High glucose/lysophosphatidylcholine levels stimulate extracellular matrix deposition in diabetic nephropathy via platelet-activating factor receptor

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Abstract. Platelet-activating factor (PAF), protein kinase C (PKC)βI, transforming growth factor (TGF)-β1 and aberrant extracellular matrix (ECM) deposition have been associated with diabetic nephropathy (DN). However, the mechanistic basis underlying this association remains to be elucidated. The present study investigated the association among the aforementioned factors in a DN model consisting of human mesangial cells (HMCs) exposed to high glucose (HG) and lysophosphatidylcholine (LPC) treatments. HMCs were divided into the following treatment groups: Control; PAF; PAF+PKCβI inhibitor LY333531; HG + LPC; PAF + HG + LPC; and PAF + HG + LPC + LY333531. Cells were cultured for 24 h, and PKCβI and TGF-β1 expression was determined using the reverse transcription-quantitative polymerase chain reaction and western blotting. The expression levels of the ECM-associated molecules collagen IV and fibronectin in the supernatant were detected using ELISA analysis. Subcellular localization of PKCβI was assessed using immunocytochemistry. PKCβI and TGF-β1 expression was increased in the PAF + HG + LPC group compared with the other groups (P<0.05); however, this effect was abolished in the presence of LY333531 (P<0.05). Supernatant fibronectin and collagen IV levels were increased in the PAF + HG + LPC group compared with the others (P<0.05); this was reversed by treatment with LY333531 (P<0.05). In cells treated with PAF, HG and LPC, PKCβI was translocated from the cytosol to the nucleus, an effect which was blocked when PKCβI expression was inhibited (P<0.05). The findings of the present study demonstrated that PAF stimulated ECM deposition in HMCs via activation of the PKC-TGF-β1 axis in a DN model.

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Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) in diabetes, with an incidence of 20-40% worldwide (1,2). DN is characterized by progressive renal interstitial fibrosis. A previous study reported that high glucose (HG) and lysophosphatidylcholine (LPC) levels were associated with the development and progression of DN (3); these two factors have been demonstrated to stimulate platelet-activating factor (PAF) expression and extracellular matrix (ECM) secretion by the mesangial cells (MCs) of the kidney (4).

Protein kinase C (PKC)βI is an isoenzyme in the PKC family and is involved in a number of biological processes, including cell proliferation, differentiation, apoptosis and angiogenesis (5), in addition to having a role in the pathogenesis of DN (6,7). PKC is aberrantly activated in the diabetic kidney, which leads to an increase in PKCβI activity and deposition of ECM proteins, including fibronectin (Fn) and collagen (Col) type IV (8,9). In addition, transforming growth factor (TGF)-β1 has an important role in ECM accumulation during renal fibrosis (10), and it has been implicated in the occurrence of DN (11-13). However, the underlying molecular mechanism between PAF, PKC, TGF-β1 and the ECM in DN remains to be elucidated. The present study investigated the association among the aforementioned factors in a DN model consisting of human (H)MCs exposed to high HG) and LPC treatments. Reverse transcription-quantitative polymerase chain reaction and western blotting was used to detect PKCβI and TGF-β1 expression, and then an ELISA assay was used to detect the

expression levels of the ECM-associated molecules collagen IV and fibronectin in the supernatant. To clarify the function of PKCβI, immunocytochemistry was used to demonstrated the subcellular localization of PKCβI. The results of the present study suggested that PAF stimulated ECM deposition in HMCs via activation of the PKC-TGF-β1 axis in a DN model.

Materials and methods

Cell culture. HMCs donated by the Zhongda Hospital affiliated with Southeast University (Nanjing, China) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere containing 5% CO_2 at 37˚C.

The cells were divided into six groups: Control (5.5 mM D-glucose; Enzo Life Sciences, Inc., Farmingdale, NY, USA); PAF ($2x10^{-8}$ M PAF C-16; Cayman Chemical Company, Ann Arbor, MI, USA); PAF + PKCβI inhibitor LY333531 (Enzo Life Sciences, Inc.; $2x10^{-8}$ M PAF and $2x10^{-7}$ M LY333531); HG + LPC (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 30 mM D-glucose and 20 mg/l LPC); PAF + HG + LPC ($2x10^{-8}$ PAF, 30 mM D-glucose and 20 mg/l LPC); and PAF + HG + LPC + LY333531 ($2x10^{-8}$ PAF, 30 mM D-glucose, 20 mg/l LPC and $2x10^{-7}$ M LY333531) (4).

ELISA analysis. The expression levels of Fn and Col IV in the cell culture supernatants were detected using specific ELISA kits (cat nos. CSB-EL005745HU and CSB-E04551h) according to the manufacturer's protocol (JingMei Biotech, Shenzheng, China). Samples were analyzed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR assay was performed using a SYBR_Premix ExTaq II kit (Takara Biotechnology Co., Ltd., Dalian, China) was performed using in the CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the relative expression levels of target genes. The sequences of forward and reverse primers: PKCβI, 5'-GGG GGCGACCTCATGTAT-3' and 5'-GCAATTTCTGCAGCG TAAAA-3'; and GAPDH, 5'-ACACCCACTCCTCCACCT TT-3' and 5'-TTACTCCTTGGAGGCCATGT-3'. Primers were designed using Premier Oligo version 5 and Primer version 6.22 (Premier Biosoft International, Palo Alto, CA, USA). The thermocycling program used was as follows: 95˚C for 30 sec, followed by 40 cycles of 60˚C for 30 sec and 72˚C for 30 sec. Relative changes in expression level were calculated using the quantification cycle ($2^{-\Delta\Delta Cq}$) method (14). Each sample was prepared in triplicate and the results are expressed as the mean of three independent experiments.

Western blotting. Cells were resuspended in lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min and sonicated for 2 min at 20 W, followed by centrifugation at 12,000 x g for 10 min at 4˚C. The supernatant was collected and 50 μg/lane protein (concentration determined using the bicinchoninic assay kit (Thermo Fisher Scientific, Inc.) was separated using SDS-PAGE on a 10% gel (Bio-Rad Laboratories, Inc.) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.), which was blocked in Tris-buffered saline/Tween-20 (TBST) with 5% non-fat milk for 1 h at 37˚C. The membrane was subsequently incubated with primary antibodies against TGF-β1 (cat no. sc-146; 1:2,000), PKCβI (cat no. sc- 209; 1:1,000) and GAPDH (cat no. sc-25778; 1:500) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Following washing with TBST, the membranes were incubated with a horseradish peroxidase-conjugated labeled goat anti-rabbit secondary antibody (cat no. sc-2004; 1:500; Santa Cruz Biotechnology, Inc.) for 1 h at 4˚C, followed by additional three washes with TBST. Protein bands were visualized by enhanced chemiluminescence (GE Healthcare, Chicago, IL, USA). The Scion Image system version 4.03 (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensity and data are expressed as the mean of three independent experiments.

Immunocytochemistry. Cells ($2x10^4$/ml) were cultured on coverslips in 24-well plates for 24 h, and subsequently fixed with 4% paraformaldehyde for 5 min at -20˚C and blocked at room temperature for 30 min in 0.2% Triton X-100 in PBS. The cells were incubated with anti-PKCβI antibody (1:50) (cat no. 07-870; EMD Millipore, Billerica, MA, USA) overnight at 4˚C, followed by fluorescein isothiocyanate-conjugated secondary antibody (1:400; cat no. K532511-8; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 h in the dark at room temperature. Following three washes in PBS, coverslips were placed on the slides and the cells were visualized using confocal microscopy. Fluorescence intensity (wavelength of 490 nm) was analyzed using Image J software (version number: 1.48u; (National Institutes of Health).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Data were analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were assessed using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PKCβI expression is upregulated in HMCs in the presence of PAF, HG and LPC. PKCβI mRNA expression level was increased in the PAF, HG + LPC, and PAF + HG + LPC groups compared with control group ($P<0.05$). The expression was increased in the PAF + HG + LPC group compared with cells treated with HG and LPC alone ($P<0.05$), this increase in PKCβI expression was reversed by treatment with the PKCβI inhibitor LY333531 ($P<0.05$; Table I; Fig. 1).

A similar trend was observed for PKCβI protein expression, which was increased in the PAF, HG + LPC and PAF + HG + LPC groups compared with control cells ($P<0.05$; Fig. 2). The observed upregulation in PKCβI expression levels was reduced following treatment with LY333531 ($P<0.05$).

TGF-β1 expression is upregulated in HMCs in the presence of PAF, HG and LPC. TGF-β1 mRNA (Table II; Fig. 3) and

Table I. PKCβI mRNA expression in each treatment group.

Group	Expression
Control	1.00±0.00
PAF	2.68±0.17[a]
PAF + LY333531	1.85±0.39[a,b]
HG + LPC	2.12±0.31[a]
PAF + HG + LPC	3.59±0.41[a]
PAF + HG + LPC + LY333531	2.76±0.57[a,c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. PAF group; [c]P<0.05 vs. PAF + HG + LPC group. PKCβI, protein kinase C βI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

Table II. TGF-β1 mRNA expression in each treatment group.

Group	Expression
Control	1.00±0.00
PAF	1.84±0.11[a]
PAF + LY333531	1.02±0.15[b]
HG + LPC	1.88±0.21[a]
PAF + HG + LPC	2.25±0.09[a]
PAF + HG + LPC + LY333531	1.95±0.11[a,c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. PAF group; [c]P<0.05 vs. PAF + HG + LPC group. TGF-β1, transforming growth factor-β1; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

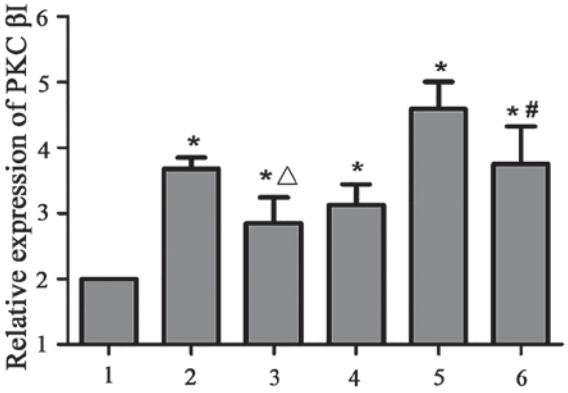

Figure 1. PKCβI mRNA expression in human mesangial cells in various treatment groups. Expression levels were determined relative to GAPDH using the reverse transcription-quantitative polymerase chain reaction. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. PKCβI, protein kinase CβI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

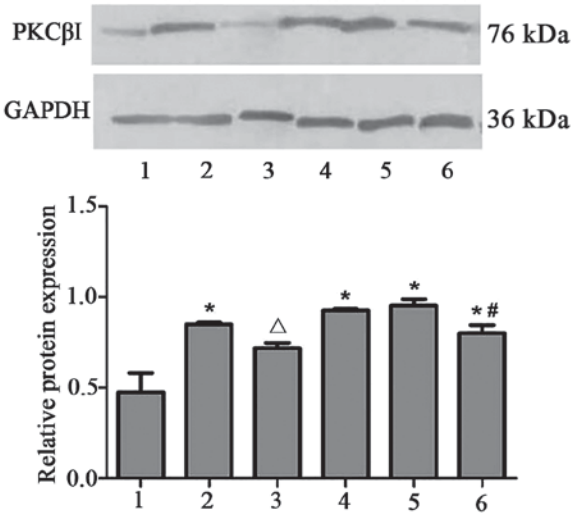

Figure 2. PKCβI protein expression in human mesangial cells under various treatment conditions. The protein expression level was determined using western blotting, with GAPDH used as a loading control. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. PKCβI, protein kinase CβI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

protein (Fig. 4) expression levels were upregulated in HMCs treated with PAF, HG and LPC, compared with the control (P<0.05). The increased expression was not observed in the presence of LY333531.

ECM production is induced in HMCs in the presence of PAF, HG and LPC. The expression levels of two ECM proteins, Fn and Col IV, in the supernatant of cultured HMCs were significantly upregulated following treatment with PAF, HG and LPC, compared with the control group (P<0.05; Fig. 5), with increased levels observed in cells treated with all three factors compared with HG and LPC group (P<0.05). This effect was reduced following treatment with LY333531 (Table III).

PKCβI protein is translocated from the cytoplasm to the nucleus of HMCs following treatment with PAF, HG and LPC. In the control group, PKCβI was diffusely distributed throughout the cytoplasm, with no membrane or nuclear localization. Treatment with PAF, HG and LPC increased PKCβI protein levels, and induced the translocation of the protein from the cytoplasm to the nucleus(P<0.05). Treatment with LY333531 did not alter in the subcellular localization of PKCβI protein (Table IV; Figs. 6 and 7).

Discussion

Diabetes mellitus is an important public health concern, especially in developed countries (15), with DN being the primary cause of ESRD worldwide (16-19). DN is caused by nerve damage resulting from ECM deposition, mesangial expansion and basement membrane thickening (20). The accumulation of Fn and Col IV underlies chronic kidney diseases, including progressive renal interstitial fibrosis (21). Metabolic disorders, such as hyperlipidemia and hyperglycemia, are associated with the occurrence and development of DN, with increased glucose and fat levels having an adverse effect on glomerular

Table III. Expression of the extracellular matrix components Fn and Col IV in the different treatment groups.

Group	Fn, mg/l	Col IV, μg/l
Control	3.90±0.43	4.54±0.74
PAF	7.05±0.05[a]	13.71±0.88[a]
PAF + LY333531	3.81±0.13[b]	5.31±0.81[b]
HG + LPC	7.89±0.34[a,c]	16.32±1.55[a,c]
PAF + HG + LPC	9.11±0.10[a]	22.89±0.34[a]
PAF + HG + LPC + LY333531	5.23±0.24[a,c]	11.40±0.72[a,c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. PAF group; [c]P<0.05 vs. PAF + HG + LPC group. Fn, fibronectin; Col IV, collagen type IV; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

Table IV. Mean fluorescence intensity of PKCβI in human mesangial cells under various treatment conditions.

Group	Mean fluorescence intensity
Control	11.80±2.57
PAF	41.14±7.21[a]
PAF + LY333531	20.19±3.60[b]
HG + LPC	48.92±7.70[a]
PAF + HG + LPC	54.45±3.57[a]
PAF + HG + LPC + LY333531	42.50±5.70[a,c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. PAF group; [c]P<0.05 vs. PAF + HG + LPC group. PKCβI, protein kinase CβI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

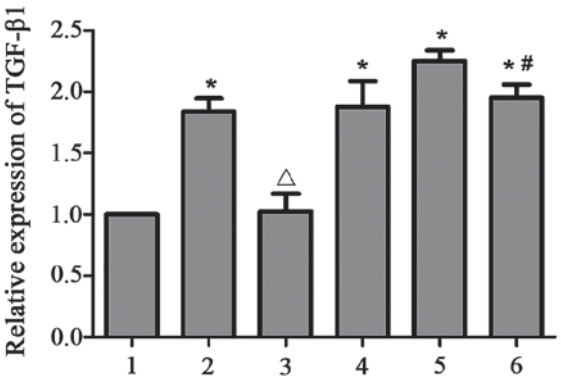

Figure 3. TGF-β1 mRNA expression in human mesangial cells under various treatment conditions. Expression levels were determined relative to GAPDH using the reverse transcription-quantitative polymerase chain reaction. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. TGF-β1, transforming growth factor-β1; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

capillary endothelial cells and MCs, in addition to podocytes in the kidney (22), via stimulation of ECM secretion (23) mediated by TGF-β/mothers against decapentaplegic homolog

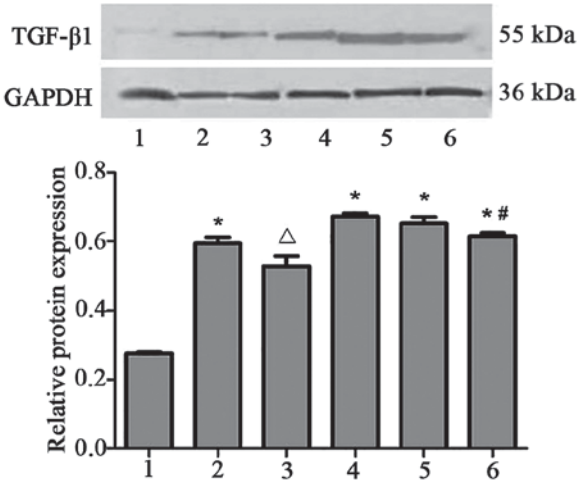

Figure 4. TGF-β1 protein expression in human mesangial cells under various treatment conditions. Protein expression level was determined using western blotting, with GAPDH used as a loading control. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. TGF-β1, transforming growth factor-β1; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

3 signaling. A HG/high fat diet may upregulate Fn and Col IV expression, which may alter the structure and function of renal tubules and lead to renal tubulointerstitial fibrosis (24). PAF is a lipid polymer, involved in the metabolism of arachidonic acid, that has a role in DN by stimulating Fn secretion (25). The present study determined that Fn and Col IV secretion were stimulated by PAF, HG and LPC, consistent with previous studies (8,26,27). The findings of the present study supported the hypothesis that HG and LPC may be risk factors for renal fibrosis and DN.

PKC is a serine/threonine kinase expressed in various mammalian tissues, which regulates a number of signaling pathways (28,29). The present study revealed that PKC was diffusely distributed throughout the cytoplasm in untreated HMCs and translocated to the nucleus in the presence of PAF, HG and LPC. DN may be delayed or prevented by inhibiting PKC (30,31); enlargement of kidney volume and renal fibrosis were rescued by PKCβI-knockout in a mouse model of DN (8).

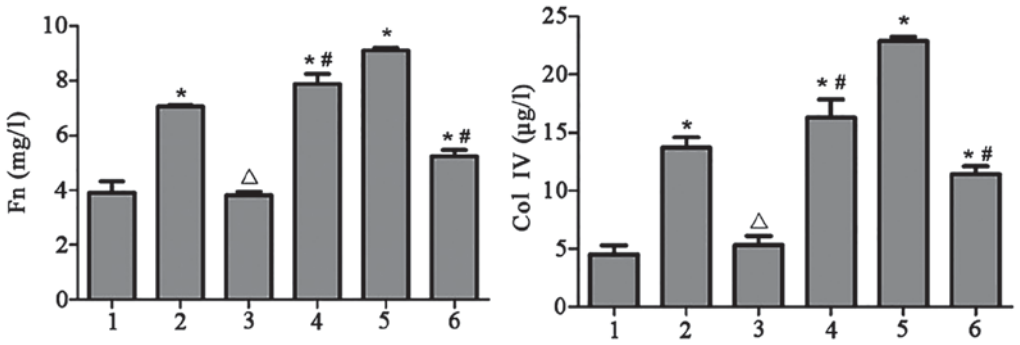

Figure 5. Fn and Col IV levels in human mesangial cell culture supernatants, as detected by ELISA analysis. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. Fn, fibronectin; Col IV, collagen type IV; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

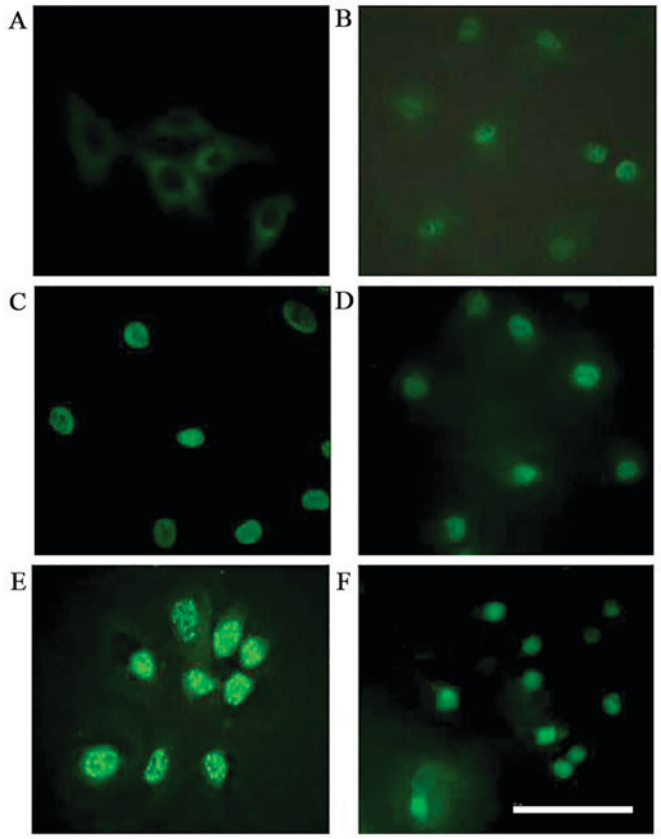

Figure 6. Immunocytochemical analysis of PKCβI localization in human mesangial cells under various treatment conditions. PKCβI was detected by immunocytochemistry and visualized by confocal microscopy in the (A) control, (B) PAF, (C) PAF + LY333531, (D) HG + LPC, (E) PAF + HG + LPC, and (F) PAF + HG + LPC + LY333531 groups. Scale bar, 30μm. PKCβI, protein kinase CβI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

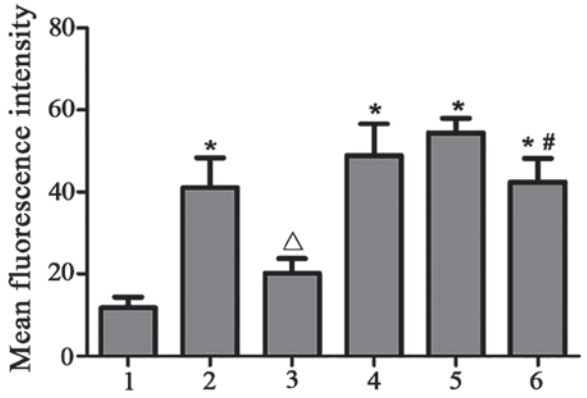

Figure 7. Subcellular localization of PKCβI protein in human mesangial cells under various treatment conditions, based on mean fluorescence intensity. 1, control; 2, PAF; 3, PAF + LY333531; 4, HG + LPC; 5, PAF + HG + LPC; 6, PAF + HG + LPC + LY333531. Data are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control group; ΔP<0.05 vs. PAF group; #P<0.05 vs. PAF + HG + LPC group. PKCβI, protein kinase CβI; PAF, platelet activating factor; LY333531, PKCβI inhibitor; HG, high glucose; LPC, lysophosphatidylcholine.

LY333531 is a Food and Drug Administration-approved inhibitor of PKC-B (32), which has been demonstrated to promote myocardial angiogenesis in diabetes (33) and improve albuminuria and other pathological features in DN rats via inhibition of PKC expression (34). Treatment with LY333531 was demonstrated to reduce mesangial matrix expansion and decrease the urinary protein excretion rate in diabetic mice (35). In the present study, LY333531 treatment prevented the nuclear localization of PKCβI protein in the presence of PAF, HG and LPC, which corresponded to the decrease in Fn and Col IV secretion. The findings of the present study suggested that PKCβI may have an important role in ECM deposition by HMCs in DN.

TGF-β1 is a TGF-β superfamily member which regulates a variety of cellular processes, including proliferation, differentiation and apoptosis (36,37). TGF-β1 has an important role in kidney hypertrophy (26), glomerular and renal tubular basement membrane thickening, and renal tubulointerstitial fibrosis (38,39), and previous studies have suggested that it may modulate ECM secretion in DN. For example, plasmacytoma variant translocation 1 was demonstrated to increase plasminogen TGF-β1 in addition to Fn expression in MCs (40), whereas TGF-β1 inhibited the expression of microRNA (miR)-26a to modulate DN progression in diabetic mice (41). ECM accumulation was increased via upregulation of miR-1207-5p in the presence of glucose and TGF-β1, which was implicated in DN pathogenesis (42). Additionally, Fn and Col IV levels were suppressed by the knockdown of TGF-β1 (43). The present study revealed that TGF-β1 mRNA and protein expression

levels were upregulated in HMCs, following treatment with PAF, HG and LPC compared with the control group, which was accompanied by increased Fn and Col IV secretion; these effects were abolished by treatment with LY333531.

In conclusion, the findings of the present study suggested that ECM deposition by MCs may be induced by HG and LPC treatment and activation of PKCβI–TGF-β1 signaling via PAF. Increased ECM deposition increases the risk of glomerular fibrosis and DN in individuals with disorders of glucose and lipid metabolism. The present findings reveal novel strategies for managing DN by targeting the PKC-TGF-β1 signaling pathway in MCs.

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