Regulation of type II transforming-growth-factor-β receptors by protein kinase C ι

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TGF-β (transforming growth factor-β) is implicated in the pathogenesis of diabetic nephropathy. We previously demonstrated that up-regulation of type II TGF-β receptor (TβRII) induced by high glucose might contribute to distal tubular hypertrophy [Yang, Guh,Yang, Lai, Tsai, Hung, Chang and Chuang (1998) J. Am. Soc. Nephrol. **9**, 182–193]. We have elucidated the mechanism by using cultured Madin–Darby canine kidney cells. Enhancer assay and electrophoretic-mobility-shift assay were used to estimate the involvement of transcription factors. Western blotting and an *in vitro* kinase assay were used to evaluate the level and activity of protein kinase. We showed that glucose (100–900 mg/dl) induced an increase in mRNA level and promoter activity of TβRII (note: 'mg/dl' are the units commonly used in diabetes studies). The promoter region -209 to -177 appeared to contribute to positive transactivation of TβRII promoter by comparing five TβRII–promoter–CAT (chloramphenicol acetyltransferase) plasmids. Moreover, the transcription factor AP-1 (activator protein 1) was significantly activated and specifically binds to TβRII promoter (-209 to -177). More importantly, we found that atypical PKC ι might be pivotal for high glucose-induced increase in both AP-1 binding and TβRII promoter activity. First, high glucose induced cytosolic translocation, activation and autophosphorylation of PKC ι. Secondly, antisense PKC ι expression plasmids attenuated high-glucose-induced increase in AP-1 binding and TβRII promoter activity; moreover, sense PKC ι expression plasmids enhanced these instead. Finally, we showed that antisense PKC ι expression plasmids might partly attenuate a high-glucose/TGF-β1-induced increase in fibronectin. We conclude that PKC ι might mediate high-glucose-induced increase in TβRII promoter activity. In addition, antisense PKC ι expression plasmid effectively suppressed up-regulation of TβRII and fibronectin in hyperglycaemic distal-tubule cells.

Key words: diabetic nephropathy, glucose, isoforms, protein kinase C, signal transduction, transforming-growth-factor-β receptor.

INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is a relatively ubiquitous cytokine that functions in an autocrine or paracrine fashion to elicit a multiplicity of effects, principally related to the accumulation of extracellular matrix [1]. Thus TGF-β has been demonstrated to play an important role in diabetic renal fibrosis [2–4]. TGF-β exerts biological effects by interacting with specific cell-surface receptors, including a type I receptor (TβRI, 53 kDa) and a type II receptor (TβRII, 75 kDa) [5,6]. In the extracellular space, TGF-β binds directly to TβRII on the cell membrane. Then the TGF-β–TβRII complex binds to the type I receptor, and finally, phosphorylation of the type I receptor occurs [7]. Phosphorylation activates several cytosolic signalling pathways and regulates multiple TGF-β functions [8]. In this respect, TβRII is essential for the TGF-β signalling pathway. Thus regulation of TβRII might be an alternative therapeutic approach to prevent diabetic renal fibrosis; however, most studies have been focused on the expression of TGF-β1 [9,10].

Hyperglycaemia is the major causal factor in the pathogenesis of diabetic nephropathy. Elevated blood glucose may mediate its adverse effects through protein kinase C (PKC). Studies in isolated glomeruli and in cultured mesangial cells have demonstrated that high ambient concentrations of glucose activate PKC and mediate many biological changes [11,12]. In fact, increased PKC expression has been observed in the diabetic kidney [13]. The family of PKCs includes at least 12 isoforms representing the major downstream targets for lipid second messengers or phorbol esters. These PKC isoenzymes vary in their tissue distribution, cellular distribution and properties [14,15]. To date, no one has examined whether there exist a similar heterogeneity in respect of the expression or role of individual PKC isoenzymes in the expression of TGF-β receptor. Despite the close association between PKC activation and the development of diabetic neuropathy, which has been extensively studied [16], very little work has been done regarding the detailed molecular mechanism of the effects of PKC isoforms in renal-distal-tubule cells under hyperglycaemic conditions.

According to our previous studies showing high-glucose-induced up-regulation of TβRII protein in renal-distal-tubule cells [17], it would be worthwhile to elucidate the potential differential involvement of PKC isoforms in the regulation of TβRII, since PKC is significantly activated in hyperglycaemic kidney as well [13]. So far, TβRII has been reported to be regulated by PKC β

Abbreviations used: AP-1, activator protein 1; CAT, chloramphenicol acetyltransferase; Cdc42, cell-division cycle 42 (a GTP-binding protein); CRE, cAMP response element; DAG, diacylglycerol; EMSA, electrophoretic-mobility-shift assay; FCS, fetal-calf serum; FKBP12, FK506 binding protein 12; HSV-TK, herpes-simplex-virus thymidine kinase; MBP, myelin basic protein; MDCK, Madin–Darby canine kidney; PKC, protein kinase C; pTAL, TATA-like promoter; RT-PCR, reverse transcription PCR; SEAP, a secreted form of human placental alkaline phosphatase; SRE, serum-responsive element; SV40, simian virus 40; TGF-β, transforming growth factor-β; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor.

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[18,19]. In the present study, we utilized PKC-isoform-specific antibodies and an *in vitro* kinase assay to evaluate the differential expression of PKC isoforms. The present study will not only help us to clarify how the TβRII is regulated, but will also provide the groundwork for potentially identifying new therapeutic strategies for diabetic nephropathy.

EXPERIMENTAL

Glucose and actinomycin were purchased from Sigma (St. Louis, MO, U.S.A.). Cell-permeant myristoylated pseudosubstrate inhibitory peptide corresponding to conserved residues in atypical PKC (Myr-SIYRRGARRWRKL) was from Quality Controlled Biochemicals (Hopkington, MA, U.S.A.). TGF-β1 was from R&D Systems (Minneapolis, MN, U.S.A.). The bisindolymaleimide PKC inhibitor Gö 6850 and Protein A/G–agarose gel were from Calbiochem. PKC isoform antibody sampler kit was from BD Transduction Laboratories (Lexington, KY, U.S.A.). CAT (chloramphenicol acetyltransferase) ELISA kit was purchased from Roche Molecular Biochemicals (Laval, Quebec, Canada). Horseradish-peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, U.S.A.). pβgal-control plasmids, Mercury™ Pathway Profiling System, Calphos™ Mammalian Transfection Kit, Great EscAPe™ SEAP kit, first-strand cDNA synthesis kit and GeneClean™ DNA elution kit was purchased from Clontech Laboratories (Palo Alto, U.S.A.). Fibronectin ELISA kit was from Biomedical Technologies Inc (Stroughton, MA, U.S.A.). MDCK (Madin–Darby canine kidney, CCL 34) cells were cultured as described in our previous study [17].

Northern-blot and RT-PCR (reverse transcription PCR)

Northern-blot analysis and RT-PCR were performed as described in our previous study [20]. cDNA probe for TβRII was purified from the RT-PCR reaction. TβRII-specific primers for RT-PCR were designed (TIB Molbiol syntheselabor, Berlin, Germany) according to the mRNA sequence of human (GenBank® accession number M85079), mouse (GenBank® accession number D32072), mice (GenBank® accession number s69114), rattus (GenBank® accession number L09653) and rat (GenBank® accession number s67770) by Primer 3 program® (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The upstream primer was:

5′-(A/T)T(G/T)TCCAC(T/C)TG(C/T)GACAACCA-3′

The downstream primer was:

5′-GCTGA(T/C)GCCTGTCACTTG(A/G)A-3′

The amplification profile was 94 °C for 1 min, 56 °C for 45 s and 72 °C for 2 min. The content of the PCR product (342 bp) was further verified by DNA sequencing. The primer pair for β-actin was based on the mouse sequence [21]. Quality control for RT-PCR was performed as described in our previous study [22].

Construct of plasmids and DNA transfection

TβRII promoter CAT constructs were kindly obtained from Dr Seong-Jin Kim (Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) with some modifications. In brief, DNA constructs were generated by PCR using genomic DNA containing promoter region of the TβRII gene as a template. Amplified DNA fragments were cloned into the promoter-less CAT expression plasmid (pGEM4-SV0CAT) [23]. The sequences of the PCR-generated portions of all constructs were verified by DNA sequencing. In the present study, these constructs were named pTβRII-*n*, where *n* is the distance in nucleotides from the transcription initiation site.The CAT level was determined by an ELISA kit. We used construct pTβRII-CAT ($-1240/+50$) to represent promoter activity for TβRII, since Dr. Kim demonstrated that $-1240/+50$ generated a level of CAT activity similar to that of the longer constructs ($-1420/+50$; $-1670/+50$; and $-1883/+50$) [23]. Transfection was performed using the Calphos™ Mammalian Transfection Kit (Clontech). To normalize the transfection efficiency, pβgal-control plasmids (Clontech), which contain the SV40 (simian virus 40) promoter and enhancer sequence and constitutively express the enzymes were co-transfected in each experiment. The expression level of β-galactosidase was used to normalize CAT expression among different treatment groups. The full-length human PKC ι cDNA was constructed as described in [24]. Cells were transfected with plasmids containing PKC ι in either orientation. Transfectants were screened and verified for PKC ι expression by immunoblotting. Since PKC isoenzymes ι and λ are the same, but from different species, we selected PKC ι for the following assays.

Enhancer assay

We used the Mercury™ Pathway Profiling System (Clontech) to assay the transactivating activity of transcription factors. Each vector contains a specific *cis*-acting DNA binding sequence [SRE (serum-responsive element), AP-1 (activator protein 1), CRE (cAMP response element) or NF-κB (nuclear factor κB)] located upstream from the TATA-like promoter (pTAL) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter followed by a sensitive reporter gene (coding for a secreted form of human placental alkaline phosphatase, SEAP). The negative control vectors (pTAL-SEAP), which contain the same HSV-TK promoter and downstream SEAP reporter gene with no enhancer elements were used to determine the uninduced background levels of enhancer activity. pCTL-SEAP, which contains the SV40 early promoter inserted upstream of the SEAP gene and the SV40 enhancer inserted downstream, was used as a positive control. To normalize transfection efficiency, pβgal-control plasmids were co-transfected in each experiment. SEAP activity was determined using the Great EscAPe™ SEAP kit (Clontech).

Electrophoretic-mobility-shift assay (EMSA)

EMSA was performed according to Xie et al. [25]. The probe was 5′-GAACTGTGTGCACTTAGTCATTCTTGAGTAAAT-3′, which was corresponding to promoter region -209 to -177 of TβRII with putative AP-1 binding site underlined (-196: TTAGTCA) [26]. To demonstrate the specific association with c-Jun protein, nuclear extract was pre-incubated with anti-c-Jun specific antibodies (Santa Cruz, Santa Cruz, CA, U.S.A.) and allowed to react at 37 °C for 2 h prior to reaction with oligonucleotide probe. We used a 100-fold concentration of unlabelled oligonucleotide probe (-209 to -177, designated '100 × cold probes') to verify the specific binding to designed probe and to see if AP-1 complex could be competed for. In addition, we used a 100-fold concentration of unlabelled non-specific scrambled oligonucleotide probe (ATGTACTGAGCTAGTGTTTGACACTGCATTAAT, according to the -209 to -177 sequence of human TβRII promoter, designated '100 × cold NS

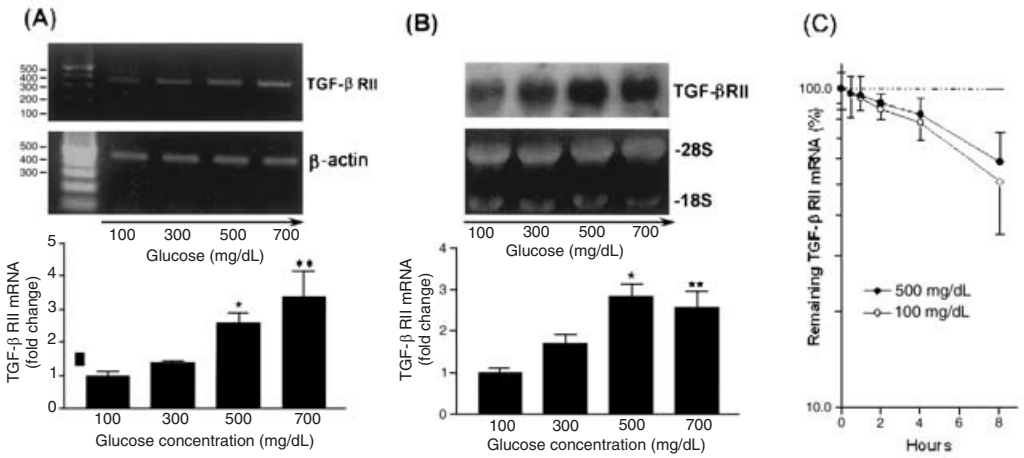

Figure 1 High-glucose culture induces an increase in the mRNA level without affecting mRNA half-life in TβRII MDCK cells

Serum-starved cells were cultured in 100, 300, 500, 700 mg/dl glucose in the presence of 10 % (v/v) FCS (fetal-calf serum) for 48 h. (**A**) RT-PCR and (**B**) Northern hybridization were performed. Expression of β-actin and 28 S rRNA was used as internal control. Results were analysed by a densitometer and are expressed as means $\pm$ S.E.M. for three independent experiments. (**C**) Cells were cultured in 100 (○) or 500 (●) mg/dl glucose for 48 h, followed by adding actinomycin D (5 μmol/l). RNA was harvested at the indicated time point, and RT-PCR analysis of TβRII mRNA was performed. Cellular mRNA levels of the TβRII were quantified, normalized to β-actin, and blotted over time after the addition of actinomycin D. $^{*}P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose.

probes') to verify the specific binding to designed probe and to see if AP-1 complex could not be competed for.

Immunoblotting

Cells were detached by using trypsin/EDTA followed by homogenizing in ice-cold lysis buffer A (20 mM Tris/2 mM EDTA/10 mM EGTA/2 mM dithiothreitol, pH 7.5) in a Polytron homogenizer at 20 000 rev./min for 2 min at 4 °C. The cytosolic fraction was collected by centrifugation with 12 000 ***g*** for 30 min at 4 °C. The pellet was resuspended with lysis buffer B (lysis buffer A supplemented with 1% Triton X-100) and homogenized with a Polytron homogenizer with the same profile. The membrane fraction was collected by centrifugation at 12 000 ***g*** for 30 min at 4 °C. A 10 μg portion of protein from both the cytosolic and the membrane fraction was subjected to SDS/10 %-(w/v)-PAGE as described for our previous studies [17,20].

In vitro kinase assay

Cells manipulated using the above-described condition were trypsinized and washed three times in ice-cold PBS. The cytosolic fraction (200 μg) was collected as described above for Western-blot procedures. Primary antibodies were incubated with the collected fraction with 50-fold (v/v) dilution at 4 °C overnight with gentle shaking. A 20 μl portion of Protein A/G–agarose gel was added and allowed to react at 4 °C for 1 h to immunoprecipitate the immunocomplex. The immunocomplex was washed twice with buffer E [buffer D (50 mM Tris/HCl/150 mM NaCl/5 mM EGTA/15 mM β-glycerophosphate/1 mM Na_3VO_4/1 mM DTT/20 μg/ml leupeptin/50 μM PMSF, pH 7.5) with 1% Triton X-100] and once with kinase washing buffer (50 mM Tris/HCl/1 μM $CaCl_2$/2.5 mM $MgCl_2$/0.03% Triton-X 100, pH 7.5). In the final step, the immunocomplex was solubilized in 50 μl of kinase buffer {kinase washing buffer supplemented with 100 μM [^{32}P]ATP (100–200 c.p.m./pmol), 30 μg/ml phosphatidylserine, 10 μM PMA and 200 μg/ml MBP (myelin basic protein)}. The kinase reaction was performed at 30 °C for 15 min, followed by adding SDS sample buffer to terminate the kinase reaction. The signal was resolved by SDS/10 %-PAGE [17,20]. For the autophosphorylation assay, the MBP substrate was not included in the kinase buffer.

Statistics

The results were expressed as the means $\pm$ S.E.M. Unpaired Student's *t* tests were used for the comparison between two groups. One-way ANOVA, followed by unpaired *t* test was used for the comparison among more than three groups. Two-way ANOVA was used to compare between the two curves in Figure 7. A *P* value of less than 0.05 was considered as statistically significant.

RESULTS

High-glucose culture induced an increase in mRNA level of TβRII in renal-distal-tubule cells

High-glucose dose-dependently increased the mRNA level of TβRII as reflected by RT-PCR (Figure 1A) and Northern hybridization (Figure 1B). Together with the results shown in Figure 1(C), we suggested that overexpression of TβRII protein might be due to the up-regulation of gene expression rather than increasing mRNA half-life.

High-glucose culture induced an increase in promoter activity of TGF-β RII in renal-distal-tubule cells

High-glucose dose-dependently increased the promoter activity of TβRII (Figure 2). Promoter region -209 to -177 appears to contribute to the positive transactivation of TβRII gene under high-glucose treatment, since TβRII–CAT ($-209/+50$) was responsive to high glucose treatment but TβRII–CAT ($-177/+50$) was not.

High-glucose induced differential involvement of transcription factors

AP-1 is the principle transcription factor activated under high-glucose treatment in MDCK cells (Figures 3A, 3B and 3C). Moreover, high-glucose treatment induced a specific binding of transcription factor AP-1 to the promoter region (-209 to -177)

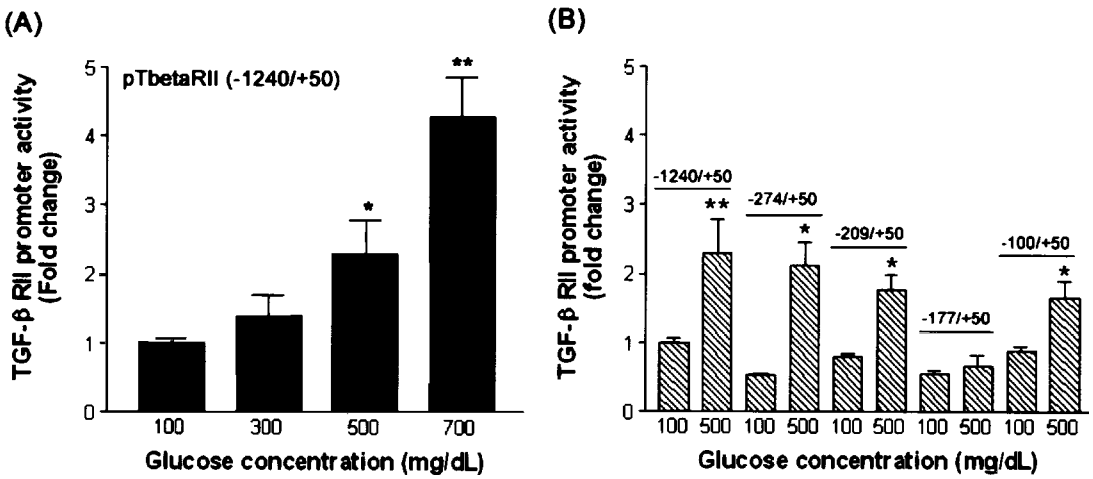

Figure 2 High-glucose culture induces an increase in promoter activity of TGF-β RII in MDCK cells

(**A**) Cells cultured in six-well plates were transfected with 3 μg of TβRII–CAT expression vector/well ($-1240/+50$), followed by serum starvation for 24 h. The cells were then cultured in 100, 300, 500 and 700 mg/dl glucose in the presence of 10 % (v/v) FCS for 48 h. Transfection efficiency in each experiment was normalized by co-transfection with pβgal control plasmids as described in the Experimental section. The CAT level was determined by ELISA kits. Results are expressed as means $\pm$ S.E.M. for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose. (**B**) CAT reporter plasmids containing different promoter regions of TβRII ($-1240/+50$, $-274/+50$, $-209/+50$, $-177/+50$, $-100/+50$) were transfected to cells followed by serum starvation and glucose treatment (100 and 500 mg/dl) as described above. Each experiment was performed in triplicate. It is evident that high glucose induced a significant increase in promoter activity of TβRII in MDCK cells; moreover, the specific promoter region -209 to -177 appears to be associated with the positive transactivation of the TβRII gene under high-glucose treatment.

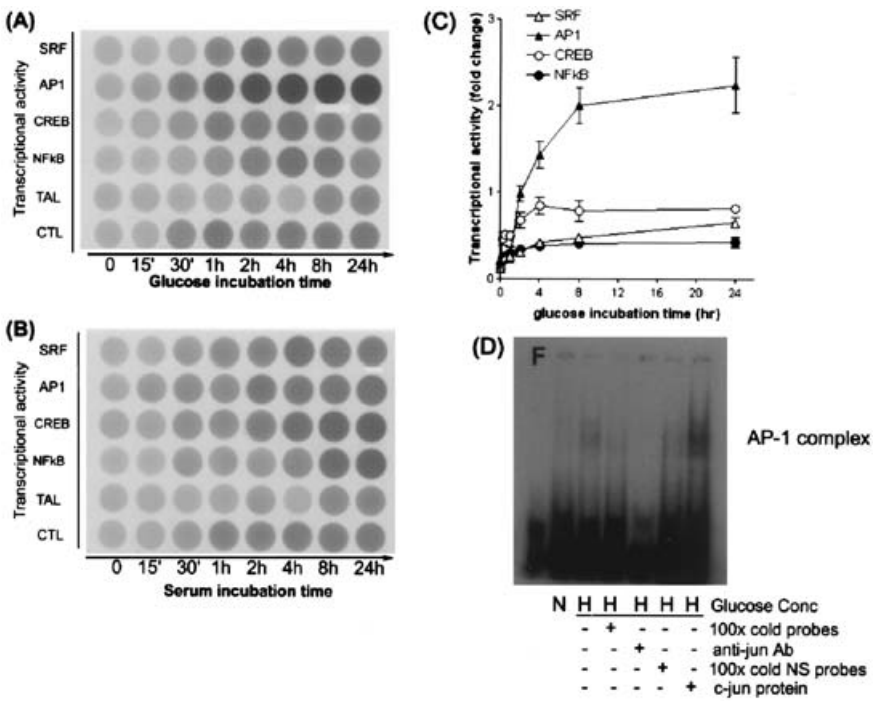

Figure 3 Differential implication of transcription factors under high-glucose treatment

MDCK cells were plated in six-well culture plates and transfected with different reporter vectors (pSRE-SEAP, pAP1-SEAP, pCRE-SEAP, pNFκB-SEAP, pTAL-SEAP and pCTL-SEAP), which contain a specific *cis*-acting enhancer element and a sensitive reporter gene (SEAP). After transfection, cells were treated with 500 (**A**) or 100 (**B**) mg/dl glucose in the presence of 10 % (v/v) FCS. A 15 μl portion of supernatant was collected at the indicated time points [0 min, 15 min (15′), 30 min (30′), 2 h, 4 h, 8 h and 24 h] from the same well. Activation of differential transcription factors was determined by SEAP activity in the culture medium. Dot density was determined by densitometry. Results in (**C**) were obtained by subtracting the respective densities in (**A**) from those in (**B**). Experiments were repeated three times, and similar results were obtained. It is evident that high glucose specifically induced activation in transcription factor AP-1 (▲) in MDCK cells as early as 2 h after glucose treatment. (**D**) A nuclear extract from MDCK cells treated with 500 mg/dl glucose (designated 'H') or 100 mg/dl glucose (designated 'N') for exactly 2 h was collected and subjected to an EMSA assay with specific TβRII promoter region (-209 to -177) as a probe. To ensure the specificity of AP-1 complex, 100 $\times$ cold probes, anti-c-Jun antibodies, 100 $\times$ cold non-specific ('NS') probes and c-Jun protein were used as described in the Experimental section. It is evident that high glucose specifically induced AP-1 binding to TβRII promoter in renal distal tubule cells. Abbreviations: CREB, cAMP-response-element-binding protein; CTL, positive control for transcription factors; NFκB, nuclear factor κB; SRF, serum-responsive factor; TAL, negative control for transcription factors.

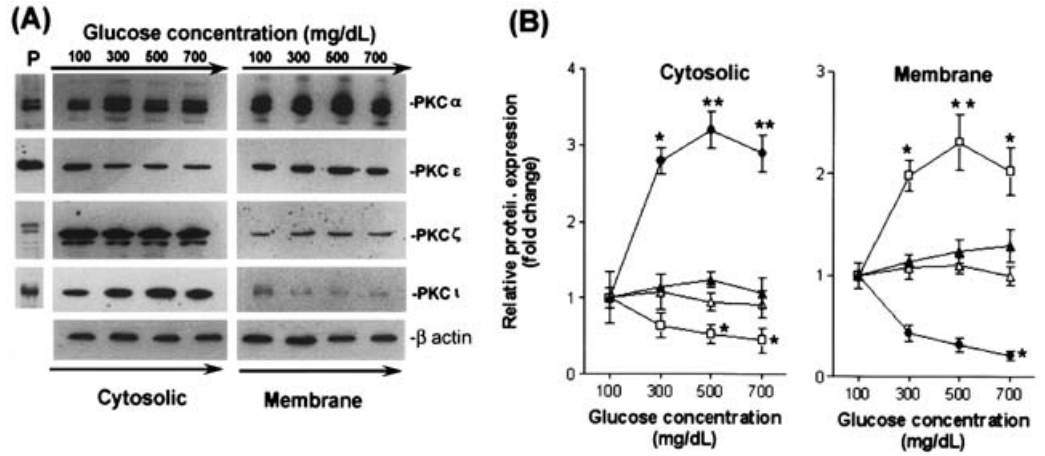

Figure 4 High glucose induces differential translocation of PKC isoenzymes in MDCK cells

Serum-starved cells were cultured in 100, 300, 500 or 700 mg/dl glucose in the presence of 10 % (v/v) FCS for 48 h. The cells were harvested as membrane and cytosolic fractions as described above. (**A**) A 10 μg portion of cell extract from cytosolic or particulate fractions were subjected to immunoblotting assay for detection of PKC α ($\triangle$), ε ($\square$), ζ ($\blacktriangle$), ι ($\bullet$) and β-actin (as loading control) with rat cerebrum lysate as a positive control (designated 'P'). (**B**) Each band was laser-scanned by densitometry and normalized to β-actin. Results are expressed as means $\pm$ S.E.M. for three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose. It is evident that high glucose induced membrane translocation of PKC ε and cytosolic translocation of PKC ι in renal-distal-tubule cells.

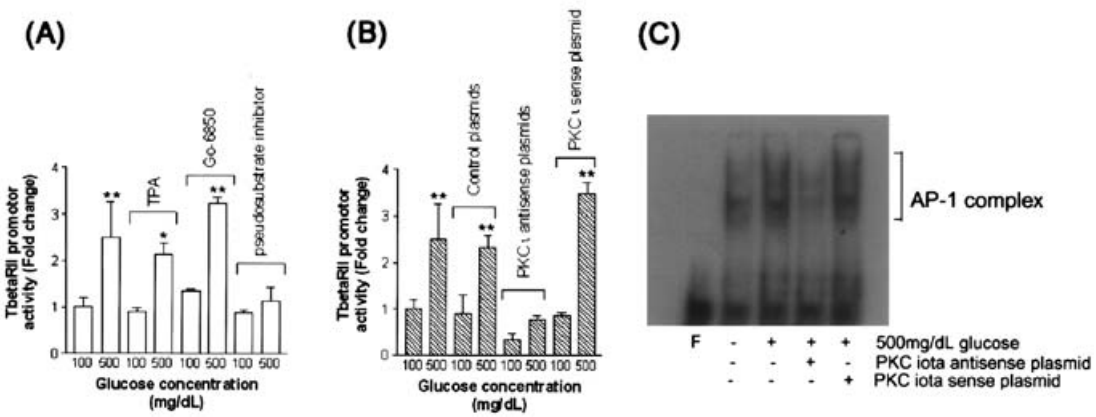

Figure 5 Effect of PKC isoenzyme on TβRII promoter activity and activation of transcription factor AP-1 on high-glucose-cultured distal-tubule cells

(**A**) Cells were cultured in six-well culture plates followed by transfection with 3 μg of TβRII–CAT expression vector ($-1240/+50$)/well. Then cells were starved in 0.5 %-FCS-containing medium, with the addition of different PKC inhibitors (500 nM PMA, 5 μM Gö 6850, and 15 μM pseudosubstrate inhibitor of atypical PKCs) during the last several hours of 24 h starvation (PMA for 18 h, Gö 6850 for 1 h and pseudosubstrate inhibitor for 2 h). Then cells were treated with normal (100 mg/dl)- or high (500 mg/dl)-glucose conditioned medium in the presence of 10 % (v/v) FCS for additional 48 h. CAT expression was determined by ELISA kits. (**B**) Cells were treated as in (**A**). Transfection of 2 μg/well sense/antisense plasmids for PKC ι or their control plasmids (pREP4) was performed at the end of starvation. Results are expressed as means $\pm$ S.E.M. for three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose. (**C**) Nuclear extract from 500 mg/dl-glucose-treated MDCK cells (for 2 h) with/without the transfection of PKC ι sense/antisense expression plasmids was collected and subjected to EMSA assay with a TβRII promoter (-209 to -177; with a consensus AP-1 site) fragment as a double-stranded-DNA probe. It is evident that high glucose activated AP-1 and TβRII promoter activity in a PKC ι-dependent manner in distal-tubule cells. F, nuclear extract is absent ('free') from the lane.

of TβRII, since 100$\times$ cold probes for TβRII could compete for the AP-1 complex and 100$\times$ non-specific probes could not. Furthermore, c-Jun appears to be involved in the up-regulation of TβRII promoter, since c-Jun-specific antibodies could inhibit the formation of AP-1 complex induced by high glucose. Moreover, addition of c-Jun protein in nuclear extract sample could mimic and enhance the formation of the AP-1 complex.

Effects of high glucose on the expression of PKC isoenzyme

We investigated PKC families, since they have been reported to be upstream regulators of AP-1 [27–29]. High-glucose dose-dependently induced membrane translocation of novel PKC ε and cytosolic translocation of atypical PKC ι/λ in renal-distal-tubule cells (Figure 4). Selective translocation of PKC isoforms suggested a distinctive role for PKC ε and ι/λ in high-glucose-induced cellular adaptation.

Involvement of atypical PKC ι in the activation of TβRII promoter

We used Gö 6850 and PMA, since Jacobson et al. [30] have shown that pretreatment with these inhibitors effectively depleted the DAG (diacylglycerol)-sensitive PKC isoform (i.e. conventional and novel PKC). As shown in Figure 5(A), 18 h pretreatment of 500 nM PMA (non-selective inhibition) or 1 h pretreatment of 5 μM Gö-6850 (selective inhibition), did not attenuate the high-glucose-induced increase in TβRII promoter activity. Thus we suggest that the high-glucose-mediated increase in TβRII

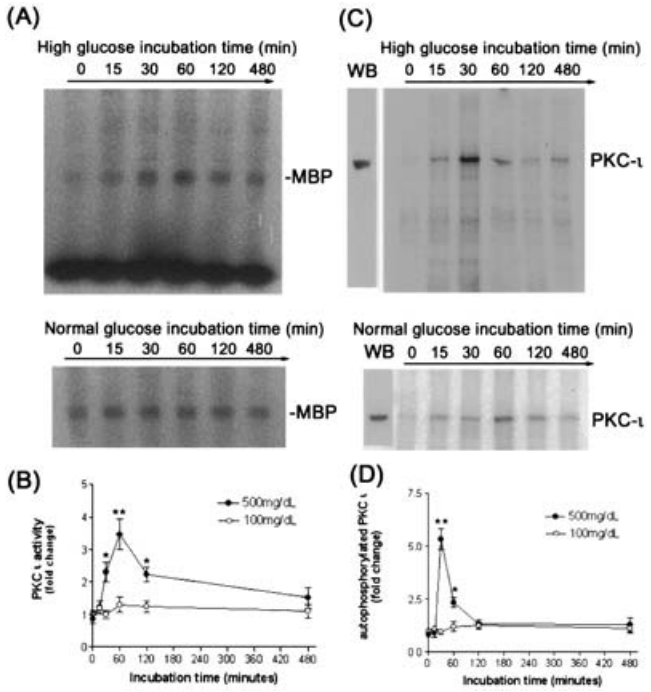

Figure 6 Effects of high-glucose culture on the activity of PKC ι

Serum-starved cells were cultured in 100 (○) or 500 (●) mg/dl glucose in the presence of 10 % (v/v) FCS for the indicated times. (**A**) A 200 μg portion of cell extract from cytosolic fractions were subjected to an *in vitro* kinase assay as described in the Experimental section. MBP was used as a pseudosubstrate for PKC ι. (**B**) Each band from (**A**) was laser-scanned by densitometry. (**C**) The procedures were performed as in (**A**), except that pseudosubstrate was removed from the kinase buffer. Western blotting for PKC ι was used as a positive control (designated WB). (**D**) Each band from (**C**) was laser-scanned by densitometry. Results are expressed as means ± S.E.M. for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose. It is evident that high glucose significantly induced autophosphorylation (maximum at 30 min) and activation (maximum at 60 min) of cytosolic PKC ι in distal-tubule cells.

promoter activity might act through a DAG-insensitive PKC isoform. Moreover, pretreatment of pseudosubstrate inhibitor of atypical PKC dramatically suppressed the high-glucose-induced increase in TβRII activity. Simultaneously, we observed that transfection with antisense PKC ι expression plasmids effectively reversed high-glucose-induced activation in AP-1 (Figure 5C) and increase in TβRII promoter activity (Figure 5B). In contrast, transfection of PKC ι sense plasmids accentuated high-glucose-induced effects instead.

Effects of high-glucose treatment on the activity of PKC ι

As shown in Figure 6(A), high glucose induced a significant increase in kinase activity of cytosolic PKC ι as compared with that treated under normal glucose conditions. Moreover, high glucose induced a dramatic increase in the autophosphorylation level of PKC ι as compared with that treated in normal glucose as shown in Figure 6(B). Simultaneously there exists a correlation in time events, since autophosphorylation of PKC ι (maximal at 30 min) occurs just before the activation of PKC ι (maximal at 60 min).

Antisense PKC ι expression plasmids attenuated TGF-β1 and glucose-induced overexpression of fibronectin in renal-distal-tubule cells

As Figure 7(A) shows, glucose dose-dependently increased the fibronectin level in the absence (○, control) or presence (●, TGF-β1) of TGF-β1. We compared the two groups (Figure 7A, ● and ○) by two-way ANOVA. We found that the glucose-induced fibronectin level was statistically different between the two groups (as shown by the # sign).

More importantly, as shown in Figure 7(B), transfection of antisense PKC ι expression plasmids effectively attenuated the increase in fibronectin expression induced by high glucose or TGF-β1. Thus we suggested that suppressing PKC ι by anti-sense technique might help to attenuate high glucose-induced accumulation of extracellular matrix, possibly by down-regulating TβRII promoter activity. The effect of anti-sense PKC ι was specific, because the antisense PKC ι plasmid attenuated the glucose-induced fibronectin level in both groups (Figure7B). In contrast, the non-specific plasmids did not affect glucose-induced fibronectin level in either group (Figure 7C).

DISCUSSION

Tubulointerstitial changes are as important as glomerulopathy in diabetic nephropathy [31]. Thus although many studies have been performed on the proximal tubules [31,20], the distal nephron has also been found to be relevant, for several reasons. First, significant changes (e.g. Armanni–Ebstein lesion, hyperplasia and hypertrophy) in the distal tubules were present in diabetes [20,31, 32]. Secondly, Na^+/K^+-ATPase is increased in both proximal- and distal-tubular cells [33]. Thirdly, urinary excretion of distal nephron markers (e.g.Tamm–Horsfall protein, epidermal growth factor and kinin–kallikrein) is increased in diabetes [34,35]. In this regard, MDCK cells have been used extensively to represent distal nephron cells. In fact, MDCK cells mimic the distal nephron cells [36], inasmuch as they show the presence of glucose transporter-1 and a lack of sodium/glucose co-transporter and glucose transporter-2 [37]. Moreover, MDCK cells mimic the distal nephron in terms of glucose metabolism [38].

Regulation of TβRII has been reported to be essential for diabetic nephropathy [39–41]. In the present study we showed that high glucose significantly increased promoter activity and the mRNA level of TβRII without affecting its half-life (Figures 1 and 2). In addition, AP-1 was highly correlated with high-glucose-induced TβRII expression (Figures 2 and 3). This observation parallels that by Wolf et al. [43], who demonstrated that the AP-1 site is necessary for transcriptional activity of TβRII in murine proximal-tubular cells under angiotensin II treatment. Moreover, the observation that AP-1 regulated the promoter activity of TβRII was compatible with the results of Bae et al. [26]. They presented an expanded sequence for the promoter region of TβRII and described the existence of two positive regulatory elements (E1: -219 to -172; E2: $+1$ to $+35$) in the TβRII promoter. Thus we supposed that high glucose might induce AP-1 activation followed by selective binding to this AP-1 binding site on the E1 enhancer of the type II TGF-β receptor.

Since little or no work has been carried out on the differential involvement of PKC isoforms in renal-distal-tubule cells, this was considered in the present study. We found that novel and atypical PKCs, instead of the classical PKC, appeared to be responsible for the high-glucose-induced effects in renal-distal-tubule cells (Figure 4). Furthermore, PKC ε (a DAG-sensitive isoform) was the main PKC isoform translocated to membrane fraction under high-glucose culture in distal-tubule cells. This finding parallels that of Sena et al. [44], since they found that treatment with phorbol ester phorbol 12,13-dibutyrate may cause the membrane translocation of PKC ε. Thus it is possible that DAG, enhanced by high glucose, might contribute to the membrane translocation of PKC ε.

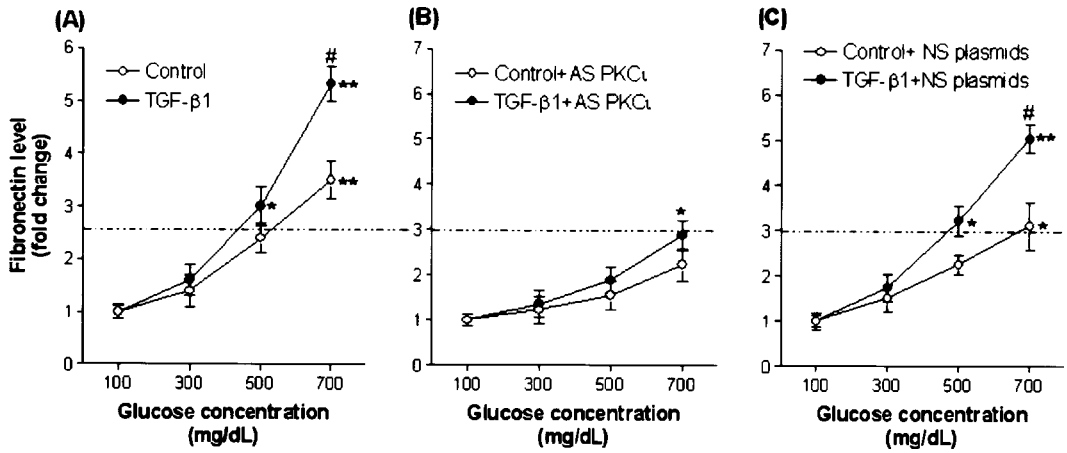

Figure 7 Effects of antisense PKC ι expression plasmids, TGF-β1 and high glucose on fibronectin expression in renal-distal-tubule cells

(A) Cells were starved in unconditioned medium with 0.5 % FCS for 24 h, followed by an additional 48 h of high-glucose treatment (100, 300, 500 or 700 mg/dl) in the presence (●) or absence (○) of 5 ng/ml TGF-β1 in 0.5 % FCS. The fibronectin level in the culture medium was evaluated by ELISA kits. (**B**) Experiment procedures were performed as (**A**), except for a transient transfection of 2 μg/well antisense PKC ι expression plasmid (AS PKC ι) before the addition of TGF-β1. (**C**) Experiment procedures were performed as in (**A**), except for a transfection of control plasmid (pREP4, designated 'NS plasmids') before the addition of TGF-β1. Results are expressed as means $\pm$ S.E.M. for four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus 100 mg/dl glucose. $^{\#}P < 0.05$ (TGF-β1 curve versus control curve) by two-way ANOVA.

Whereas the classic and novel members of the PKC family are expected to participate in the signal transduction that triggers the generation of DAG by activating phospholipase C, the mode of activation and the function of the atypical members are much less clear [45]. In the present study we found that high glucose significantly induced cytosolic translocation of atypical PKC (including PKC ι/λ instead of ζ). Moreover, cytosolic translocated PKC ι exhibited a marked increase in kinase activity and autophosphorylation level after high-glucose treatment (Figure 6). This observation is unique, since it is the first time that PKC ι has been found to be correlated with diabetic renal disease. In fact, some studies have revealed that an atypical PKC might be as essential in regulating gene expression as are other isoforms. Idris et al. [46] showed that atypical PKC is involved in the regulation of glucose transport and oxidation. Furukawa et al. [47] demonstrated that activation of atypical PKC ζ by high glucose could result in transcriptional activation of the human insulin gene promoter. However, little work has been done in elucidating roles of atypical PKC in the pathogenesis of diabetic nephropathy and renal fibrosis.

The cytosolic translocation and activation of PKC ι under high glucose (Figures 4 and 6) was compatible with the following two observations. First, Zoukhri et al. [48] demonstrated PKC ι/λ was mainly located at a cytosolic compartment under stimulation. Secondly, Nishizuka [49], and Hug and Sarre [50] showed that the atypical members of the PKC family are biochemically unable to (and are not needed to) to bind and to respond to phorbol esters and membrane-bound DAG. In other words, membrane translocation was not a prerequisite for atypical PKC to achieve its maximum activation. In fact, a significant increase in kinase activity of atypical PKC ι was induced in the cytosolic compartment under high-glucose treatment (Figure 6). In addition, we speculate that cdc42 (cell-division cycle 42, a GTP-binding protein) might be involved in the cytosolic activation of PKC ι, since we observed that glucose dose-dependently (5.5–38.5 mM) induced increases in GTP-bound cdc42 in MDCK cells (S.-F. Liu, J.-S. Huang, Y.-L. Huang, C.-F. Lin and Yu.-L. Yang, unpublished work), and cdc42 has been shown to be associated with, and result, in the translocation of PKC ι from the nucleus into the cytosol [51].

More importantly, we hereby show, by transfection of sense/antisense PKC ι expression plasmids, a causal relationship between PKC ι and TβRII expression (Figure 5). To our knowledge we show, for the first time, that activation of atypical PKC ι (cytosolic translocation, activation and autophosphorylation) is involved in the regulation of TβRII gene expression. In addition to PKC, a number of cytoplasmic proteins, e.g., FKBP12 (FK506-binding protein 12), STRAP (serine/threonine-kinase-receptor-associated protein) and TRAP-1 (tumour-necrosis-factor-receptor-associated protein 1), can interact and regulate the kinase activity of the TGF-β receptors [52]. Among these proteins, FKBP12 appears to be correlated with PKC, since FKBP12 has been shown to have sequence similarity to an endogenous inhibitor PKC; moreover, FKBP12 significantly inhibits PKC activity [53]. However, it is presently unclear whether high glucose induced the activation of PKC ι/λ and up-regulation of TβRII is associated with FKBP12.

By transfection of PKC ι expression plasmids, we observed that promoter activity of TβRII appeared to be concomitant with the specific binding of AP-1 to the TβRII promoter (-209 to -177), as shown in Figures 5(B) and 5(C). Thus it is possible that PKC ι might mediate high-glucose-induced up-regulation of the TβRII promoter by AP-1. However, it is presently not clear whether specific AP-1 binding of the TβRII promoter is directly associated with gene expression. It is possible that: (i) AP-1 binding directly activated the TβRII gene; (ii) AP-1 activated a second series of genes whose products were responsible for activation of TβRII gene; or (iii) AP-1 is irrelevant to TβRII expression and other protein (e.g. PKC ι) directly mediate these effects. In this regard, further studies need to be performed on the interaction between specific binding of AP-1 and regulation of TβRII gene expression under high-glucose status.

Type II TGF-β receptors have been considered an additional control point in mediating the glucose-induced increase in extracellular-matrix deposition [41]. Thus, in the present study, we wondered whether regulating the expression of PKC ι might be helpful in suppressing high-glucose- or TGF-β1-induced accumulation of extracellular matrix. As shown in Figure 7, treatment with antisense PKC ι expression plasmids partly attenuated high-glucose- and TGF-β1-induced increases in the level of fibronectin.

Taken together with the observations shown in Figure 5, we believe that modulating gene expression of PKC ι would be a feasible approach for treating diabetic renal fibrosis. However, this observation should be further elucidated in an animal model. The present paper provides a basis for understanding the molecular events and interactions between atypical PKC ι and the expression of type II TGF-β receptor in hyperglycaemic renal-distal-tubule cells.

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