High glucose-induced mesangial cell altered contractility: role of the polyol pathway

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Summary Glomerular mesangial cells cultured in high glucose conditions display impaired contractile responsiveness. It was postulated that glucose metabolism through the polyol pathway leads to altered mesangial cell contractility involving protein kinase C. Rat mesangial cells were growth-arrested for 24 h with 0.5 % fetal bovine serum in either normal (5.6 mmol/l) or high (30 mmol/l) glucose concentrations or high glucose plus the aldose reductase inhibitor, ARI-509 (100 µmol/l). The reduction of cell planar surface area (contraction) in response to endothelin-1 (0.1 µmol/l), or to phorbol 12-myristate 13-acetate (50 pmol/l), was studied by videomicroscopy. In response to endothelin-1, mesangial cells in normal glucose contracted to 52 ± 3 % of initial planar area. In high glucose, the significantly ($p < 0.05$) smaller cell size and no contractile responsiveness to endothelin-1 were normalized with ARI-509. Membrane-associated diacylglycerol, measured by a kinase specific ^{32}P-phosphorylation assay, in high glucose was unchanged after 3 h, but significantly increased ($p < 0.05$) after 24 h which was normalized with ARI-509. Protein kinase C activity, measured by in situ ^{32}P-phosphorylation of the epidermal growth factor receptor substrate was: increased by 32 % at 3 h of high glucose, unchanged by ARI-509; and decreased significantly ($p < 0.05$) at 24 h compared to cells in normal glucose, normalized by ARI-509. Total cellular protein kinase C-alpha, -delta and -epsilon, analysed by immunoblotting, were unchanged in high glucose at 24 h. Only protein kinase C-epsilon content was reduced by ARI-509 in both normal and high glucose. Therefore, high glucose-induced loss of mesangial cell contractility, diacylglycerol accumulation and altered protein kinase C activity are mediated through activation of the polyol-pathway, although no specific relationship between elevated diacylglycerol and protein kinase C activity was observed. In high glucose, altered protein kinase C function, or another mechanism related to the polyol pathway, contribute to loss of mesangial cell contractile responsiveness. [Diabetologia (1998) 41: 507–515]

Keywords Sorbitol, aldose reductase inhibitor, protein kinase C, diacylglycerol.

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Abbreviations: AA, Arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; ET-1, endothelin-1; EGF, epidermal growth factor; FBS, fetal bovine serum; F, filamentous actin; DAG, diacylglycerol; PDD, phorbol 12, 13-didecanoate; PMA, phorbol 12-myristate 13-acetate; PA, phosphatidic acid; PL, phospholipase; PKC, protein kinase C -alpha (-α), -delta (-δ), epsilon (-ε); STZ, streptozotocin; PBS, phosphate buffered saline.

Hyperglycaemia is necessary for the development of progressive diabetic nephropathy in susceptible individuals with insulin-dependent diabetes mellitus [1]. Multiple cellular mechanisms are likely involved in the pathogenesis of progressive glomerulosclerosis in diabetic humans and animal models. Loss of normal renal afferent arteriolar contractility resulting in raised intraglomerular pressure is observed in the streptozotocin (STZ) diabetic rat model of nephropathy [2, 3]. Glomerular mesangial cells respond to raised hydrostatic pressure by increasing transforming growth factor-β_1 and collagen synthesis [4]. Mes-

angial cells are contractile and resemble vascular smooth muscle cells with respect to their signalling and cytoskeletal responsiveness to vasopressor hormones [5, 6]. Glomeruli isolated from the hyperglycaemic STZ rat demonstrate little or no contractile response of mesangial cells to potent vasoconstrictor peptides including angiotensin II and endothelin-1 (ET-1) [7–9]. Furthermore, no contraction is elicited from STZ rat glomeruli during exposure to the calcium ionophore A23187, confirming the lack of mesangial cell cytoskeletal responsiveness to raised mesangial cell cytosolic calcium [8]. As extracellular glucose concentration increases between 5.6 and 18 mmol/l, cultured rat mesangial cells display progressively reduced contractility to ET-1, despite preservation of normal calcium signalling [8, 9].

Recently, we have reported that mesangial cells cultured in high glucose, and the cells of glomeruli isolated from the STZ rat, demonstrate disassembly of filamentous (F-) actin which appears to be protein kinase C (PKC)-dependent [10, 11]. It is likely that partial disassembly of F-actin accounts for the smaller size of mesangial cells cultured in high glucose compared to those in normal glucose [10, 11]. Mesangial cells cultured in normal glucose display partial F-actin disassembly in response to a vasopressor stimulus [12], creating a favourable arrangement for myosin light chain cross-bridging [13]. The normal contractile response involves both F-actin and myosin light chain phosphorylation stimulated by signalling events including a calcium transient and cytoskeletal protein-specific kinase activation [14]. Vasopressor peptide activation of kinase cascades, including those mediated by PKC, also play a role in cytoskeletal protein phosphorylation. Myosin light chain and caldesmon are phosphorylated by phorbol ester activation of PKC [15, 16]. Therefore, altered mesangial and renal arteriolar smooth muscle cell contractile function in high glucose may involve PKC-mediated cytoskeletal dysfunction.

Enhanced PKC signalling in high glucose is implicated in the complications of diabetes [17, 18], including nephropathy [19, 20]. One mechanism linking increased intracellular glucose to altered PKC activity is through the polyol pathway. Raised intracellular glucose is converted to sorbitol by aldose reductase. The subsequent synthesis of fructose from sorbitol generates increased NADH which is then available for the conversion of the glycolytic intermediate glycerol-3-phosphate into dihydroxyacetone phosphate, leading to de novo synthesis of diacylglycerol (DAG) [21, 22]. Membrane accumulation of DAG in high glucose is reported in many cell types including cultured mesangial cells [23] and the cells of isolated STZ diabetic rat glomeruli [24]. DAG in turn, may activate several PKC isoforms. Controversy exists about the time course of increased PKC activity demonstrated by mesangial cells cultured in high glu-

cose. Ayo et al. [23], reported increased PKC activity as early as 30 min, and reduced activity at 24 h. Williams and Schrier [25] report a small, but significant increase in mesangial cell PKC activity at both 3 and 24 h during exposure to high glucose which also correlates with an increase in PKC-dependent phospholipase (PL-)A_2 activity and arachidonic acid (AA) release.

The purpose of the present study was to identify a cause and effect relationship between loss of mesangial contractile responsiveness to ET-1 and high glucose metabolism through the polyol pathway contributing to accumulation of DAG and altered PKC activity. Since mesangial cells synthesize a number of PKC isoforms [26], it was further postulated that high glucose concentrations may alter the synthesis and activity of DAG sensitive PKC isoforms which are expressed by mesangial cells and recently reported to accumulate in both vascular smooth muscle [27] and mesangial cells [28]. Metabolism of intracellular glucose through the polyol pathway was monitored by measuring accumulation of sorbitol. The aldose reductase inhibitor ARI-509 (Wyeth-Ayerst, Princeton, N.J., USA) was utilized to prevent the conversion of glucose to sorbitol. In growth-arrested rat mesangial cells after 24 h exposure to normal glucose (5.6 mmol/l), high glucose (30 mmol/l), or high glucose plus ARI-509, the contractile responsiveness to ET-1, membrane-associated DAG accumulation, in situ PKC activity, AA release as a measure of (PL)-A_2 activity and PKC-alpha(-α), -delta(-δ) and epsilon (-ε) contents were compared.

Materials and methods

Mesangial cell culture. The chemicals were purchased from ICN, Pharmaceuticals (Montreal, PQ, Canada) or otherwise stated. Glomeruli were isolated under sterile conditions by selective sieving of finely chopped renal cortex from male Sprague-Dawley rats weighing 200 g. Mesangial cells were cultured from isolated glomeruli in minimal essential medium (MEM) with D-valine (Gibco BRL, Mississauga, ON, Canada) at 37 °C containing 20 % fetal bovine serum (FBS), 5 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Cells were harvested by washing two times in phosphate buffered saline (PBS) without calcium and magnesium and briefly exposed to 0.16 % trypsin (Gibco BRL). The effect of trypsin was terminated by adding excess Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) followed by replating. Mesangial cells were identified by their positive immunofluorescence staining for desmin and vimentin, negative staining for cytokeratin and Factor VIII and their positive contractile response to vasopressin [9]. In the following experiments, mesangial cells at passage 10 to 20 were utilized.

Measurement of intracellular glucose, and sorbitol. Intracellular glucose and sorbitol levels were measured as their aldonitril and alditol acetate derivatives by capillary gas liquid chromatography. Mesangial cells were cultured to confluence in DMEM (FBS 20 %), in either normal or high glucose, with or

without the aldose reductase inhibitor ARI-509 (100 µmol/l) on 100 mm culture dishes. The cells were then washed with PBS, harvested by scraping, suspended in 1.0 ml of distilled water and homogenized at 4 °C. Aliquots of the homogenate were treated with an equal volume of 0.3 N $ZnSO_4$ to precipitate the protein. The solution was neutralized with $Ba(OH)_2$ and centrifuged at $1000 \times g$ for 10 min at 4 °C. The samples were then lyophilized, extracted with ether, derivatized with hydroxylamine hydrochloride, 4-(dimethylamino)pyridine and acetic anhydride, and injected into an HP-5890 A chromatograph equipped with a SP-2100 fused silica capillary column. Sorbitol was quantitated by flame-ionization detection. Carbohydrate measurements were calculated on the basis of an 8-point standard curve constructed by addition of varying amounts of glucose and sorbitol to homogenates of mesangial cells cultured in normal glucose containing media. The processing and analysis of these samples were identical to those used for the unknown samples. Alpha-methyl mannoside was used as an internal standard. With this technique, the instrument response was linear for concentrations ranging from 6 to 230 nmol per sample. Measurements are expressed as nmol per mg cell protein.

Mesangial cell planar surface area. Rat mesangial cells were cultured for 4 days in DMEM (20 % FBS), with or without the aldose reductase inhibitor, ARI-509 (100 µmol/l), and then transferred to 35 mm dishes. The cells were cultured for 24 h in 2 ml DMEM (plus 0.5 % FBS) with either normal (5.6 mmol/l) glucose, high (30 mmol/l) glucose, normal glucose (5.6 mmol/l) plus mannitol (25 mmol/l), normal glucose plus 50 pmol/l phorbol 12-myristate 13-acetate (PMA) (Sigma Diagnostic, St. Louis, Mo., USA) or high glucose plus 100 µmol/l ARI-509. Then, the dishes were mounted on a heated stage of an inverted light microscope (Bausch and Lomb, New York, N. Y., USA) and change in mesangial cell planar surface area in response to 0.1 µmol/l ET-1 or 50 pmol/l PMA, was observed at 30 °C pH 7.4 and 5 % CO_2, as previously described [8]. Using a video camera attached to the microscope, 10–14 cell images per experiment were captured and digitized at 0 min. Then, either ET-1 or PMA were added directly to the medium to create the above concentrations. Images of the same cells were digitized serially at time intervals from 5 to 60 min. The perimeter of the individual cells with clearly defined borders was outlined ($\times 2$) and planar surface area calculated using a 486SX PC and JAVA software (Jandel Scientific, San Rafael, Calif., USA). The decrease in planar surface area compared to original size was calculated for each cell. The planar surface area, mean ± SEM, was determined for each time point.

Cellular membrane-associated DAG. Mesangial cells cultured to confluence in DMEM (20 % FBS) with or without 100 µmol/l ARI-509, were treated for 24 h with medium containing 0.5 % FBS, and either normal (5.6 mmol/l), high (30 mmol/l) glucose or high glucose plus ARI-509. DAG mass was estimated using a modified procedure described by Preiss et al. [29] based on the total conversion of extracted DAG to [^{32}P]phosphatidic acid (PA) in the presence of [γ-^{32}P]ATP and the enzyme sn-1,2-DAG kinase. The cells were washed with PBS harvested by scraping and the cellular lipids were extracted in 3 ml of chloroform:methanol 100 %: PBS (1:1:0.4, vol/vol). The phases were split by addition of 1 ml of chloroform and 1 ml NaCl 1.0 mmol/l. The lower chloroform phase was washed twice with 2 ml NaCl 1.0 mmol/l and evaporated under N_2. Each sample was solubilized in 20 ml of 7.5 % octyl-β-D-glucoside, 5 mmol/l cardiolipin, in 1 mmol/l DETAPAC (Amersham Life Science, Oakville, ON, Canada) and then sonicated for 2 min. The solubilized lipids were mixed with 70 µl

of a reaction solution containing (mmol/l) 78.5 imidazole-HCl buffer (pH 6.6), 71.4 NaCl, 17.8 $MgCl_2$, 1.4 EGTA, 0.8 DETA-PAC, 2.9 dithiothreitol, 0.7 potassium phosphate, 1.4 % glycerol (vol/vol), 0.1 mercaptomethanol, and 0.015 U sn-1,2-DAG kinase (Amersham Life Science). Samples were preincubated in the reaction solution for 5 min at 25 °C. The reaction was initiated by addition of 10 µl of 5 mmol/l [γ-^{32}P]ATP, (specific activity 100,000 cpm/nmol, Amersham Life Science). After 30 min, the reaction was stopped by adding 20 µl of 1 % perchloric acid (vol/vol) (Sigma Diagnostic) and 450 µl of chloroform:methanol (1:2 vol/vol). After incubation for 10 min at room temperature and centrifugation at 2000 g for 1 min, 150 µl of chloroform and 150 µl 1 % perchloric acid, were added to extract the lipids. The lower chloroform phase was washed with 1 ml of 1 % perchloric acid. After the second washing, the chloroform phase was applied to Amprep Si columns 100 mg, (Amersham Life Science), preconditioned with 2 ml of hexane (Anachemia Science, Mississauga, ON, Canada). The columns were washed with 2 ml of chloroform followed by 2 ml of ethyl acetate 15 %, in hexane (vol/vol). PA was eluted from the columns with 2 ml of solvent system consisting of chloroform: methanol: acetic acid (65:10:10 vol/vol). The radioactivity attributable to [^{32}P]PA was counted. The sn-1,2-DAG content was calculated from the amount of [^{32}P]PA recovered and specific activity of the [γ-^{32}P]ATP. A standard curve for the conversion of 1,2-dioleolyl-sn-glycerol 10 to 1000 pmol (Amersham Life Science) to [^{32}P]PA, was established and found to be linear in this range. The protein concentration of the chloroform phase was measured in a separate sample by the Lowry method using BSA (Bio-Rad, Hercules, Calif., USA) as the standard.

Measurement of PKC activity. Total PKC activity was measured by in situ phosphorylation of the PKC-specific substrate derived from the epidermal growth factor (EGF) receptor (VRKRTLRRL). This substrate was synthesized, in accordance with the published amino acid sequence [30], with an automated solid phase peptide synthesizer and its purity was estimated to be 99 % by HPLC. This method was originally developed by Heasley and Johnson [30] and used to study cultured mesangial cell PKC activity by Williams and Schrier [25].

Mesangial cells were plated onto 96-well flat-bottom microtitre plates at densities of 20,000 cells per well. The cells were cultured for 3–4 days until confluent in DMEM supplemented with 20 % FBS, 5.6 mmol/l glucose, and with or without 100 µmol/l ARI-509. The monolayers were then incubated for 24 h in DMEM supplemented with 0.5 % FBS, normal (5.6 mol/l) or high (30 mmol/l) glucose. After incubation, the medium was aspirated and replaced with fresh medium supplemented with or without 50 pmol/l PMA, the inactive phorbol 12, 13-didecanoate (PDD) 0.1 µmol/l (Calbiochem, San Diego, Calif., USA), the cAMP-dependent kinase inhibitor HA1004 20 µmol/l (Calbiochem) or the PKC inhibitor calphostin C 0.2 µmol/l (Calbiochem) for 20–30 min. The medium was aspirated and replaced with 40 µl of the buffered reaction solution containing 50 µg/ml digitonin, (mmol/l) 137 NaCl, 5.4 KCl, 10 $MgCl_2$, 0.3 sodium phosphate, 0.4 potassium phosphate, 25 β-glycerophosphate, 5.6 or 30 glucose, 5 EGTA, 1 EDTA, 100 µmol/l ATP, [γ-^{32}P]ATP (specific activity 860 cpm/pmol, Amersham Life Science), 20 Hepes, and the PKC-specific peptide substrate 100 µmol/, (pH 7.2, 37 °C). After 10 min incubation at 30 °C, the reaction was terminated by the addition of 10 µl ice cold trichloroacetic acid 25 % (wt/vol), (Caledon). Aliquots (45 µl) of the acidified reaction mixtures were spotted onto 2 cm phosphocelluose paper circles (P81, Gibco BRL) and washed batchwise in three changes (500 ml each) of 1 %

phosphoric acid, 0.1 % Tween 20 and one change of 75 mmol/l sodium phosphate, (pH 7.5). Due to the basicity of the PKC substrate peptide, it is retained on the phosphocellulose paper at neutral pH while the excess $[\gamma\text{-}^{32}P]$ATP bound to the filters at acidic pH is removed [30]. To determine background phosphorylation, the assay was performed in the absence of VRKRTLRRL.

AA. Rat mesangial cells were grown to confluence in DMEM (20 % FBS). Two days prior to the experiment, cells were split into 60 mm diameter dishes DMEM (20 % FBS). Confluent monolayers were preincubated for 24 h with 0.2 μCi of ^{3}H-arachidonic acid (^{3}H-AA) (Amersham Life Science) in 1 ml DMEM, 0.5 % FBS. Radioactive medium was aspirated 24 h later and cells were washed gently ($\times 5$) with 2 ml of washing medium which consisted of DMEM (glucose 5.6 mmol/l), 0.5 % FBS and 5 mg/ml BSA. After washing, cells were incubated for 3 or 24 h at 37 °C in 2 ml of incubation medium which consisted of DMEM, 0.5 % FBS, 1 mg/ml BSA and either normal or high glucose. After incubation, the medium was aspirated and cells were washed five times with washing medium as above and 1 ml of corresponding incubation medium was added for an additional 30 min incubation at 37 °C. A 750 μl sample of medium was removed without touching the cells. The sample was centrifuged at 8000 rev/min for 10 min, and used for scintillation counting. Results were multiplied by 4/3 to determine the amount of ^{3}H-AA released by the monolayer into 1 ml incubation medium over a 30 min period.

To compare EGF-stimulated ^{3}H-AA response, radiolabelled monolayers were washed with washing medium, as above, then the cells were exposed to 1 ml of incubation medium with normal (5.6 mmol/l) glucose supplemented with 100 nmol/l EGF (Sigma Diagnostic) for 20 min plus calcium ionophore A23 187 (Calbiochem) 2 μmol/l for 5 min as a positive control, or the acetic acid vehicle (100 nmol/l) for 25 min as a negative control, 37 °C. The dimethyl sulfoxide (DMSO) vehicle was added for 5 min at a final concentration 0.5 %, to the negative control.

After removal of radioactive medium, cells were washed five times with PBS and lysed with 1 ml per well of 0.1 mol/l NaOH for 1 h. The wells were scraped and the entire lysate was collected, vortexed, and 50 μl aliquots were removed for scintillation counting. The results were standardized by expressing the total amount of ^{3}H-AA released as a percentage of total ^{3}H-AA initially incorporated by the cells.

Immunoblot analysis of PKC isoforms. Rat mesangial cells were cultured to confluence in 60 mm diameter dishes and then exposed for 24 h to normal (5.6 mmol/l) or high (30 mmol/l) glucose in 0.5 % FBS, with or without 100 μmol/l ARI-509. The cells were washed with PBS ($\times 2$), scraped in 300 μl lysis buffer, (mmol/l) 1 NaHCO$_3$, 5 MgCl$_2\cdot$6H$_2$O, 50 Tris-HCl, 10 EGTA, 2 EDTA, 25 μg/ml leupeptin, 1 % 2-β-mercaptoethanol, 0.6 phenylmethylsulfonyl fluoride, 1 % Triton-X-100, and 1 % sodium dodecyl sulfate. DNA was removed with a DNA filter (SQ-Easy Filters, Porex Technologies, Fairburn, Ga., USA). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Mississauga, ON, Canada) and 50 μg of total cell protein/lane along with molecular weight markers were loaded onto an Immobilon PVDF membrane (Millipore, Bedford, Mass., USA) for 2 h at 250 mA using a Bio-Rad Transblot apparatus. The membrane was blocked for 2 h in blocking buffer (5 % non-fat dry milk powder, 5 % BSA, 10 mmol/l Tris HCl, 150 mmol/l NaCl, and 0.05 % Tween-20, [vol/vol]), at room temperature. Then the membrane was incubated first with monoclonal PKC-α, -δ, or -ε antisera 0.5 μg/ml (Bio/Can Scientific, Missis-

sauga, ON, Canada) in incubation buffer without BSA, and second with horseradish peroxidase-conjugated goat anti-mouse IgG, diluted 1:5000 (Bio/Can Scientific). The membranes were thoroughly washed after each incubation with 10 mmol/l Tris-HCl, 150 mmol/l NaCl, and 0.05 % Tween-20 and the final wash was in the absence of detergent. A chemiluminescence kit (Canadian life Technologies, Burlington, ON, Canada) and Kodak X-Omat AR film (Eastman Kodak, Rochester, N. Y., USA) were used for development. The PKC-isoforms were identified using molecular weight markers and positive controls (Bio/Can Scientific). To determine the density of the single bands, these images were digitized using the White/UV transilluminator, Gel Documentation System and UV Image Store 5000 (UVPm Diamed Lab Supplies, Mississauga, ON, Canada). Then the area of each specific band was calculated using JAVA software and multiplied by its mean pixel intensity (grey scale 1 to 255) to obtain total density. Background intensity was subtracted from each band.

Statistical analyses were performed using analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. All data are presented as mean values ± SEM.

Results

Effects of high glucose and ARI-509 on mesangial intracellular glucose and sorbitol levels. The intracellular glucose level ($n = 7$ per group) increased from 1095 ± 208 nmol/mg cell protein in normal glucose to 7215 ± 491 nmol/mg cell protein in high glucose ($p < 0.01$ vs normal glucose) which was unchanged in high glucose plus ARI-509, 6816 ± 203 nmol/mg cell protein. The sorbitol level in normal glucose was 5.1 ± 0.9 nmol/mg cellular protein and increased in high glucose to 17.0 ± 1.3 nmol/mg cellular protein, ($p < 0.01$ vs normal glucose). The sorbitol content in both normal and high glucose was significantly reduced by 100 μmol/l ARI-509, to 3.7 ± 0.5 ($p < 0.01$ vs normal glucose without ARI-509) and 7.1 ± 1.1 nmol/mg cell protein ($p < 0.01$ vs high glucose without ARI-509), respectively, indicating that mesangial cell aldose reductase was inhibited.

Effect of high glucose, ARI-509 and phorbol ester on mesangial cell contraction. Figure 1 demonstrates the change in planar surface area of mesangial cells incubated for 24 h in normal glucose stimulated with 0.1 μmol/l ET-1. The planar surface area of mesangial cells in normal glucose prior to stimulation with ET-1 at zero time was 3109 ± 131 μm^2, ($n = 31$ cells). During ET-1 exposure, surface area decreased over 60 min to 52 ± 3 % of the original surface area ($p < 0.05$ vs no agonist). Cells ($n = 20$) incubated in normal glucose plus mannitol demonstrated similar planar area, 3274 ± 132 μm^2 compared to cells in normal glucose and contracted in response to ET-1 to 72 ± 5 % of original volume over 60 min. The cells incubated in high glucose demonstrated a smaller initial planar surface area of 2547 ± 148 μm^2, ($n = 41$ cells, $p < 0.05$ vs normal glucose) and did not respond
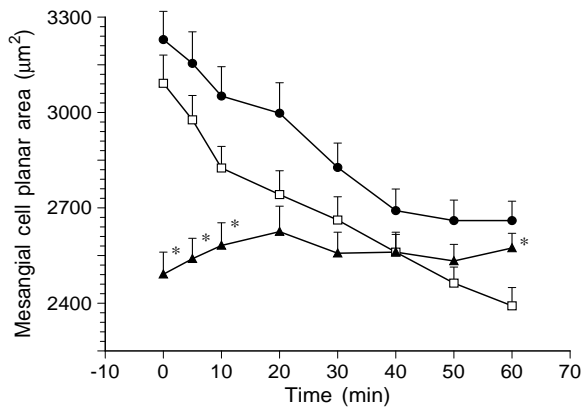
Fig. 1. Effect of high glucose (HG) and ARI-509 (ARI) on endothelin-1–stimulated mesangial cell contraction and change in planar surface area. Mesangial cells were cultured 4 days in either normal glucose (NG) 5.6 mmol/l (□), HG 30 mmol/l (▲) or HG + ARI 100 µmol/l (●), in DMEM supplemented with 20% FBS, then transferred to 20 mm dishes and rendered quiescent for 24 h in DMEM, 0.5% FBS in either NG ($n = 31$ cells), HG 30 mmol/l ($n = 24$), or HG + ARI ($n = 42$ cells). At time 0 min, 0.1 µmol/l endothelin-1 was added to the medium and cell planar surface area was measured serially. Data pooled from three separate experiments are presented as the mean ± SEM for each time point. *$p < 0.05$ vs NG
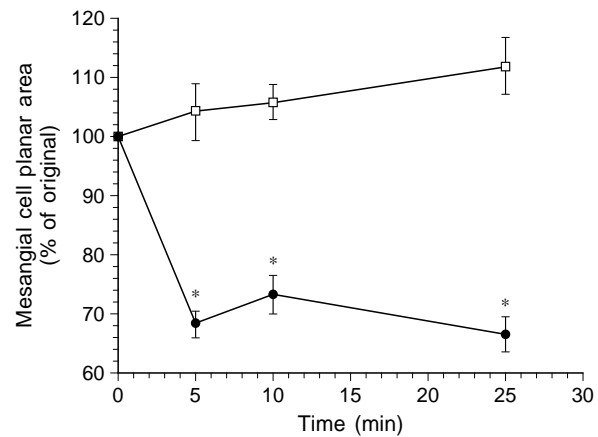

Fig. 2. Phorbol 12-myristate 13-acetate (PMA)-stimulated contraction of mesangial cells. Mesangial cells were cultured 4 days in normal glucose (NG) 5.6 mmol/l, in DMEM supplemented with 20% FBS, then transferred to 20 mm dishes for 24 h. Cell planar area was analysed at the time intervals indicated following addition of 50 pmol/l PMA (●) ($n = 15$ cells) or its vehicle DMSO (□) at final concentration 0.25% (vol/vol) ($n = 20$ cells). The percent of original surface area (100%) was calculated for each cell. Data pooled from two separate experiments are expressed as mean ± SEM for each time point. *$p < 0.05$ vs NG + DMSO

to ET-1 stimulation, see Figure 1. The initial planar surface area of the cells incubated in high glucose plus ARI-509 was 3274 ± 132 µm², ($n = 42$ cells). Treatment with ARI-509 normalized the contractile response to ET-1 of the cells cultured in high glucose which demonstrated a decrease in surface area to $70 \pm 4\%$ of original ($n = 42$ cells, $p < 0.05$ vs no agonist).

Figure 2 indicates that mesangial cells cultured for 24 h in normal glucose, when stimulated acutely with 50 pmol/l PMA, demonstrated decreased surface area to $66 \pm 3\%$ of original ($p < 0.05$ vs no agonist, $n = 15$ cells) over 60 min.

To determine whether ET-1 stimulation of contraction requires PKC, mesangial cells ($n = 30$) were cultured for 24 h in normal glucose, supplemented with 50 pmol/l PMA. Figure 3 demonstrates that when PKC was downregulated, no contractile response to ET-1 was observed. Also, the planar surface area in normal glucose supplemented with 50 pmol/l PMA for 24 h was 2513 ± 125 µm² ($n = 34$) resembling the cells cultured in high glucose.

Cell membrane DAG accumulation. The membrane-associated DAG content of cells incubated in normal glucose for 24 h was 688 ± 113 pmol/mg protein DAG ($n = 11$). No significant change in DAG content was observed at 3 h of high glucose exposure. After 24 h in high glucose, the DAG content was elevated to 1110 ± 49 pmol/mg protein, ($n = 16$, $p < 0.05$ high vs normal glucose). Treatment with ARI-509 prevented
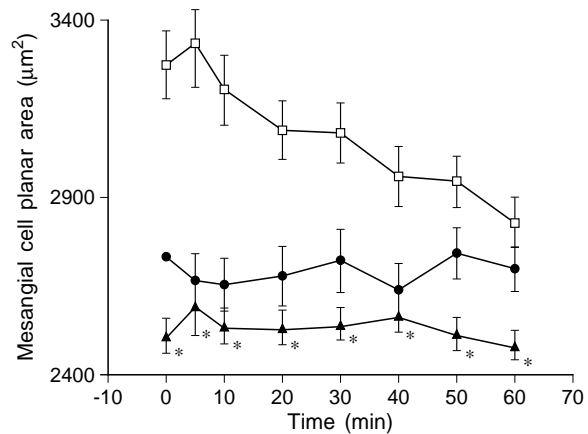

Fig. 3. Endothelin-1–stimulated contraction and planar area of mesangial cells, effect of phorbol 12-myristate 13-acetate (PMA) exposure for 24 h. Mesangial cells were cultured 4 days in normal glucose (NG) 5.6 mmol/l, in DMEM supplemented with 20% FBS, then transferred to 20 mm dishes for 24 h in either NG (□) ($n = 30$ cells), high glucose (HG) 30 mmol/l (●) ($n = 20$ cells), or NG + PMA 50 pmol/l (▲) ($n = 34$ cells). At time 0 min 0.1 µmol/l endothelin-1 was added to the medium and the cell planar area was analysed serially. Data pooled from three separate experiments are presented as the mean ± SEM for each time point. *$p < 0.05$ vs NG

high glucose-mediated increase in DAG measured at 776 ± 168 pmol/mg protein ($n = 6$).

Effect of high glucose, and phorbol ester on PKC activity. As illustrated in Figure 4A, in normal glucose incubation for 15 min with 50 pmol/l PMA increased
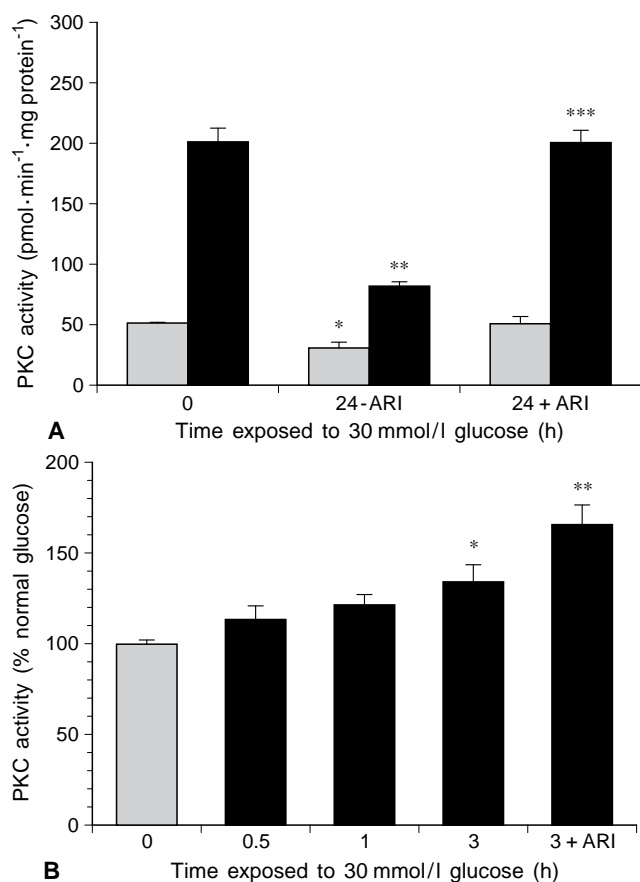
Fig. 4 A, B. Effect of high glucose (HG) on mesangial cell protein kinase C (PKC) activity. Confluent mesangial cells were incubated for 24 h in DMEM (0.5 % FBS) in either normal glucose (NG) 5.6 mmol/l ($n = 40$), HG 30 mmol/l ($n = 40$), or HG + ARI 100 μmol/l ($n = 40$). Following this incubation and additional incubation with 50 pmol/l phorbol 12-myristate 13-acetate (PMA) for 10 min ($n = 20$ from each group), PKC activity was measured in situ in digitonin-permeabilized cells by detecting the phosphorylation (pmol $\cdot$ min^{-1} $\cdot$ mg total cell protein^{-1}) of a PKC-specific peptide substrate (VRKTLRRL). **A** At 24 h of high glucose, basal □ and PMA-stimulated ■ PKC activities were significantly reduced and normalized with ARI-509. **B** High glucose exposure at 3 h caused an increase of basal □, but not PMA-stimulated ■ PKC activity (not shown) compared to normal glucose (0 min). ARI-509 did not alter increased PKC-activity at 3 h. Data are expressed as mean $\pm$ SEM. *$p < 0.05$ vs basal 0 h, **$p < 0.05$ vs PMA-stimulated at 0 h, ***$p < 0.05$ vs PMA-stimulated at 24 h

phosphorylation of the PKC-specific EGF-receptor substrate from 50 ± 3 (basal) to 200 ± 15 pmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}. Exposure to 0.1 μmol/l PDD, an inactive structural analogue of PMA, did not stimulate PKC activity. Exposure of mesangial cells to 0.2 μmol/l calphostin C, an inhibitor of PKC, for 30 min prevented PMA activation of PKC. Similarly, 24 h exposure of mesangial cells to PMA reduced basal activity to 20 ± 7 pmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1} which was unchanged when stimulated with ET-1. Preincubation of cells with 20 μmol/l HA1004, which inhibits cAMP-dependent kinase, for 30 min

did not affect PMA-mediated PKC activation (data not shown). In high glucose at 24 h, basal and PMA-stimulated PKC activities decreased significantly ($p < 0.05$, vs time 0) and remained normal in the presence of ARI-509 (Fig. 4A).

Figure 4B indicates that high glucose exposure for 3 h increased basal PKC activity to 132 ± 7% above normal glucose ($p < 0.05$ vs time 0). No effect of ARI-509 on high glucose-stimulated basal activity was observed.

3H-AA release. In normal glucose, 3H-AA release in the presence of 100 nmol/l EGF plus A23187, as a positive control, was 13.8 ± 0.6% of total cellular 3H-AA and was significantly different from 3H-AA release in the presence of the vehicle, 1.8 ± 0.3% ($n = 7$, $p < 0.05$). When the cells were incubated for 3 h in either normal or high glucose, the unstimulated, basal release was 2.0 ± 0.5% and 1.2 ± 0.3% of total 3H-AA, respectively, ($n = 9$, no significant difference). Cells incubated for 24 h in either normal or high glucose released 1.9 ± 0.4% and 2.1 ± 0.4% of total 3H-AA, respectively ($n = 9$, no significant difference).

Immunoblot mesangial cell PKC-isoforms. Figure 5 illustrates the representative immunoblots of mesangial cell total PKC-α, -δ and -ε in normal and high glucose, with or without ARI-509. Protein loading of 50 μg/lane, observed by Coomassie blue staining, was identical in all lanes. The densitometry data pooled from 5–6 individual experiments are shown. No significant differences in total PKC-α (Fig. 5A), or PKC-δ (Fig. 5B) were observed in high glucose after 24 h or in the presence of ARI-509. No change in total PKC-ε was observed in high glucose after 24 h (Fig. 5C). ARI-509 significantly reduced the content of PKC-ε in both normal and high glucose conditions ($p < 0.05$ vs no ARI-509). No effect of mannitol on PKC-α, -δ or -ε was observed and exposure to PMA for 24 h reduced mesangial PKC-α, -δ and -ε to approximately 50 % of normal recovery on immunoblot (data not shown).

Discussion

Metabolism of excess glucose through the polyol pathway is implicated in the pathogenesis of diabetic complications [31, 32]. The findings of our study link this mechanism to acute changes in mesangial cell contractile function which may have relevance to the early glomerular hemodynamic changes observed in the STZ rat. It was postulated that in high glucose altered PKC function may contribute to polyol pathway-dependent loss of normal mesangial cell contractile responsiveness. In high glucose, the loss of mesangial cell normal contraction in response to ET-1,
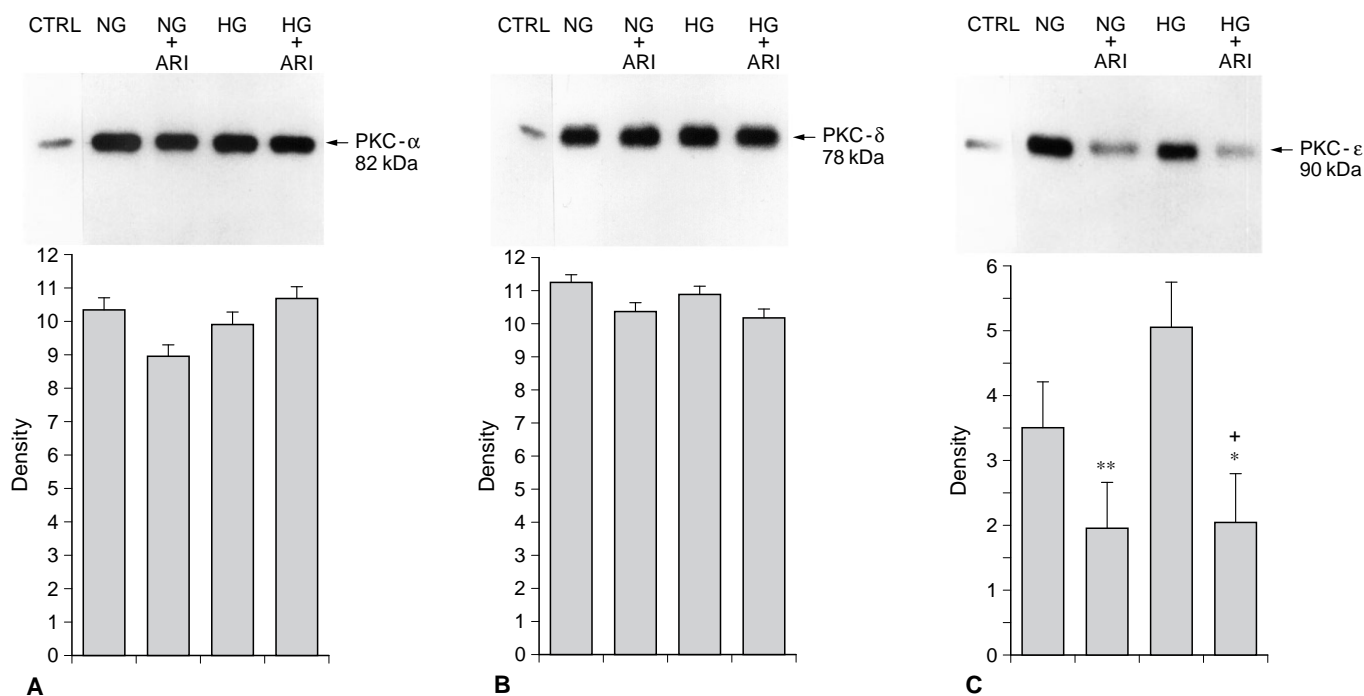
Fig. 5. Effect of high glucose (HG) and aldose reductase inhibition on PKC-isoform accumulation. Mesangial cells were cultured to confluence in normal glucose (NG) 5.6 mmol/l or HG 30 mmol/l, with or without 100 µmol/l ARI-509, then rendered quiescent for 24 h in 0.5 % FBS. Total PKC-isoform content was analysed by Western analysis using primary monoclonal antibodies. Representative immunoblots are shown above and positive PKC-isoforms were used to identify the antibody specificity along with the determination of size from the simultaneously electrophoresed molecular markers. A single band of the molecular size indicated was observed in each blot. The histogram represents the mean ± SEM of band densitometry measured in 5–6 separate experiments. **A** Total PKC-α remained unchanged in high glucose and with ARI-509. **B** Total PKC-δ remained unchanged in high glucose and with ARI-509. **C** Total PKC-ε remained unchanged in high glucose. In the presence of ARI-509, total PKC-ε decreased in both normal and high glucose. *$p < 0.05$ vs HG,**$p < 0.05$ vs NG, [+] not significantly different from NG + ARI

decrease in PKC activity and accumulation of DAG at 24 h are all prevented by aldose reductase inhibition. Early activation of mesangial cell PKC following 3 h exposure to high glucose appears to be independent of the polyol pathway. No alteration in membrane-associated DAG, at least within the sensitivity of our DAG assay, was observed at 3 h suggesting no specific cause and effect relationship between increased PKC activity and de novo synthesis of DAG. In normal glucose, downregulation of PKC activity by phorbol ester rendered mesangial cells smaller and unresponsive to ET-1, similar to the high glucose state. However, the immunoblot data revealed no change in total cellular PKC-α, -δ, and -ε following high glucose exposure for 24 h despite reduced PKC

activity. Therefore, either the moderately increased PKC activity at 3 h was insufficient to downregulate PKC-α, -δ, and -ε, or expression of these isoforms was simultaneously enhanced in high glucose. ARI-509 significantly reduced mesangial cell PKC-ε in both normal and high glucose, with no significant alteration in PKC-α or -δ suggesting a selective effect of the polyol pathway on PKC-ε. Since, in high glucose ARI-509 reduces PKC-ε but restores PKC activity to normal, the EGF-receptor substrate phosphorylation assay may reflect PKC activity excluding PKC-ε. The complex and time-dependent action of high glucose on DAG accumulation and altered PKC activity, as well as the unknown PKC isoform specificity of the PKC-activity assay, may have prevented the identification of a strict cause and effect relationship between high glucose-enhanced DAG synthesis and PKC activity. Nevertheless, the data do support a relationship between the polyol pathway and PKC-dependent altered mesangial cell contractility in high glucose.

The loss of normal glomerular afferent arteriolar tone in diabetes is due to the effect of high glucose on vascular smooth muscle cells. The normalization of STZ rat glomerular arteriolar contractile function in the presence of an aldose reductase inhibitor, has been reported by Bank et al. [33]. More recently polyol-dependent afferent arteriolar dilatation in the galactose-fed hydronephrotic rat has been demonstrated using direct videomicroscopy by Forster et al. [34]. We have also observed that tolrestat-treated hyperglycaemic STZ rats have normal glomerular filtration and renal plasma flow, no increase in microalbuminuria and no glomerular hypertrophy in the first 12 weeks of diabetes [35]. Glomeruli isolated

from tolrestat-treated STZ rats contract normally in response to ET-1 [35]. These in vivo findings implicate the polyol pathway in the mediation of the high glucose effect on renal microcirculation.

Our study examines the status of mesangial cells exposed to high glucose up to 24 h. The time duration was chosen because we focused on the analysis of contractility in vitro. The mesangial cells take 24 h to adhere and spread onto the culture dishes, a requirement for the videomicroscopy measurement of change in cell size. DAG and PKC were analysed in relation to this functional outcome.

Mesangial cell contractility in vitro is well characterized. Mesangial cells in culture retain many important phenotypic characteristics found in vivo, including vasopressor peptide receptor expression, signalling cascades and cytoskeletal protein responses. However, cultured mesangial cells assume a flattened, almost two-dimensional form in contrast to their three-dimensional shape and smaller size in vivo. To observe mesangial cell contraction in response to reorganization and shortening of the cytoskeleton in vitro, the mesangial cells must also partially detach from the substratum. In this study, the finding of smaller mesangial cells unresponsive to ET-1 when cultured in high glucose could be due to a number of changes which are not mutually exclusive. These include partial disassembly of F-actin, as we have observed previously [11], other cytoskeletal changes, such as myosin light chain "pre"-phosphorylation (independent of a signalled event), less cellular attachment or decreased activity of DAG-sensitive PKC-isoform(s) which are necessary for a normal contractile response. ARI-509 normalized mesangial cell size and contractile response to ET-1 in high glucose (Fig. 1A, B), indicating a mechanism related to glucose metabolism through the polyol pathway.

The stimulation of mesangial cells with a vasoactive agent, such as ET-1, initiates a series of signalling events including raised intracellular calcium which is unchanged in high glucose in our hands [8]. Calcium-calmodulin stimulates myosin light chain kinase [36] which phosphorylates myosin light chain. The resultant cross-bridging of myosin light chain to F-actin causes cytoskeletal shortening and cell contraction [14]. Another consequence of vasopressor signalling is disassembly of F-actin from a bundled to more of a network pattern [11]. This complex process involves several reactions including calcium-dependent severing of F-actin filaments, and activation of actin-ATPase [14]. Another potential link between polyol-dependent de novo synthesis of signalling molecules is via lysophosphatidic acid (LPA), a precursor of DAG [37]. LPA stimulates fibroblasts through a G-protein coupled Rho pathway [38]. Rho-dependent inhibition of myosin light chain phosphatase, which indirectly enhances myosin light chain phosphorylation, was reported recently by Kimura et al. [39].

Pre-phosphorylation of myosin light chain, subsequent precontraction and loss of contractile response to ET-1, all prevented by aldose reductase inhibition, could explain the results reported here.

No change in $[^3H]$-AA release in high glucose at 24 h was observed. These data imply that high glucose-induced loss of contractility within 24 h is unlikely to be mediated by AA vasoactive metabolites. The difference between our data and the results of Williams and Schrier [25] who reported a small, but significant increase in $[^3H]$-AA in high glucose, may be explained by differences in experimental conditions. We have studied the effects of high glucose in the growth-arrested state assuming that cell signalling may be modified by the multiple growth factors present in higher concentrations of FBS. As well, the passage of our cells ranged from 10 to 20, compared to less than 10 reported by Williams and Schrier [24]. Also, clonal selection could influence phenotypic expression of signalling factors. In the STZ diabetic rat, increased glomerular (PL)-A_2 activity leads to enhanced synthesis of vasodilatory prostaglandin E_2 [40]. Recently, Trachtman et al. [41] reported that mesangial cells cultured in high glucose demonstrate a decline in nitric oxide and cyclic GMP generation, which could potentiate the vasoconstrictor effect of ET-1. Previously [8], our laboratory reported that preincubation of mesangial cells with either indomethacin or N^G-nitro-L-arginine methyl ester had no effect on high glucose-induced loss of contractility, indicating that altered synthesis of autocrine vasodilatory agents are probably not involved in contractile dysfunction.

In summary, this study identifies the importance of the polyol pathway in the mediation of early high glucose effects on mesangial cells. Aldose reductase inhibition prevents high glucose-induced loss of the mesangial cell contractile response to ET-1. Through the polyol pathway, high glucose also causes mesangial cell DAG accumulation, altered PKC activity and reduced PKC-ε content, one or more of which may contribute to altered mesangial cell contractility. A functional relationship between de novo DAG and increased PKC activity following 3 h exposure to high glucose was not observed. More detailed examination of the effects of high glucose on mesangial cell DAG-sensitive PKC isoform expression and function are required.

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