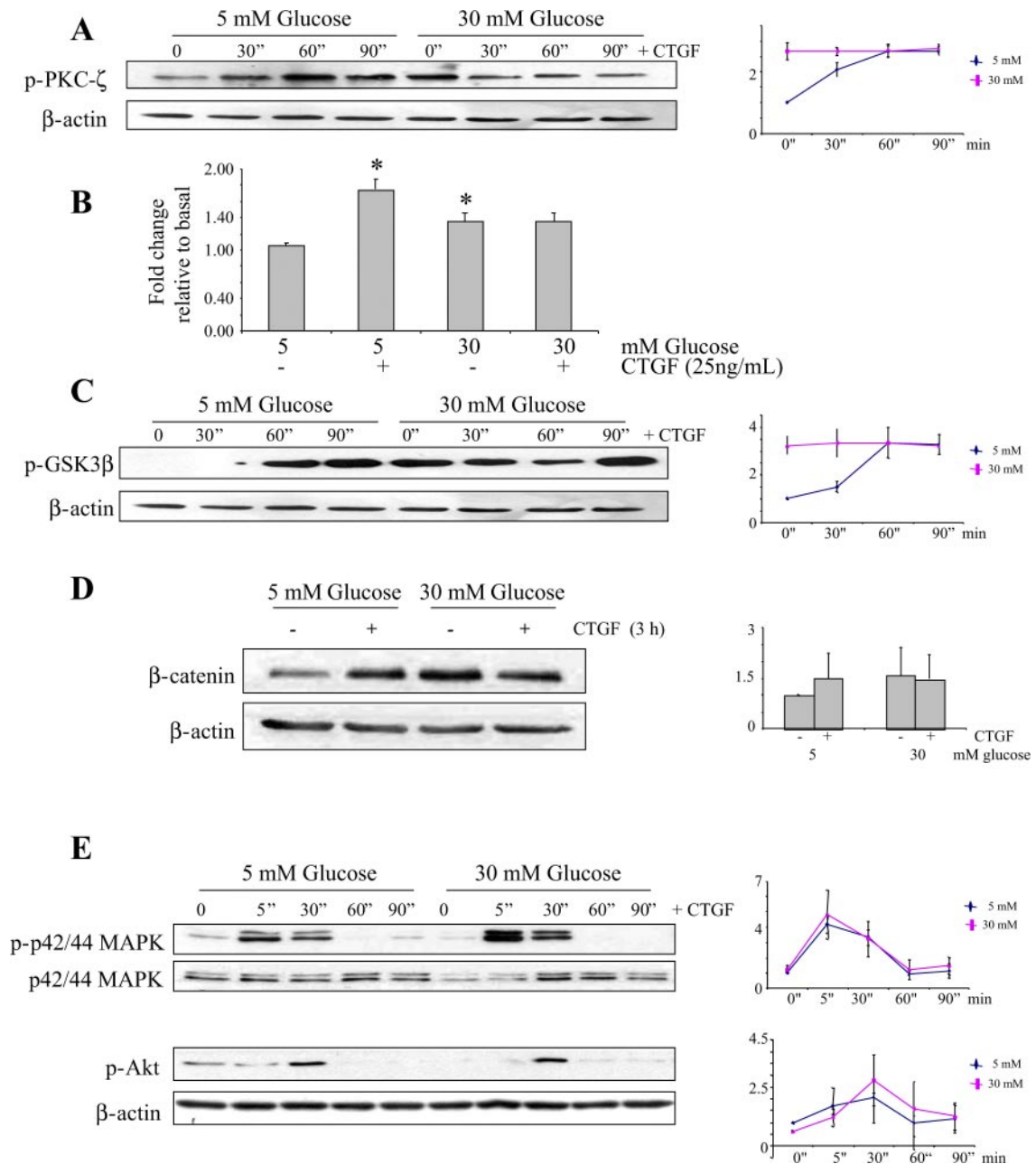


Fig. 3. Chronic exposure to high glucose causes sustained phosphorylation of PKC- ζ and GSK3 β in HMCs; CTGF fails to further increase phosphorylation of these proteins under these conditions. **A**: Western blot analyses for phospho-PKC- ζ (Thr410/403) and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium containing 5 or 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 0–90 min. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$).] **B**: PKC- ζ kinase activity in RIPA lysates prepared from HMCs cultured as in **A**. RIPA lysates prepared after cell lysis were normalized for protein concentration and were immune-precipitated with anti-PKC- ζ antibody. Samples were then analyzed for PKC activity using a PKC assay kit containing a specific substrate peptide for PKC- ζ . Then, 32 P incorporated into substrate for each sample was separated from the residual 32 P[ATP] using p81 phosphocellulose paper and measured by scintillation counting (means \pm SE, $n = 4$, * $P < 0.05$, bar 2 vs. bar 1, bar 3 vs. bar 1). **C**: Western blot analyses for phospho-GSK3 β (Ser9) and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium containing 5 or 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 0–90 min. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$).] **D**: Western blot analyses for β -catenin and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium containing 5 or 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 3 h. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$).] **E**: Western blot analyses for phospho-p42/44 MAP kinase (Thr202/Tyr204), total p42/44 MAP kinase, phospho-Akt (Ser473), and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium containing 5 or 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 0–90 min. [Alterations in phosphorylation levels were measured semiquantitatively by using densitometric analysis ($n = 3$).]

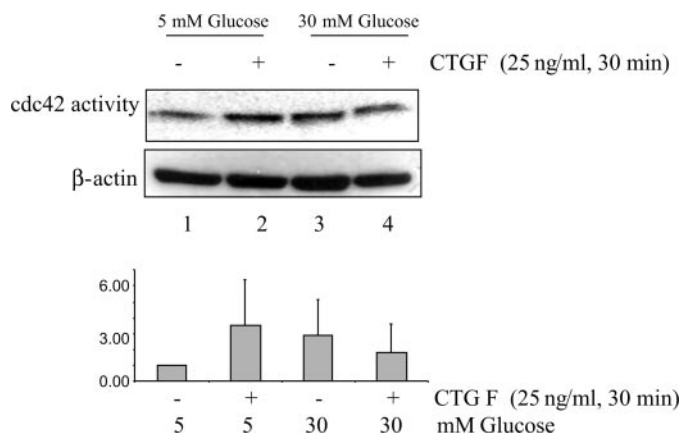
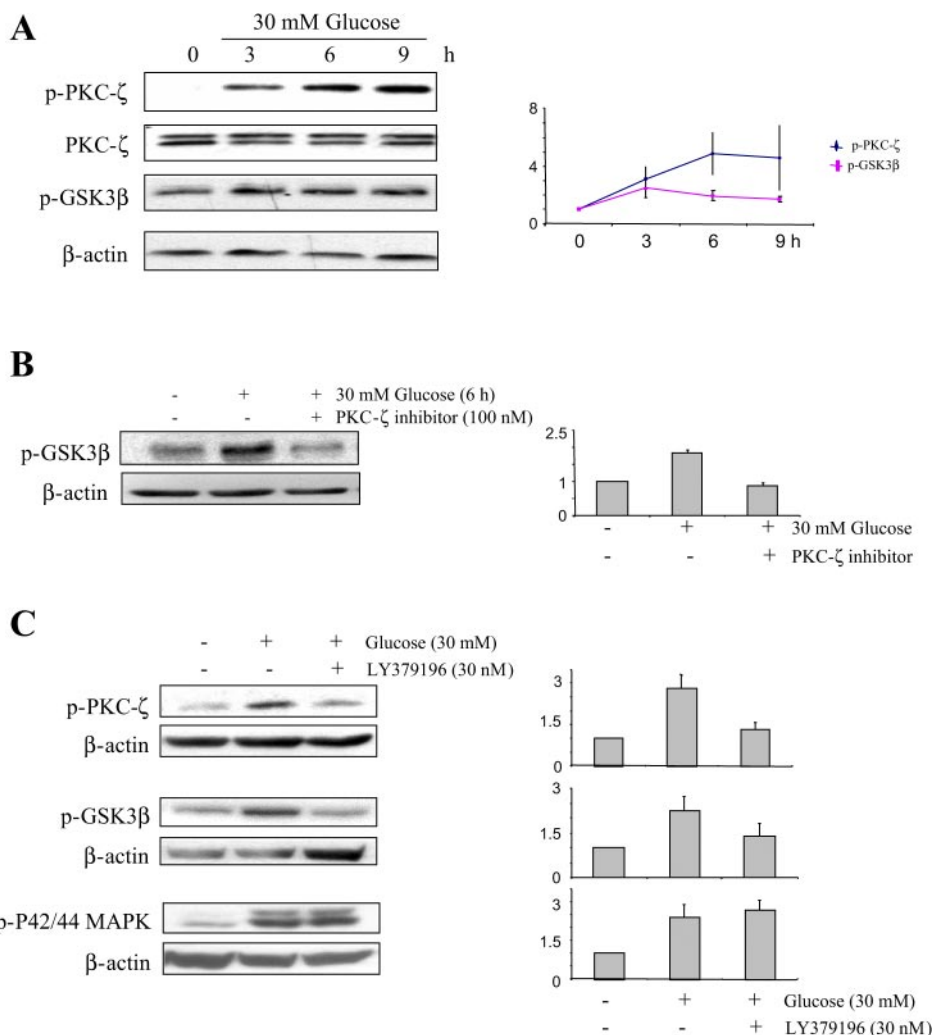


Fig. 4. Activation of cdc42 by CTGF is compromised in cells exposed to high extracellular glucose. cdc42 activity in RIPA lysates prepared from HMCs cultured in serum-free medium containing either 5 or 30 mM glucose for 48 h and then stimulated with CTGF for 30 min. RIPA lysates were normalized for protein concentration and cdc42 activity was determined by an activated Pak-1 pull-down assay (see METHODS). [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 4$.)]

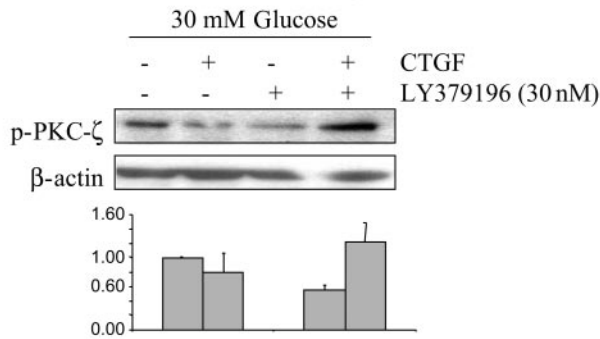
that PKC- β inhibition might protect from the pathological effects of high glucose in our model. We tested PKC- β inhibition with LY379196, an analog of LY333531 and specific PKC- β inhibitor (9, 31, 36), in our model (Fig. 5C). Glucose-induced PKC- ζ and GSK-3 β phosphorylation was reduced in the presence of the PKC- β inhibitor while glucose-induced p42/44 MAP kinase phosphorylation occurred independently of PKC- β . Phosphorylation of p42/44 MAP kinase in high glucose has been shown to be regulated by PKC- δ (21) and our data would suggest that LY379196 is not inhibiting such other PKC isozymes. Decreased PKC- ζ phosphorylation in cultures exposed to high glucose and pretreated with LY379196 was not due to effects of LY379196 on total GSK3 β and PKC- ζ protein levels (data not shown). These data demonstrate that PKC- β inhibition can reduce glucose-induced PKC- ζ and GSK3 β phosphorylation (Fig. 5C).

If inappropriate phosphorylation of PKC- ζ contributes to glucose-induced HMC dysfunction that leads to loss of HMC motility, then lowering phospho-PKC- ζ levels by modulating PKC- β activity should reverse the inhibition of CTGF-induced HMC migration. We first showed that PKC- β inhibition reduced PKC- ζ phosphorylation in HMCs exposed to high glucose for 48 h (Fig. 6A, compare lane 1 and 3). Furthermore, CTGF stimulation of HMCs exposed to high glucose and

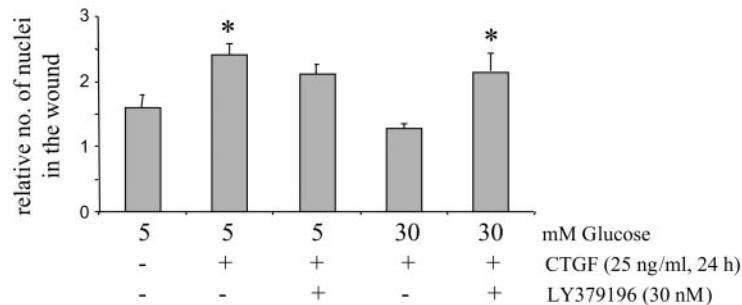
Fig. 5. Acute exposure to high extracellular glucose alone can elicit the sustained phosphorylation of GSK3 β and PKC- ζ . A: Western blot analyses for phospho-PKC- ζ (Thr410/403), total PKC- ζ , phospho-GSK3 β (Ser9), and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium for 24 h and then exposed to glucose (30 mM) for 0–9 h. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$.)] B: effect of the myristoylated PKC- ζ pseudosubstrate inhibitor on high glucose-induced GSK3 β phosphorylation. Western blot analyses for phospho-GSK3 β (Ser9) and β -actin on RIPA lysates prepared from HMCs cultured in serum-free medium for 24 h and then exposed to glucose (30 mM) for 6 h. Cells were pretreated with the PKC- ζ pseudosubstrate inhibitor (100 nM) 1 h before glucose stimulation. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$.)] C: effect of PKC- β inhibition on high glucose induced phospho-PKC- ζ (Thr410/403), phospho-GSK3 β (Ser9), and phospho-p42/44 MAP kinase (Thr202/Tyr204). Western blot analyses of HMCs serum starved for 24 h and then treated with 30 mM glucose for 6 h. HMCs were pretreated with LY379196 (30 nM) 1 h before CTGF and glucose exposure. β -Actin Western blot was used as a loading control. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$.)]



A



B



C

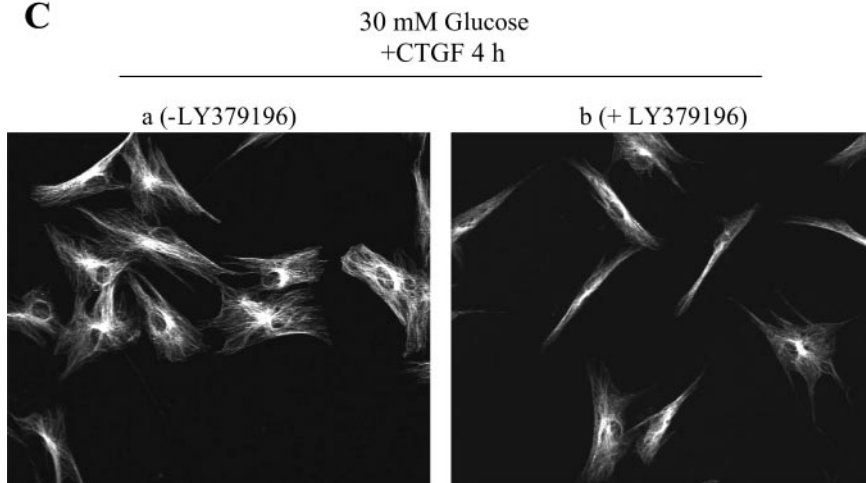


Fig. 6. Modulation of PKC- β signal transduction restores CTGF-induced HMC migration in glucose-treated cells. **A:** Western blot analyses for phospho-PKC- ζ (Thr410/403) on RIPA lysates prepared from HMCs cultured in serum-free medium containing 30 mM glucose for 48 h and then stimulated with CTGF (25 ng/ml) for 60 min. HMCs were incubated with LY379196 14 h before CTGF stimulation. β -Actin Western blot was used as a loading control. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$).] **B:** HMCs were cultured in serum-free medium containing either 5 or 30 mM glucose for 48 h before treatment. LY379196 (30 nM) was added 14 h before treatment. A scratch was then applied to the monolayer of cells and the cells were exposed to CTGF (25 ng/ml) for 24 h under the same conditions of culture. Fixed cells stained with Hoechst 33432 were photographed at $\times 10$ magnification and 10 fields of each sample were taken. The cells in the wound were counted and the relative number of cells that had migrated into the wound was estimated. Results were graphed as relative number of migrating cells (means \pm SE, $n = 3$ independent experiments, $*P < 0.05$, bar 2 vs. 1, bar 5 vs. 4). **C:** HMCs were cultured subconfluently in serum-free medium containing 5 or 30 mM glucose for 48 h and then exposed to CTGF (25 ng/ml) for 4 h. The cells were fixed in 3.7% paraformaldehyde and stained with anti-tubulin antibody. HMCs were incubated with LY379196 14 h before CTGF stimulation. Stained cells were visualized with an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and AxioVision 3.0.6 software (Carl Zeiss); $\times 20$ magnification. Images presented are representative of 3 independent experiments.

treated with LY379196 now resulted in induction of PKC- ζ phosphorylation (Fig. 6A, lane 4). We next tested the effect of PKC- β inhibition on CTGF-induced HMC migration by scratch wound assay. CTGF-induced migration in HMCs cultured in 5 mM glucose was reduced but not significantly by PKC- β inhibition (Fig. 6B). However, treating high-glucose-exposed HMCs with LY379196 resulted in a significant increase in the number of HMCs migrating into the wound in response to CTGF ($P < 0.05$). PKC- β inhibition in cultures exposed to 30 mM glucose also now permitted microtubule polarization in response to CTGF (Fig. 6C, compare *b* with *a*). Last, we independently verified that modulation of PKC- β activity in high glucose-exposed HMCs could re-establish the motile phenotype in glucose-stressed HMCs. In these experi-

ments, we used siRNA directed against PKC- β . We successfully reduced PKC- β protein levels in cells transfected with siRNA directed against PKC- β as demonstrated by Western blot (Fig. 7, top). We analyzed the ability of HMCs cultured in 30 mM glucose to polarize their microtubule network in response to CTGF after PKC- β knockdown with siRNA. HMCs were cotransfected with fluorescent siRNA (siGlo) and specific PKC- β siRNA. The microtubules of siGlo-transfected cells were then analyzed. Again, we could show that reduction of PKC- β levels with siRNA in HMCs exposed to high glucose resulted in CTGF-induced polarization-associated microtubule reorientation (Fig. 7, compare *a* with *b*). The data presented demonstrate that high ambient glucose inappropriately drives the phosphorylation of PKC- ζ and impairs HMC motility in

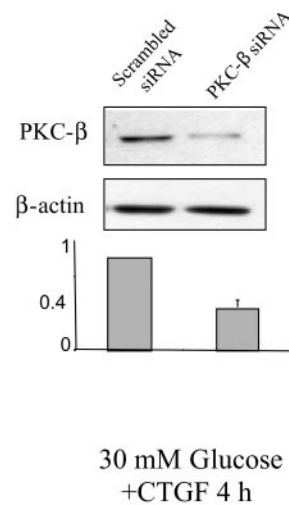
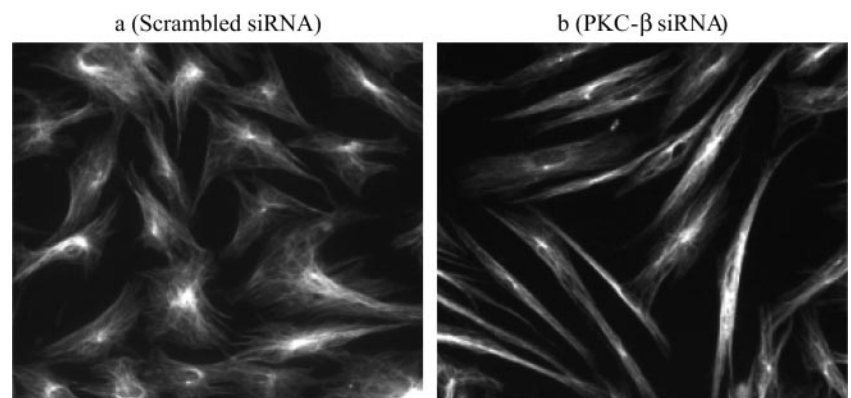


Fig. 7. Modulation of PKC- β signal transduction with PKC- β siRNA in mesangial cells exposed to high glucose. *Top*: PKC- β Western blot analysis on RIPA lysates from HMCs transfected with either scrambled or PKC- β siRNA and maintained in growth medium for 48 h. [Alterations in phosphorylation levels were measured semiquantitatively using densitometric analysis (shown are mean fold change \pm SE relative to unstimulated control values, $n = 3$).] *Bottom*: immunocytochemical analysis of HMCs transfected with siGlo or cotransfected with siGlo and PKC- β siRNA and maintained in serum-free medium containing 30 mM glucose for 48 h before stimulation with CTGF for 4 h. The cells were fixed in 3.7% paraformaldehyde and stained with anti-tubulin antibody. Stained cells were visualized with an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and AxioVision 3.0.6 software (Carl Zeiss); $\times 20$ magnification. Images presented are representative of 3 independent experiments.



response to CTGF. By constraining glucose stress on HMCs by PKC- β inhibition PKC- ζ was resensitized. This restored sensitivity to CTGF and enabled CTGF-induced HMC migration. Overexpression of PKC- β in HMCs cultured in 5 mM glucose did not induce this pathological response (data not shown). This suggests that there is a very tight regulation of PKC- β activation in the mesangial cell and PKC- β activation by high glucose stress is core to its deleterious effects.

DISCUSSION

Despite recognition that hyperglycemia is the major cause of DN, the mechanisms by which high ambient glucose leads to deleterious changes in diabetic kidneys are not understood. A number of functions of CTGF have been studied in mesangial cells cultured in normo-glucose; however, in the diabetic milieu, the mesangial cell phenotype is altered by high ambient glucose and understanding the regulation of intracellular signaling networks in this context is necessary. In this study we examined intracellular signaling events associated with mesangial cell dysfunction caused by exposure to high glucose levels. We identified that glucose-stressed mesangial cells are no longer capable of migration in response to CTGF stimulation. It would appear that dysregulated intracellular signaling caused by exposure to high glucose results in the sustained activation of components of a intracellular signaling axis required for cell polarization.

In 5 mM glucose, CTGF regulates HMC migration through facilitative actin and microtubule cytoskeleton rearrangement,

which is mediated by activation of cdc42 and phosphorylation of PKC- ζ and GSK3 β (13). In our analysis, we found that high glucose exposure alters the levels of PKC- ζ -GSK3 β signaling axis activity downstream of cdc42 activation. Importantly, precise spatial and temporal modulation of Rho GTPases is required to control the actin cytoskeletal and microtubule network changes that are necessary to bring about the complex gross cellular changes that regulate events such as cell migration. For example, constitutive activation or dominant negative inhibition of cdc42 abolished astrocyte cell migration (16, 40). The activation state of cdc42, PKC- ζ , and GSK3 β is inappropriately stimulated by high glucose and has inhibited their further selective stimulation by CTGF in our model. The altered responses of high glucose-exposed mesangial cells to growth factors have already been shown to contribute to mesangial cell dysfunction (14, 20, 21).

Acute high glucose exposure triggered increases in phospho-GSK3 β and phospho-PKC- ζ levels which were sustained on prolonged treatment leading to the HMCs becoming refractory to further stimulation by CTGF. In normal cells, CTGF stimulates PKC- ζ -dependent GSK3 β phosphorylation (13). Loss of CTGF responsiveness in HMCs exposed to high extracellular glucose could occur due to the endogenous overproduction of CTGF by the cells (32). However, CTGF-treated HMCs demonstrated a rapid and transient phosphorylation of p42/44 MAPK, and Akt in both 5 or 30 mM glucose making this unlikely. Loss of CTGF-induced PKC- ζ and GSK3 β phosphorylation is not due to a global loss in cellular responsiveness to

CTGF. Interestingly, CTGF-driven p42/44 MAPK and Akt phosphorylation are required for fibronectin production in HMCs (12). Such mesangial ECM overproduction is the hallmark of glomerulosclerosis (28). Activation of p42/44 MAPK and Akt phosphorylation by CTGF in high glucose suggest that activation of this profibrotic response to CTGF persists in HMCs exposed to high glucose stress.

Recent studies have revealed that PKC- ζ activation is responsible for F-actin disassembly in glucose-stressed mesangial cells (14, 29). Modulation of this PKC isozyme may be of therapeutic value in resolving mesangial cell dysfunction. However, the precise mechanism of high glucose-induced PKC- ζ activity is not known. Entry of glucose into intermediate metabolic pathways stimulates the activation of PKC isozymes directly and indirectly. De novo synthesized diacylglycerol (DAG) may directly activate conventional and novel PKC isozymes. Phosphatidic acid (PA), a precursor of DAG, was reported to activate PKC- ζ (23). Generation of reactive oxygen species (ROS) which also occurs due to excess glucose metabolism by the cells was also shown to activate the PKC isozymes (18, 19). More specifically, Kwan et al. (29) showed that in high glucose ROS generation was dependent on PKC- ζ activation and that conversely, PKC- ζ activation was dependent on ROS generation. These experiments suggest that a positive feedback loop occurs between ROS and the activation of PKC- ζ in a high glucose milieu (29). Moreover, generation of ROS arising from oxidative stress was shown to be enhanced in diabetic glomeruli due to a PKC- β -dependent activation of NADPH oxidase (25). Here, we show a novel link between high glucose-induced PKC- β activation and PKC- ζ activation. Whether it is direct or indirect or ROS dependent requires further study.

Studies in a range of experimental models have implicated PKC- β as the key PKC isozyme responsible for high glucose-induced cell damage (1, 27). Ruboxistaurin (LY333531) has been evaluated in a number of animal models of DN (22, 24, 26, 27) and Ruboxistaurin is currently being assessed in studies on humans with diabetic kidney disease (39). We analyzed the effects of PKC- β inhibition on cell signaling downstream of CTGF and CTGF-induced migration in HMCs preexposed to high glucose and treated with either the specific PKC- β inhibitor LY379196 or PKC- β siRNA. Modulation of PKC- β signal transduction in HMCs cultured in high extracellular glucose restored CTGF-induced PKC- ζ phosphorylation and HMC migration. Regulation of PKC- ζ by PKC- β in this instance may establish PKC- ζ as a target for constraining the progression of mesangial cell dysfunction in the pathogenesis of DN.

ACKNOWLEDGMENTS

We acknowledge the support of the Health Research Board, Ireland, the Wellcome Trust and Science Foundation Ireland. The authors also acknowledge Fibrogen for the gift of rh CTGF and Eli Lilly and Company for the gift of LY379196.

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