Mesangial cell filamentous actin disassembly and hypocontractility in high glucose are mediated by PKC-ζ

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ENDOTHELIN-1 (ET-1) is a potent vasoconstrictor that is synthesized by glomerular endothelial cells (33), epi-

thelial cells (45), and mesangial cells (40). ET-1-stimu-

lated mesangial cell contraction can regulate glomerular capillary surface area and filtration rate (3). However, mesangial cells cultured in high glucose (8, 17) and glomeruli isolated from streptozotocin (STZ)-diabetic rats fail to contract in response to ANG II, (24), ET-1, or raised intracellular Ca^{2+} (17). In dia-

betics, high glucose-induced loss of preglomerular affer-

ent arteriolar contractile response to vasoconstrictors leads to elevated intraglomerular pressure, accelerat-

ing the progression of glomerulosclerosis (58).

The precise mechanisms by which high glucose alters the phenotype of mesangial cell and vascular smooth muscle cell (VSMC) responsiveness are not known. Reduced Ca^{2+} signaling mediated by either high glucose-induced protein kinase C (PKC) inhibition of inositol 1,4,5-trisphosphate (IP_3) release (34) or via high glucose-induced inhibition of receptor-operated channels (35) have been proposed. Work from our laboratory has demonstrated that isolated glomeruli and mesangial cells cultured in 25 mM (high) glucose display normal Ca^{2+} signaling to ET-1, ANG II, and arginine vasopressin (AVP) as measured by ^{45}Ca^{2+} efflux and fura 2 spectrofluorimetry, respectively (53). The normal IP_3 response observed in high glucose is attributed to upregulation of myo-inositol transport, which prevents mesangial cell myo-inositol deple-

tion (4).

The initial Ca^{2+} transient induced by vasoconstric-
tor peptides in VSMCs and mesangial cells is accom-
panied by Ca^{2+}/calmodulin (CaM)-dependent activa-
tion of myosin light chain kinase (MLCK) and the phosphorylation of the regulatory 20-kDa myosin light chain (MLC20) at serine-19 and threonine-18. This leads to the activation of the actin-activated myosin ATPase, interaction of filamentous (F)-actin and myo-
sin, and cellular contraction (42, 46). PKC, a ubiqui-
tous serine-threonine protein kinase, is also implicated in the regulation of contraction in both smooth muscle and mesangial cells (8, 9, 50, 52). Activation of mesan-
gial cell PKC isozymes by hyperglycemia in diabetes represents an important pathway potentially contrib-

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PKC can directly phosphorylate MLC\textsubscript{20} at serine-1, serine-2, and threonine-9 without activating contraction (52) and indirectly stimulates MLC\textsubscript{20} phosphorylation at serine-19 and threonine-18 by inhibiting myosin light chain phosphatase (MLC-PP). PKC can also stimulate contraction, independent of MLC\textsubscript{20} phosphorylation, by phosphorylating the actin regulatory proteins calponin and caldesmon (15, 16).

Although we have previously shown that ET-1-stimulated mesangial cell contraction involves the activation of PKC-\(\zeta\), \(-\epsilon\), and, to a lesser extent, \(-\alpha\) in normal glucose (9), little is known about PKC isozyme regulation of mesangial cell MLC\textsubscript{20} phosphorylation. In high glucose, mesangial cell PKC isozyme expression patterns, subcellular distribution, and activity are altered (1, 2, 20, 21, 23, 55). In mesangial cells, Kikkawa et al. (23) and, recently, Amiri and Garcia (1) demonstrated membrane translocation of PKC-\(\zeta\) and \(-\alpha\) after 72 and 120 h of high glucose. Therefore, high glucose-induced activation of selective PKC isozymes that mediate cytoskeletal restructuring may be implicated in the mechanism of reduced contractility. We have observed that mesangial cells in high glucose for 48 h demonstrate F-actin disassembly, which is reversed by inhibition of the polyol pathway and appears to be mediated through a PKC-dependent mechanism (61). Loss of mesangial cell contractility to ET-1 is also restored with aldose reductase inhibition (8).

In this study we postulated that high glucose may alter mesangial cell Ca\textsuperscript{2+} signaling, MLC\textsubscript{20} phosphorylation, or PKC isozyme activity, mediating cytoskeletal dysfunction and hypocontractility. We examined the effects of high glucose on ET-1-stimulated mesangial cell planar area reduction (contraction), Ca\textsuperscript{2+} signaling, MLC\textsubscript{20} phosphorylation, PKC isozyme distribution, and F-actin disassembly. The use of a myristoylated PKC-\(\zeta\) peptide inhibitor (myr-RRGAR-RWRK; ZI) was used to determine the role of the PKC-\(\zeta\) isozyme in the regulation of contraction and F-actin assembly in high glucose.

**Materials and Methods**

**Materials**

DMEM, penicillin, streptomycin, and tryptophin were purchased from Gibco BRL Life Technologies (Burlington, ON). Fetal bovine serum (FBS) was purchased from Wisent (St. Bruno, PQ). 12 Phorbol-13-myristate (PMA), ET-1, leupeptin, pepstatin A, aprotinin, benzamidine, Tween 20, so-
dium orthovanadate, dithiothreitol (DTT), and polyclonal anti-PKC-\(\alpha\), \(-\delta\), \(-\epsilon\), and \(-\gamma\) antibodies were purchased from Sigma (St. Louis, MO). Polyclonal anti-PKC-\(\beta\) and \(-\zeta\) antibodies and protein G-Sepharose were from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine-phalloidin and FITC-DNase 1 were from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-labeled goat anti-rabbit and goat anti-mouse secondary antibodies were from Bio-Rad (Hercules, CA) and Jackson Immunoresearch Laboratories (West Grove, PA), respectively. Polyclonal anti-MLC\textsubscript{20} antibody and a monoclonal anti-phospho-Ser\textsuperscript{19}MLC\textsubscript{20} antibody were kindly supplied by Drs. Subah Packer (Indiana University, IN) and Yasuharu Sasaki, (Asahi Chemical, Shizuoka, Japan), respectively. Dr. Michael P. Walsh (University of Calgary, Calgary, AB) generously provided purified purified chicken gizzard MLC\textsubscript{20}. The myristoylated PKC-\(\zeta\) inhibitor ZI was synthesized by the Hospital for Sick Children Peptide Synthesis Laboratory (Toronto, ON). The purity of the peptide was >95% as determined by high-pressure liquid chromatography and mass spectroscopy. All other chemicals were of analytic or electrophoresis grade and purchased from BDH (Toronto, ON).

**Mesangial Cell Planar Area Measurements**

Mesangial cells (passages T5–T10) were cultured as previously described (18) in 20% FBS-DMEM in 5.6 mM (normal) glucose, pH 7.4, supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10 mM HEPES.

Mesangial cells (100,000 cells/dish) were directly growth arrested for 24 h on 35-mm plastic dishes (Falcon, Becton-Dickinson Laboratories, Lincoln Park, NJ) in either 2 ml 0.5% FBS-DMEM with 5.6 or 30 mM (high) glucose. The dishes were transferred to the heated stage of an inverted phase-contrast light microscope (Bausch and Lomb, New York, NY) and maintained in 5% CO\textsubscript{2} in air at 30°C. The images were captured by a Hitachi KP-113 solid-state television camera (Hitachi Denshi, Tokyo, Japan). After visualization, the image was digitized to represent time 0. To downregulate PKC in normal glucose, cells were pretreated for 24 h with 0.1 \(\mu\)M PMA. To inhibit PKC-\(\zeta\), cells were pretreated for 24 h with 10 \(\mu\)M of ZI. This peptide has been shown to inhibit PKC-\(\zeta\) activity in Xenopus laevis oocytes (10) and in pancreatic islet \(\beta\)-cells (47). ET-1 was added directly to the medium to achieve a final concentration of 0.1 \(\mu\)M for cells in normal or high glucose in the absence or presence of ZI. To control for osmolarity, cells were growth arrested in 0.5% FBS in normal glucose supplemented with 24.4 mM mannitol. The same group of cells \((n = 10–20/group)\) was digitized serially for the next 60 min. Images were captured on a 486 DX PC and digitized with Sigma Scan morphometric software (Jandel Scientific, San Rafael, CA). Only those cells with a clearly defined border perimeter were used for planar surface area measurement. Changes in individual cell surface planar area were calculated for all of the cells at each time point.

**Confocal Imaging of Intracellular Ca\textsuperscript{2+}**

Cells were sparsely plated on glass coverslips to minimize overlap of individual cells. With the use of our previously published methods (54), growth-arrested cells were loaded with 2.5 \(\mu\)M fluo 3 in DMEM (containing CaCl\textsubscript{2}) with 0.02% Pluronic F-127 and 1 mg/ml BSA for 30 min at 37°C. The cells were washed once in DMEM without fluo 3 and then incubated in the dark in DMEM for 30 min at room temperature (RT). The coverslip was mounted in a chamber on the stage of a Zeiss confocal microscope (LSM 410, Duesseldorf, Germany), and live cells were imaged at 30°C in response to 0.1 \(\mu\)M ET-1. An argon laser was focused with an inverted objective lens (Axiovert 100, \(\times 10\)), and the pixel resolution was set at 512 \(\times\) 512 with a gray-scale level of 0 (minimum) to 255 (maximum). A section through the maximum diameter of each cell was digitized at consecutive time points after the introduction of ET-1. To standardize the fluorescence intensity measurement between experiments, the image contrast and brightness levels were adjusted optimally and then kept constant for the experiments in the study. The pinhole (size = 25), scanning time (0.546 s), zoom (1), and magnification of the confocal scanning system were identical for each.
experiment. The digitized confocal images were analyzed using National Institutes of Health (NIH) Image software (version 1.62) for the Macintosh (NIH, Bethesda, MD), without enhancement.

**Analysis of MLC\(_{20}\) Phosphorylation**

Phosphorylation of the 20-kDa regulatory MLC\(_{20}\) was measured using a modified method from Perschini et al. (36). The separation of unphosphorylated, monophosphorylated, and diphosphorylated forms of MLC\(_{20}\) was accomplished with glyceral PAGE followed by electrophoretic transfer of protein to nitrocellulose membranes and immunoblot analysis.

Mesangial cells were growth arrested for 24 h on 100-mm plastic dishes in 0.5% FBS-DMEM containing 5.6 or 30 mM glucose. Cells were treated with either 0.1 \(\mu\)M ET-1, PMA, or ionomycin for 0, 2, 5, 10, 20, and 40 min at 30°C. To inhibit PKC-dependent MLC\(_{20}\) phosphorylation, cells were exposed to 0.1 \(\mu\)M PMA for 24 h to downregulate PKC. Trypsin-detached cells served as a negative control. After treatment, cells were rinsed with PBS and cellular proteins were precipitated in 600 \(\mu\)l of 10% trichloroacetic acid containing 2 mM DTT at 4°C. Precipitates were scraped from the plates and centrifuged at 15,000 \(g\) for 15 min. The supernatant fraction was decanted, and the pellet was extracted three times in 6 ml of diethyl ether containing 2 mM DTT at 4°C for 2 min, followed by air drying at room temperature (RT) for 30 min to remove residual ether. The pellet was then dissolved in 100 \(\mu\)l of sample buffer containing 8 M urea, 20 mM Tris base, 23 mM glycine, and 2 mM DTT, pH 8.6. After centrifugation and protein determination, 100 \(\mu\)g of the urea-solubilized samples were electrophoresed at 400 V for 1.5 h at 10°C in 1.0-mm minigels containing 10% polyacrylamide, 0.5% bisacrylamide, 40% glycerol, 20 mM Tris base, and 23 mM glycine. The reservoir buffer contained (in mM) 20 Tris, 23 glycine, 1 sodium thioglycolate, and 1 DTT. Separated proteins were then electrobotted to 0.22-\(\mu\)m nitrocellulose (Bio-Rad) membranes at 24 V for 3 h, employing a 20 mM Tris-23 mM glycine-2% methanol transfer buffer, pH 7.6.

After transfer, membranes were blocked in 5% skim milk in Tris-buffered saline plus Tween 20 (TTBS) and then incubated for 1 h at RT in a rabbit polyclonal anti-MLC\(_{20}\) antibody (1:1,000) in 5% skim milk in TTBS. For chemiluminescent detection, the membranes were incubated with an HRP-conjugated anti-rabbit secondary antibody, dipped into luminol substrate solution for 1 min, and developed on film, using National Institutes of Health (NIH) Image software (version 1.62) for the Macintosh (NIH, Bethesda, MD), with constant for all image analysis. The specificity of primary antibody interaction with the sample was eliminated in the presence of the peptide that was used to generate the antibody.

**PKC Immunoblotting**

PKC isozymes were probed in cytosolic, membrane, and particulate fractions of 24-h-growth-arrested mesangial cells in normal and high glucose in the absence and presence of 0.1 \(\mu\)M ET for 10 min at 30°C. As described previously (9, 21), cellular fractions were obtained using sequential ultracentrifugation in the absence (cytosolic) or presence of the detergents 1% Triton X-100 (membrane) and 10% SDS (particle). Total PKC isozyme expression was determined in cells lysed in 2\(\times\) SDS sample buffer at 100°C. Protein content was measured using a Bio-Rad Dc Lowry protein assay (Bio-Rad). Fifteen microliters of protein were subjected to SDS-PAGE on 10% gels followed by overnight transfer onto PVDF membranes. Membranes were then exposed to polyclonal anti PKC\(-\alpha, \beta_1, \beta_2, \gamma, \delta, \epsilon, \gamma, \zeta, \xi, \eta, \zeta\) antibodies for 1 h at 1:40,000 dilution for PKC-\(\alpha\); 1:15,000 for PKC-\(\zeta\); 1:10,000 for PKC-\(\delta\) and -\(\epsilon\); 1:8,000 for PKC-\(\gamma\); and 1:1,000 for PKC-\(\beta_1\). This was followed by a 20-min incubation with a HRP-conjugated affinity-purified goat anti-rabbit IgG antibody (Jackson Immunochemicals) diluted 1:5,000. The secondary antibody was detected by chemiluminescence (Kirkegaard & Perry Labs, Gaithersburg, MD), and the membranes were developed on Kodak X-Omat Blue film (Eastman Kodak, Rochester, NY). Densitometry was performed using NIH Image 1.62 analysis software. The specificity of primary antibody interaction with the sample was eliminated in the presence of the peptide that was used to generate the antibody.

**Cell Fractionation and PKC Immunoblots**

Mesangial cells were cultured on glass coverslips under experimental conditions described above. The cells were fixed with 3.7% formaldehyde for 15 min at RT followed by plasma membrane and nuclear membrane permeabilization with 100% methanol at -20°C for 10 min, as previously published (61). After being washed three times with PBS, the cell proteins were blocked with 1% goat serum plus 0.1% BSA in PBS for 60 min at RT. For Ser\(^{19}\)-MLC\(_{20}\), cells were permeabilized with 0.1% Triton X-100 for 30 min. Rabbit polyclonal anti-PKC-\(\alpha, \beta_1, \beta_2, \gamma, \delta, \epsilon, \gamma, \zeta\), and monoclonal anti-Ser\(^{19}\)-MLC\(_{20}\) were diluted to 1:100 (1:10 PKC-\(\beta_1\)) in blocking solution and added to each coverslip for 60 min at 37°C. After being washed three times with PBS, FITC-conjugated anti-rabbit or anti-mouse secondary antibodies were diluted 1:160 in blocking solution and added for 60 min at 37°C. The coverslips were mounted on glass slides with Aqua-Polymount (Polysciences, Warrington, PA) and SlowFade antifade reagent. The cells were visualized with an oil-immersion, inverted objective lens (Axiovert 100, \(\times63\)), and the image pixel resolution was 512 \(\times\) 512 with a gray-scale level of 0 (minimum) to 255 (maximum) intensity. To standardize the fluorescence intensity for all the experimental preparations, the confocal image contrast and brightness levels were adjusted optimally for each PKC isozyme and then kept constant for all image analysis.

Mesangial cell F- and G-actin were simultaneously labeled with the fluorescent probes rhodamine-phalloidin (F-actin) and FITC-DNase 1 (G-actin) according to our previously published methods (60). Cells were cultured on glass coverslips under experimental conditions described above. After being washed in PBS (4°C), the cells were fixed in 3.7% formaldehyde in PBS for 15 min at RT and permeabilized with either a monoclonal anti-Ser\(^{19}\)-MLC\(_{20}\) antibody or a sheep polyclonal anti-ET\(_{A}\) receptor antibody (US Biologicals, Swampscott, MA). Chemiluminescent detection and densitometry were performed as described above.
Measurement of PKC-ζ and PKC-δ Activity

PKC-ζ activity was determined in membrane fractions using immunoprecipitation and 32P phosphorylation of Ser159-PKC-δ, PKC-ε pseudosubstrate peptide (44). To inhibit PKC-ζ, cells were pretreated with 10 μM ZI for 24 h. Cells cultured in normal glucose supplemented with 24.4 mM mannitol for 24 h served as an osmotic control. Membrane fractions were obtained as previously described (9, 21). The supernatant was precleared by incubation with protein G-Sepharose (100 μl/lysate) at 4°C for 20 min on a rocker, followed by centrifugation at 4°C at 15,000 g for 10 min. Samples of the lysate were removed for protein assay (Bio-Rad, Dc protein assay), and 2 μg of rabbit polyclonal anti-PKC-ζ (Santa Cruz Biotechnology) were added to 300 μg of total cellular protein. The samples were allowed to rock at 4°C for 3 h, followed by immunoprecipitation with 40 μl of protein G-Sepharose for 1 h. The immunocomplexes were centrifuged at 15,000 g for 10 min at 4°C and then washed three times with ice-cold lysis buffer, followed by three washes in kinase buffer. The kinase activity of immunoprecipitated PKC-ζ was assayed in 40 μl of kinase buffer that contained (in mM) 50 Tris, pH 7.5, 5 MgCl2, 2 NaF, 0.1 Na3VO4, and 0.1 Na2P2O7·10 H2O, as well as 5 μg of Ser159-PKC-149–164ε pseudosubstrate peptide (Biomol Research Laboratories, Plymouth Meeting, PA), 40 μM ATP, 5 μCi of [γ-32P]ATP, and 10 nM microcystin. After 15 min at 30°C, the reaction was terminated by the addition of 25 μl of ice-cold quenching buffer containing 0.1 mM ATP and 100 mM EDTA, pH 8.0. Fifty microliters of the reaction mixture were spotted on P81 filter disks and washed four times in 75 mM phosphoric acid followed by a final wash in 80% ethanol. The disks were air dried and placed in 10 ml of Ready Protein scintillant (Beckman Industries); then, 32P radioactivity was counted in a Beckman LSC5 scintillation counter.

PKC-δ activity was determined in total cell lysates using immunoprecipitation and 32P phosphorylation of Ser25-PKC-δ, PKC-α pseudosubstrate peptide. To determine the specificity of the PKC-ζ inhibitor, cells were pretreated with 10 μM ZI for 24 h. Cells were lysed in 500 μl of lysis buffer containing (in mM) 150 NaCl, 25 HEPES, 1 EDTA, 2 EDTA, 10 NaF, 50 β-glycerophosphate, 1 Na3VO4, 2.5 bensamidine, and 1 phenylmethylsulfonyl fluoride, as well as 10 μg/ml leupeptin, pepstatin, and aprotonin, 10 mM microcystin, and 1% Triton-X-100, pH 7.5. Lysates were triturated five times through a 25-gauge needle, placed on ice for 30 min, and centrifuged at 15,000 g for 5 min to remove cellular debris. Three hundred microliters of total cellular protein were immunoprecipitated with 2 μg of rabbit polyclonal anti-PKC-δ (Santa Cruz Biotechnology) for 3 h, followed by the addition of 40 μl protein G-Sepharose for 1 h. The immunocomplexes were centrifuged, washed in lysis buffer, followed by three washes in kinase buffer. Immunoprecipitated PKC-δ was assayed in 40 μl of kinase buffer that contained 5 μg of Ser25-PKC-19–31α pseudosubstrate peptide (GIBCO BRL Life Technologies) but was otherwise identical to that used for determining PKC-ζ activity. The reaction was terminated by the addition of quenching buffer, followed by spotting on P81 filter disks, subsequent washing in 75 mM phosphoric acid, and scintillation counting, as described above. Data for PKC-ζ and PKC-δ activities are expressed as the percentage of those observed in normal glucose.

Statistical Analyses

All results are expressed as means±SE. Statistical analysis was performed using InStat 2.01 statistics software (Graph Pad, Sacramento, CA). The means of two groups were compared using an unpaired Student’s t-test. The means of three or more groups were compared by one-way ANOVA with Bonferroni’s multiple comparison. To compare multiple groups with basal normal glucose, Dunnett’s multiple comparison was utilized. Differences described as significant are P < 0.05 unless stated otherwise.

RESULTS

Effects of High Glucose and Chronic Phorbol Ester on Mesangial Cell Planar Area

Figure 1 demonstrates the change in planar surface area of mesangial cells growth arrested for 24 h in 5.6 (normal) and 30 mM (high) glucose and stimulated with 0.1 μM ET-1 at 30°C. Basal planar surface area in cells cultured in 5.6 mM glucose was 3,952 ± 225 μm2 (n = 53 cells). Cells incubated in 30 mM glucose displayed a smaller basal planar area of 2,608 ± 135 μm2 (n = 31 cells, P < 0.01 vs. normal glucose at time 0). This was not due to osmolality as cells cultured in normal glucose supplemented with 24.4 mM mannitol...
were no different from cells in normal glucose alone (3,424 ± 238 μm², n = 28 cells).

In normal glucose, administration of ET-1 for 60 min decreased planar area to 77 ± 3% of the original surface area (P < 0.05 vs. normal glucose at time 0) (Fig. 1B). Mannitol in normal glucose did not alter mesangial cell responsiveness to ET-1 at 60 min (81 ± 2% of basal area).

Effects of High Glucose on ET₄ Receptor Expression

To determine whether the loss of ET-1 responsiveness in high glucose was due to reduced ET₄ receptor expression, mesangial cells were cultured in 0.5% FBS-DMEM in 5.6 or 30 mM glucose for up to 7 days, and total cell lysates were examined by immunoblot for the presence of the ET₄ receptor. In the representative immunoblot shown in Fig. 2, the antibody generated against a 39-kDa cytosolic fragment of the ET₄ receptor reacted with cellular proteins of 64-, 50-, and 39-kDa molecular mass. The intensities of the three dominant bands were not altered by high glucose. Equal protein loading was confirmed by staining the PVDF membrane with Ponceau S.

Confocal Fluorescence Imaging of ET-1-Stimulated Intracellular Ca²⁺ Signaling

In normal glucose, fluo 3-loaded cells displayed perinuclear and cytosolic Ca²⁺ fluorescence staining at time 0 (Fig. 3A). The administration of 0.1 μM ET-1 stimulated an increase in cytosolic and nuclear Ca²⁺ beginning at 20 s and peaking at 40–60 s. Fluorescence intensity returned to basal levels at 80–100 s. This rapid Ca²⁺ response to ET-1 was seen in >90% of cells examined (n = 44 cells in 3 independent experiments).

In Fig. 3B at time 0, cells in high glucose displayed a basal perinuclear and cytosolic staining pattern that was no different from that seen in normal glucose. However, an increase in cytosolic and nuclear Ca²⁺ did not occur until 40 s after the addition of 0.1 μM ET-1 and reached a peak at 70–80 s. Fluorescence intensity returned to basal levels at 120–200 s. Although the Ca²⁺ response was delayed compared with that for normal glucose, peak fluorescence pixel intensity (130 ± 13 in normal glucose vs. 117 ± 12 in high glucose) and the number of cells responding to ET-1 (>90%, n = 44 cells) were not different.

Phosphorylation of MLC₂₀

Effects of ET-1, phorbol ester, and ionomycin on MLC₂₀ phosphorylation. The change in ET-1-induced mesangial cell planar surface area was used as an indirect measurement of contraction. Glycerol-urea PAGE was used to measure MLC₂₀ phosphorylation, the key regulatory step preceding smooth muscle cell contraction. As shown in Fig. 4, at time 0 the majority of MLC₂₀ was unphosphorylated [81 ± 5 (SE)%], with 13 ± 4 and 6 ± 2% mono- and diphosphorylated, respectively. In comparison, trypsinized suspended cells contained 97 ± 5 un- and 3 ± 1% monophosphorylated MLC₂₀. ET-1 at 2 min significantly stimulated MLC₂₀ phosphorylation. Mono- and diphosphorylated MLC₂₀ increased to 60 ± 9 and 24 ± 11% of total, respectively (P < 0.05 vs. time 0, n = 3), whereas unphosphorylated MLC₂₀ decreased to 16 ± 7% of total.
Effects of high glucose on phosphorylated Ser²⁹-MLC₂₀. The use of glycerol-urea PAGE effectively separated the un-, mono-, and diphosphorylated MLC₂₀. However, this method cannot distinguish which serine or threonine is phosphorylated. To determine whether Ser²⁹-MLC₂₀, the dominant site phosphorylated by MLCK and Rho kinase, was the monophosphorylated MLC₂₀, we immunoblotted total cell lysates with a monoclonal phospho-specific Ser²⁹-MLC₂₀ antibody. As shown in Fig. 6A, basal levels of phosho-Ser²⁹-MLC₂₀ were not altered by high glucose (92 ± 8% of normal glucose, n = 8).

Confocal imaging of ET-1-stimulated phosphorylated Ser²⁹-MLC₂₀. Basal phospho-Ser²⁹-MLC₂₀ cellular distribution in mesangial cells in normal glucose is shown in Fig. 6B. Fluorescence was localized primarily to the cell membrane along with a fibrillar, cytosolic staining pattern. ET-1 administration failed to significantly alter the staining pattern. In high glucose, the intensity and staining pattern were not different from that in normal glucose. In high glucose, ET-1 also failed to change the distribution and fluorescence intensity of the membrane staining.

Mesangial Cell PKC Isozymes

Effects of high glucose on basal and ET-1-stimulated PKC isozyme distribution. In normal glucose, ET-1 stimulates the translocation of PKC-α, -δ, and -ε isozymes, but not PKC-ζ, from cytosolic to membrane and particulate fractions (9). To determine whether high glucose alters ET-1-stimulated PKC isozyme translocation, we immunoblotted cytosolic, membrane, and particulate cellular fractions with PKC isozyme-specific antibodies. As shown in Fig. 7, PKC-α, -δ, and -ε were present in all three fractions in both normal and high glucose and translocated in response to ET-1.

Effects of high glucose on phosphorylation of MC myosin light chain (MLC₂₀-P) in NG at 30°C. Representative immunoblots show the separation of un-, mono- and diphosphorylated (diphospho) MLC₂₀ in response to 0.1 μM ionomycin (IONO) or 0.1 μM 12-phorbol 13-myristate acetate (PMA) for 0, 2, 5, 10, 20, and 40 min. To downregulate protein kinase C (PKC), cells were pretreated for 24 h with 0.1 μM PMA (24P). Downregulated cells were treated for 10 min with ET-1 (24P + ET). Trypsinized cells in suspension (SUS) were used as a negative control. *P < 0.05 vs. NG at time 0, n = 3.

As shown in the representative immunoblots, 0.1 μM acute PMA did not stimulate phosphorylation of MLC₂₀, whereas 0.1 μM ionomycin stimulated phosphorylation to lower levels than seen with ET-1.

Effects of high glucose on ET-1-stimulated MLC₂₀ phosphorylation. Densitometric analysis of ET-1-stimulated MLC₂₀ phosphorylation in high glucose is shown in Fig. 5. At time 0, high glucose significantly increased monophosphorylated MLC₂₀, whereas it decreased unphosphorylated MLC₂₀ (42 ± 16 and 49 ± 15% of total, respectively, vs. 13 ± 3 and 80 ± 4% of total in normal glucose at time 0, P < 0.05, n = 3). ET-1 at 2 min significantly stimulated diphosphorylation of MLC₂₀. Mono- and diphosphorylated MLC₂₀ increased to 62 ± 20 and 28 ± 11% of total, respectively (P < 0.05 vs. time 0, n = 3), whereas unphosphorylated MLC₂₀ decreased to 10 ± 1% of total (P < 0.05 vs. time 0, n = 3). The effect of ET-1 on diphosphorylated MLC₂₀ was sustained at 40 min, with 34 ± 20% of total MLC₂₀ diphosphorylated (P < 0.05 vs. time 0). Downregulation of PKC with 24-h PMA did not alter basal MLC₂₀ phosphorylation in high glucose (54 ± 20, 37 ± 19, and 9 ± 4% un-, mono-, di-phosphorylated, respectively) and did not affect ET-1-stimulated phosphorylation at 10 min (20 ± 10, 39 ± 13, and 41 ± 21% un-, mono-, diphosphorylated, respectively, vs. 24 ± 11, 40 ± 6, and 36 ± 15% with no PMA).

Fig. 4. Effects of 0.1 μM ET-1 on phosphorylation of MC myosin light chain (MLC₂₀-P) in NG at 30°C. Representative immunoblots show the separation of un-, mono- and diphosphorylated (diphospho) MLC₂₀ in response to 0.1 μM ionomycin (IONO) or 0.1 μM 12-phorbol 13-myristate acetate (PMA) for 0, 2, 5, 10, 20, and 40 min. To downregulate protein kinase C (PKC), cells were pretreated for 24 h with 0.1 μM PMA (24P). Downregulated cells were treated for 10 min with ET-1 (24P + ET). Trypsinized cells in suspension (SUS) were used as a negative control. +, Positive smooth muscle MLC₂₀ control. *P < 0.05 vs. NG at time 0, n = 3.

Fig. 5. Effects of 0.1 μM ET-1 on MC MLC₂₀-P in HG at 30°C. Representative immunoblot shows the separation of un-, mono- and diphosphorylated MLC₂₀ in response to ET-1 for 0, 2, 5, 10, 20, and 40 min in HG. To downregulate PKC, cells were pretreated for 24 h with 0.1 μM PMA (24P). Downregulated cells were treated with 0.1 μM ET-1 for 10 min (24P + ET). +, Positive smooth muscle MLC₂₀ control. *P < 0.05 vs. NG at time 0, n = 3.
from cytosolic to membrane and particulate fractions to a similar extent. In response to ET-1 in normal vs. high glucose, PKC-α distribution in membrane and particulate fractions, respectively, was 87 ± 17 and 133 ± 10% of that in basal normal glucose (P < 0.05) vs. in high glucose 92 ± 20 and 142 ± 6% of basal normal glucose levels (P < 0.05, n = 4). PKC-δ distribution in membrane and particulate fractions, respectively, was 300 ± 76 and 134 ± 3% of that in basal normal glucose (P < 0.01 and P < 0.05, respectively) vs. in high glucose 287 ± 74 and 137 ± 24% of that in basal normal glucose (P < 0.01 and P < 0.05, respectively). PKC-ε distribution in membrane and particulate fractions, respectively, was 205 ± 42 and 133 ± 16% of that in basal normal glucose (P < 0.01 and P < 0.05, respectively) vs. in high glucose 184 ± 52 and 190 ± 56% of that in basal normal glucose (P < 0.01 and P < 0.05, respectively, n = 4). Cells treated with 0.1 μM PMA for 10 min served as a positive control and demonstrated the disappearance of PKC-α, -δ, and -ε from the cytosolic fraction (12 ± 9, 2 ± 1, and 4 ± 1% of distribution in basal normal glucose, respectively, P < 0.01, n = 4). This was accompanied by enhanced recovery of isozymes in membrane (289 ± 97, 513 ± 90, and 267 ± 43% of basal normal glucose, respectively, P < 0.01, n = 4) and particulate fractions (376 ± 46, 205 ± 33, and 244 ± 70% of basal normal glucose, respectively, P < 0.01, n = 4).

Subcellular distribution of PKC-ζ and -β₁ are also shown in Fig. 7. Both isozymes were present in all three fractions but did not respond to ET-1 in either normal or high glucose. PKC-ζ was PMA unresponsive, whereas PKC-β₁ demonstrated translocation from cytosolic to membrane and particulate fractions (2.6 ± 1, 295 ± 117, and 322 ± 111% of that in cytosolic and membrane and particulate fractions with basal normal glucose, respectively, P < 0.01, n = 4). High glucose alone enhanced recovery of PKC-ζ in the membrane fraction (294 ± 106% of basal normal glucose, P < 0.01, n = 4) with no change in either the cytosolic or particulate fractions (96 ± 7 and 91 ± 7%, respectively, of basal normal glucose, n = 4).

Effects of high glucose on total PKC isozyme content. In high glucose, it was postulated that the enhanced recovery of PKC-ζ in the membrane might be attributed to enhanced total PKC expression. Therefore, we determined the effects of 24-h high glucose on total PKC expression by immunoblotting total cell lysates (Fig. 8). PKC-α, -δ, -ε, -ζ, and -β₁ were expressed as a single band in mesangial cells, but PKC-γ was not expressed. High glucose stimulated a significant 2.7-fold increase in PKC-δ (272 ± 75%, P < 0.05, vs. normal glucose, n = 6), whereas PKC-α (125 ± 12%, n = 6), -ε (90 ± 10%, n = 6), -ζ (102 ± 2%, n = 6), and -β₁ (90 ± 12%, n = 6) remained unchanged.

PKC-ζ and High Glucose-Induced Loss of Mesangial Cell Contraction to ET-1

Effects of high glucose on immunoprecipitated PKC-ζ and PKC-δ activity. Figure 9 shows the effects of high glucose on membrane PKC-ζ activity in the absence and presence of the ZI. High glucose increased PKC-ζ activity to 190 ± 18% of normal glucose (P < 0.01, n = 4). Twenty-four-hour pretreatment with 10 μM ZI in either normal or high glucose significantly inhibited activity to 45 ± 10 and 73 ± 4% of normal glucose, respectively (P < 0.01, n = 4). Mannitol did not significantly affect PKC-ζ activity (99 ± 11% of normal glucose, n = 4).

In total cell lysates, high glucose increased PKC-δ activity to 241 ± 41% of normal glucose (P < 0.01, n = 3). Twenty-four-hour pretreatment with 10 μM ZI in either normal or high glucose did not alter total PKC-δ activity (110 ± 22 and 256 ± 37% of normal glucose, respectively, n = 3). Mannitol did not significantly affect PKC-δ activity (129 ± 12% of normal glucose, n = 4).

Effects of ZI on mesangial cell planar area and ET-1-stimulated contraction. Basal planar surface area in cells cultured in normal glucose was 3,952 ± 225 μm² (n = 53 cells) (Fig. 10A). In comparison, cells incubated in 30 mM glucose displayed a smaller basal planar area of 2,608 ± 135 μm² (n = 31 cells, P < 0.01 vs. normal glucose at time 0). This was not due to osmo-
larity as cells cultured in normal glucose supplemented with 24.4 mM mannitol were no different from cells in normal glucose (3,624 ± 238 μm², n = 28 cells). Pretreatment of cells in high glucose with 10 μM ZI for 24 h restored the area to values seen in normal glucose (3,875 ± 213 μm², n = 40 cells). In normal glucose, pretreatment with ZI did not alter basal planar area (3,871 ± 277 μm², n = 23 cells). ZI pretreatment in normal glucose did not alter MC responsiveness to ET-1 at 60 min (69 ± 5% vs. 77 ± 3% of basal area in normal glucose) as shown in Fig. 10B. Cells in high glucose did not respond to ET-1. In high glucose, pretreatment with ZI restored the ET-1 response, decreasing planar area to 80 ± 2% of basal area, similar to the change in normal glucose.

Effects of ZI on mesangial cell F-/G-actin in high glucose. In Table 1, the average pixel intensity/cell values for F-actin and G-actin are presented. F-/G-actin ratio was calculated for each cell and the mean ± SE is reported for each group. In high glucose, the F-/G-actin ratio was significantly reduced to 1.5 ± 0.2 (P < 0.05 vs. normal glucose, n = 44 cells) from a value of 1.9 ± 0.1 in normal glucose. The addition of 0.1 μM ET-1 in normal glucose resulted in an F-/G-actin ratio that was not different from that seen in the basal high glucose state (1.5 ± 0.1, n = 44). In high glucose, ET-1 did not alter the mesangial cell F-/G-actin ratio (1.4 ± 0.1, n = 36). Mannitol (normal glucose +24.4 mM mannitol for 24 h) had no effect on the F- or G-actin content or F-/G-actin ratio (2.0 ± 0.2, n = 26 cells).
The lack of a contractile response to ET-1 in mesangial cells in high glucose may involve reorganization of the cellular cytoskeleton. In normal glucose, F-actin staining filled the cytoplasm and was organized in a bundled stress fiber pattern (Fig. 11). G-actin was homogeneously distributed in the cytosol and followed a granular staining pattern. In high glucose, F-actin intensity was reduced, whereas cytosolic and perinuclear G-actin fluorescence intensity was increased. In normal glucose, ZI pretreatment had no affect on F-/G-actin staining pattern or intensity. However, pretreatment of cells in high glucose with ZI resulted in a staining pattern that was seen in normal glucose.

**DISCUSSION**

In this study, we examined the signaling events related to mesangial cell contractile and cytoskeletal dysfunction in high glucose. PKC has been implicated as an underlying mechanism of high glucose-induced VSMC dysfunction (25). In high glucose, lack of mesangial cell contractility in response to ET-1 and F-actin disassembly is not due to altered Ca\(^{2+}\) signaling or phosphorylation of MLC\(_{20}\) but is PKC-\(\zeta\)-mediated.

The observations that after exposure to 24-h high glucose mesangial cells are smaller in size, unresponsive to ET-1 (8), and display PKC-mediated F-actin disassembly (61) resemble our earlier studies in higher passage cells. We reported previously that mesangial cells growth arrested for 24 h in NG, HG, NG + 24.4 mM mannitol (Mann), NG + 24 h, PKC-\(\zeta\) inhibitor (NG + ZI), or HG + ZI. *P < 0.05 vs. NG (n = 23–46 cells). B: effects of 0.1 \(\mu\)M ET-1 on change in MC planar surface area in NG or HG with or without ZI. *P < 0.05 vs. NG.

**Table 1. Mesangial cell F- and G-actin fluorescence intensities determined by dual-channel confocal microscopy**

<table>
<thead>
<tr>
<th>Group</th>
<th>F-Actin</th>
<th>G-Actin</th>
<th>F-/G-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>125 ± 6</td>
<td>67 ± 3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>HG</td>
<td>93 ± 9</td>
<td>71 ± 12</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Mann</td>
<td>136 ± 7</td>
<td>68 ± 4</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>NG + ET-1</td>
<td>94 ± 7</td>
<td>67 ± 7</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>HG + ET-1</td>
<td>96 ± 4</td>
<td>69 ± 5</td>
<td>1.4 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 23–55 cells. NG, normal glucose (5.6 mM); HG, high glucose (30 mM); Mann, NG + 24 mM mannitol; ET-1, 0.1 \(\mu\)M endothelin-1. *P < 0.05 vs. NG.
cell reduced planar area, responsiveness to ET-1, and F-actin disassembly were reversed by tolrestat, an aldose reductase inhibitor (21). Aldose reductase inhibition of the polyol pathway prevented the de novo synthesis of diacylglycerol (DAG) (8, 29, 49) and thus activation of DAG-sensitive PKC isozymes at 48 h (21). At 48 h, high glucose stimulated the membrane translocation of PKC-δ and -ε (21). In the present study, total PKC-δ but not PKC-ε was increased by 24-h high glucose, whereas PKC-ζ was increased in the membrane fraction only. Although the exact mechanisms of PKC-ζ activation are not known, phosphatidic acid (PA), a precursor of DAG, is reported to activate PKC-ζ (32). In our earlier studies (21), tolrestat, through inhibition of de novo PA synthesis, may have reduced PKC-ζ activity concomitantly with either PKC-δ and/or -ε.

It is possible that the diminished responsiveness of mesangial cells to ET-1 in high glucose may be mediated by reduced endothelin-receptor A (ET_A) expression (19). Hargrove et al. (14) demonstrated in cultured rat mesangial cells that high glucose for 24 h upregulates the production of ET-1 and ET-1 mRNA twofold. Increased ET-1 production may cause downregulation and decreased expression of mesangial cell ET_A receptors (19). By contrast, glomerular ET_A receptor expression was upregulated in glomeruli from alloxan-diabetic rabbits (22), whereas hyperglycemia did not alter kidney ET_A receptor expression in humans or in STZ-diabetic rats (41). In our study, 1–7 days of high glucose did not alter mesangial cell ET_A receptor expression. This suggests that reduced mesangial cell ET_A receptor expression does not likely account for the high glucose-induced contractile dysfunction in response to ET-1.

As observed in Fig. 3, A and B, in high glucose ET-1 stimulated intracellular Ca^{2+} to similar levels observed in normal glucose. Although peak intensity of fluo 3-labeled release was not different, a time delay in the measured response in high glucose was consistently observed. We have previously shown that under the identical culture conditions used in this study (9), acute PMA does not elicit a Ca^{2+} signal. This suggests that PKC activation does not directly stimulate Ca^{2+} signaling in mesangial cells. However, others have shown that prior PKC activation may inhibit signaling. Interaction may be at the level of G protein coupling (57) or the cytoskeleton (37). Disruption of F-actin with cytochalasin D in platelets inhibits Ca^{2+} entry (39). Treatment with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), also inhibits Ca^{2+} entry (39). PI 3-kinase is a potent activator of atypical PKC-ζ (59). In our hands, cytochalasin D pretreatment of mesangial cells did not affect either timing or intensity of ET-1-stimulated Ca^{2+} release (data not shown). This suggests that the time delay observed in Ca^{2+} signaling in response to ET-1 in high glucose is not attributable to mesangial cell F-actin disassembly. The potential role of PI 3-kinase in high glucose-induced activation of mesangial cell PKC-ζ and stress fiber disassembly was not explored.

Stress fibers are composed not only of actin but also contain myosin, tropomyosin, and α-actinin and require the small GTP-binding protein Rho for their formation (38). Stress fiber assembly is usually preceded by MLC_{20} phosphorylation, whereas stress fiber disassembly is a consequence of MLC_{20} dephosphorylation (5). In mesangial cells, Kreisberg et al. (28) demonstrated that cAMP-mediated stress fiber disassembly preceded MLC_{20} dephosphorylation. In our study, basal MLC_{20} phosphorylation was markedly increased in high glucose, whereas F-actin was disassembled, resembling the effects of agonist stimulation. Fukuda et al. (11) reported increased basal MLC_{20} phosphorylation in platelets from type 2 diabetic patients, but the organization of actin was not studied. Because MLC_{20} phosphorylation is a balance between kinase and phosphatase activities (42), it is possible that high glucose may enhance MLCK activity or increase PKC- or Rho-mediated inhibition of phosphatase activity (31, 43). In this study, neither acute nor chronic PMA, in either normal or high glucose, affected

![Fig. 11. Dual-channel confocal fluorescence imaging of filamentous-(F)-actin labeled with rhodamine-phalloidin and globular-(G)-actin labeled with FITC-DNase 1 in 5.6 mM glucose NG or HG in the absence or presence of 10 μM ZI for 24 h. Images are representative of 3 separate experiments.](http://ajprenal.physiology.org/)

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basal MLC\textsubscript{20} phosphorylation. The lack of MLC\textsubscript{20} phosphorylation in response to acute PMA suggests that the PMA-induced mesangial cell contraction observed in our earlier studies (8, 9) must be MLC\textsubscript{20} phosphorylation independent. In our earlier study, chronic PMA inhibited contraction by downregulating PKC-\(\alpha\), -\(\delta\), and -\(\epsilon\) in the cytosolic, membrane, and particulate fractions (9), thus reducing DAG-sensitive PKC isozyme content. PKC isozymes may stimulate contraction by initiating a kinase cascade involving the phosphorylation of the actin regulatory proteins calponin, caldesmon (16), and/or myristoylated alanine-rich C-kinase substrate (48).

In high glucose, ET-1 stimulated phosphorylation of MLC\textsubscript{20} to levels seen in normal glucose, which was not accompanied by cell contraction. This might be explained by the preferential phosphorylation of PKC-specific sites serine-1, serine-2, or threonine-9 of the MLC\textsubscript{20} by ET-1 in high glucose. Phosphorylation of MLC\textsubscript{20} at these sites does not result in myosin-ATPase activation and contraction (52). If high glucose enhanced PKC-mediated inhibition of MLC-PP, phosphorylation of Ser\textsuperscript{19}-MLC\textsubscript{20} would increase. This was not detected by either immunoblotting or confocal imaging with a Ser\textsuperscript{19}-MLC\textsubscript{20} antibody. The effects of high glucose on either Rho expression or activity are not known. Therefore, in high glucose, MLC\textsubscript{20} phosphorylation does not appear to correlate with either F-actin disassembly or lack of contraction in response to ET-1. High glucose must be exerting its actions at another cytoskeletal target.

In this study, high glucose for 24-h enhanced membrane accumulation of PKC-\(\zeta\). This was much earlier than the 5-day time point reported by others (1, 24). This was not accompanied by a concomitant decrease in cytosolic and/or particulate fraction and may be explained by the fact that the relative abundance of PKC-\(\zeta\) in these fractions may hinder the detection of small compartmental changes in distribution. Immuno-complexed PKC-\(\zeta\) membrane activity was significantly increased by high glucose and was normalized by pretreatment with the myristoylated ZI. ZI was specific for the PKC-\(\zeta\) isozyme, as pretreatment with ZI did not affect immunoprecipitated PKC-\(\delta\) activity in either normal or high glucose. Although total protein expression and activity of PKC-\(\delta\) were increased by high glucose, this was not accompanied by changes in fractional distribution. PKC-\(\delta\) may be activated by PKC-\(\zeta\)-mediated Ser\textsuperscript{560} phosphorylation (62). Furthermore, overexpression of constitutively active PKC-\(\zeta\) results in phosphorylation of PKC-\(\delta\) coexpressed in HEK-293 cells (62). In our study, high-glucose-induced PKC-\(\zeta\)-mediated activation of PKC-\(\delta\) is unlikely, as pretreatment of cells in high glucose with the ZI, which inhibited PKC-\(\zeta\) activity, did not inhibit PKC-\(\delta\) activity. In COS-7 cells, H\textsubscript{2}O\textsubscript{2} causes phosphorylation of Tyr\textsuperscript{311} and Tyr\textsuperscript{229}, which results in prolonged activation of PKC-\(\delta\) (26). Recent studies by Konishi et al. (27) using phosphorylation-site specific antibodies and mass spectrometric analyses have shown that H\textsubscript{2}O\textsubscript{2}-induced PKC-\(\delta\) activation requires phosphorylation of Tyr\textsuperscript{311} and is not accompanied by membrane translocation. Mesangial cells cultured in 30 mM glucose for as little as 1 h generate H\textsubscript{2}O\textsubscript{2}, as detected by dichlorofluorescein (13), but it is not known whether this is associated with PKC isozyme tyrosine phosphorylation and activation.

The observations that 24-h high glucose-stimulated, PKC-\(\zeta\)-dependent F-actin disassembly was reversed by ZI, which also reversed cell size and response to ET-1, suggest that the smaller mesangial cell planar area is the result of F-actin disassembly. The mechanism underlying PKC-\(\zeta\)-mediated disassembly is not known. PKC-\(\zeta\) can directly associate with the actin cytoskeleton in fibroblasts (12). In NIH-3T3 fibroblasts, atypical PKC-\(\zeta\) and -\(\lambda\) are reported to mediate Cdc42-mediated stress fiber disassembly (6) and have also been implicated in regulating Ras-mediated stress fiber disassembly (51). In mesangial cells, high glucose for 48 h does not affect the expression of Ras but increases membrane associated Ras activity (56). It is not known whether high glucose-induced Ras activation results in increased PKC-\(\zeta\) activity and F-actin disassembly. The recent findings of Cortes et al. (7) that perfused glomeruli from 9-mo-old STZ-diabetic rats display F-actin disassembly and loss of stress fibers strongly suggest that the F-actin disassembly we have observed in cultured mesangial cells in high glucose mimic the in vivo findings.

In summary, this study identifies the importance of PKC-\(\zeta\) in the mediation of high glucose-induced loss of mesangial cell responsiveness to ET-1. Loss of the contractile response to ET-1 is not due to reduced Ca\textsuperscript{2+} signaling, phosphorylation of MLC\textsubscript{20}, or altered DAG-sensitive PKC isozyme activation but may be related to PKC-\(\zeta\)-dependent F-actin disassembly. This cytoskeletal disorganization alters the mesangial cell phenotype and may account for the early effects of high glucose-induced cellular dysfunction.

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