

Characterization of Protein Kinase C β Isoform Activation on the Gene Expression of Transforming Growth Factor- β , Extracellular Matrix Components, and Prostanoids in the Glomeruli of Diabetic Rats

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Abstract

Induction of protein kinase C (PKC) pathway in the vascular tissues by hyperglycemia has been associated with many of the cellular changes observed in the complications of diabetes. Recently, we have reported that the use of a novel, orally effective specific inhibitor of PKC β isoform (LY333531) normalized many of the early retinal and renal hemodynamics in rat models of diabetes. In the present study, we have characterized a spectrum of biochemical and molecular abnormalities associated with chronic changes induced by glucose or diabetes in the cultured mesangial cells and renal glomeruli that can be prevented by LY333531. Hyperglycemia increased diacylglycerol (DAG) level in cultured mesangial cells exposed to high concentrations of glucose and activated PKC α and β 1 isoforms in the renal glomeruli of diabetic rats. The addition of PKC β selective inhibitor (LY333531) to cultured mesangial cells inhibited activated PKC activities by high glucose without lowering DAG levels and LY333531 given orally in diabetic rats specifically inhibited the activation of PKC β 1 isoform without decreasing PKC α isoform activation. Glucose-induced increases in arachidonic acid release, prostaglandin E_2 production, and inhibition of Na^+ - K^+ ATPase activities in the cultured mesangial cells were completely prevented by the addition of LY333531. Oral feeding of LY333531 prevented the increased mRNA expression of TGF- β 1 and extracellular matrix components such as fibronectin and α 1(IV) collagen in the glomeruli of diabetic rats in parallel with inhibition of glomerular PKC activity. These results suggest that the activation of PKC, predominately the β isoform by hyperglycemia in the mesangial cells and glomeruli can partly contribute to early renal dysfunctions by alteration of prostaglandin production and Na^+ - K^+ ATPase activity as well as the chronic pathological changes by the overexpression of TGF- β 1 and extracellular matrix components genes. (*J. Clin. Invest.* 1997. 100:115–126.) Key words: arachidonic acid release • PGE_2 • TGF- β 1 • α 1(IV) collagen • fibronectin

Introduction

Diabetes now accounts for 35% of all new cases of end-stage renal disease in the United States (1). The causal factors for its development are most likely metabolic since the results of Diabetes Control and Complication Trial (DCCT) have clearly established that the strict maintenance of euglycemia by intensive insulin treatment can delay the onset and progression of diabetic nephropathy and other microvascular diseases (2). Amongst the various metabolic changes induced by diabetes, hyperglycemia is likely the single most important causal factor for the development of vascular dysfunctions in the microvessels (2–7).

Multiple cellular consequences of glucose-induced activation in cultured vascular cells have been described including increases in TGF- β , activation of cytosolic phospholipase A_2 (cPLA $_2$),¹ and inhibition of Na^+ - K^+ ATPase activities (3, 4, 8–10). Multiple biochemical mechanisms have been proposed to explain the adverse effects of hyperglycemia (3–7). We and others have reported that a consistent effect of hyperglycemia is the activation of the diacylglycerol (DAG)–protein kinase C (PKC) pathway which have been associated with the regulation of many vascular functions (11–18). Activation of DAG–PKC pathways in the vascular tissues has been associated with early abnormal hemodynamic functions in diabetic rats (11, 13). Amongst the many isoforms of PKC, PKC β 2 isoform was predominately activated by hyperglycemia chronically in the retina, aorta, and heart of diabetic rats (12, 13).

However, direct consequences of DAG–PKC activation especially the β isoform have been difficult to establish due to the lack of a PKC β isoform selective inhibitor for in vivo studies. We have recently described a macrocyclic bisindolylmaleimide compound (LY333531) which can inhibit PKC β isoform at concentrations 50 times less than for many other PKC isoforms and 1,000 times less than many serine–threonine and tyrosine kinases (11, 19). Oral treatment with LY333531 in rats with streptozotocin (STZ)-induced diabetes prevented the onset of early abnormalities in retinal and renal hemodynamics including the decrease in retinal blood flow, increase in GFR, and urinary albumin excretion rate (11).

In the present study, we have used this PKC β isoform selective inhibitor LY333531 to determine the role of PKC β isoform activation in causing alterations of biochemical parameters and gene expressions associated with chronic and pathological changes in diabetic glomerulopathy. These parameters are prostanoid production, Na^+ - K^+ ATPase activities, gene ex-

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1. Abbreviations used in this paper: cPLA $_2$, cytosolic phospholipase A_2 ; DAG, diacylglycerol; ECM, extracellular matrix; PA, phosphatidic acid; PKC, protein kinase C; PLC, phospholipase C; STZ, streptozotocin.

pressions of extracellular matrix components and TGF- β 1 in cultured mesangial cells and renal glomeruli.

Methods

Animals. Male Sprague-Dawley rats (200 grams; Taconic Farms, Germantown, NY) were placed in one of four groups: control, control treated with LY333531 (10 mg/kg body wt), 12 wk of diabetes, and 12 wk of diabetes treated with LY333531 (10 mg/kg body wt). For 4-wk study, only diabetic group was treated with 10 mg/kg body wt LY333531. Control group received 1 ml/kg body wt of sterile 20 mM citrate buffer (pH 4.5) by intraperitoneal injection. Diabetes was induced by a single intraperitoneal injection of sterile STZ (65 mg/kg body wt, Sigma Chemical Co., St. Louis, MO) in citrate buffer. The diabetic state was confirmed 48 h after STZ injections by blood glucose levels exceeding 300 mg/dl. Control and diabetic rats were randomly divided into four groups and LY333531 were given in the diet at a concentration of 10 mg/kg body wt. Blood glucose concentration was determined weekly in all animals. All experiments were conducted in accord with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Isolation of glomeruli from rats and the culture of mesangial cells. Rat mesangial cells were obtained from kidneys of male Sprague-Dawley rats (100–150 grams) as previously described with a few modifications (20). Kidneys were dissected from killed rats under aseptic conditions. After removing the capsules, the entire kidneys were homogenized in ice-cold DME and passed through sieves of various sizes to isolate the glomeruli. Glomeruli were then washed twice with DME and plated onto 10-cm tissue culture dish (Costar, Cambridge, MA) in 10 ml of DME containing 5 μ g/ml each of insulin, transferrin, and selenium (Gibco BRL, Grand Island, NY), 5.5 mM glucose, 100 U/ml penicillin, and 100 μ g/ml streptomycin with 20% fetal bovine serum (Gibco BRL). Mesangial cells from human glomeruli were obtained by the same method except the kidneys were provided by National Disease Research Interchange (NDRI, Philadelphia, PA). Identification of mesangial cells was performed as previously described (21). Mesangial cells of passage 2–6 were used for cell studies. For the assays of PKC activity, 86 Rb uptake, immunoblot analysis, arachidonic acid release, PGE₂ production, and in situ 32 P orthophosphate labeling, glomeruli from each group of rats were isolated after perfusion of kidneys with 20 ml of DME containing 20 mM Hepes, pH 7.4.

Assay of total DAG contents. Subconfluent rat mesangial cells in 6-well culture dishes (Costar) were exposed to 5.5 or 22 mM glucose for 4 d with daily changes of media. The cells were washed twice and pre-incubated for 1 h in serum-free DME containing various concentrations of LY333531. After washing the cells, total DAG contents were measured with a radioenzymatic assay kit (Amersham Corp., Arlington Heights, IL) using DAG kinase (Calbiochem Corp., San Diego, CA) that quantitatively converts DAG to [32 P]phosphatidic acid (PA) in the presence of [γ - 32 P]-ATP (New England Nuclear, Boston, MA). In brief, total cellular lipids were extracted twice according to the methods of Bligh and Dyer (22) and total DAG was measured as described by manufacturer's instructions. The resulting [32 P]PA was separated by silica gel G thin layer plates (EM Separations, Gibbstown, NJ) in a chamber containing chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) according to the method of Priess et al. (23). PA was visualized by autoradiography and identified by comigration with radio-labeled PA derived from DAG standard from 31 to 2,000 pmol. The spots were scraped and radioactivity was counted by liquid scintillation counter (CS6500; Beckman Instruments, Fullerton, CA). The values of total DAG contents were normalized by cellular proteins measured as described by Bradford (24).

Assay of protein kinase C activity. Subconfluent mesangial cells in 12-well culture dishes (Costar) were exposed to 5.5 or 22 mM glucose for 4 d with daily changes of media. The cells were washed twice and preincubated for 1 h in serum-free DME in the presence or ab-

sence of LY333531. PKC activity in mesangial cells was determined with a modified method described by Heasley and Johnson (25). Briefly, cells were rinsed twice with 2 ml of DME containing 20 mM Hepes (pH 7.4) and then with 2 ml of a salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose, 10 mM MgCl₂, 25 mM β -glycerophosphate, 5 mM EGTA, 2.5 mM CaCl₂, and 20 mM Hepes, pH 7.4). The cells were preincubated with the salt solution for 10 min at 37°C and incubated for another 15 min in the presence or absence of 100 μ M PKC-specific peptide substrate, RKRTLRL (26) with 50 μ g/ml digitonin and 100 μ M ATP mixed with [γ - 32 P]ATP (< 1,500 cpm/pmol; New England Nuclear, Boston, MA). The reaction was terminated with 5% TCA (final concentration). Aliquots of the reaction mixture were spotted on 3 \times 3 cm phosphocellulose papers (P-81; Whatman Instruments, Maidstone, United Kingdom) and washed with three changes of 75 mM phosphoric acid and one change of 75 mM sodium phosphate (pH 7.5). The radioactivity of phosphorylated substrate was determined by liquid scintillation counting. Protein contents were measured by Bradford's method (24).

AA release and assay of prostaglandin E₂. Subconfluent mesangial cells in 12-well plates were maintained in media containing 5.5 or 22 mM of glucose for 4 d, and then were labeled with 0.5 μ Ci/ml [3 H]arachidonic acid (221 Ci/mmol; New England Nuclear) for 20 h at 37°C. After the removal of the radioactive medium, the cells were washed four times with DME containing 0.1% BSA and preincubated in the presence or absence of LY333531 for 1 h. For the determination of glomerular AA release, isolated glomeruli were labeled with 5 μ Ci/ml [3 H]arachidonic acid for 3 h at 37°C and then washed three times with the same buffer after centrifugation at 800 g for 5 min. The medium was replaced with 0.5 ml of fresh medium and incubated for an additional 1 h. The media was then collected and the amount of [3 H] arachidonic acid released by cells or glomeruli for 1 h was determined by scintillation counting. The cells or glomeruli were washed again and solubilized with 0.5 ml of 1 M NaOH. A 100- μ l aliquot was retained for protein determination and the remaining was subjected to scintillation counting to determine the [3 H]arachidonic acid content of the cells or glomeruli. The data of [3 H]arachidonic acid release were presented as percentage of total counts (radioactivity in the released fraction + those in the solubilized cells) released for 1 h. There was no difference in the amount of incorporation of [3 H]arachidonic acid between the cells exposed to 5.5 and 22 mM glucose for 4 d, and between glomeruli isolated from control rats and those from diabetic rats.

The amount of PGE₂ produced by mesangial cells and renal glomeruli was measured in nonextracted cellular and glomerular supernatants using ELISA kit (Amersham Corp.) following the manufacturer's instructions. The data were normalized by protein concentrations as described.

Assay of Na⁺-K⁺ ATPase activity. Na⁺-K⁺ ATPase activity was estimated by 86 Rb uptake as previously described (8). Mesangial cells were grown in 12-well dishes and exposed to medium containing 5.5 or 22 mM glucose for 4 d. The cells were washed twice and preincubated for 1 h in serum-free DME in the presence or absence of the stated concentrations of LY333531. The cells were then washed with DME and incubated with 86 Rb (67.8 μ Ci/mmol, New England Nuclear) at a final concentration of 1 μ Ci/ml with or without 2 mM ouabain (Sigma Chemical Co.) for 10 min at 37°C. 86 Rb uptake was terminated by aspirating the medium and rapidly washing four times with ice-cold 100 mM MgCl₂. The cells were extracted with 0.5 ml of 10% TCA to measure radioactivity incorporated into the cells by scintillation counting. The ouabain-sensitive fraction of 86 Rb uptake was calculated by subtracting the fraction of the cellular uptake observed in the presence of ouabain from total 86 Rb uptake. The 86 Rb uptake into glomeruli isolated from each group was also measured as described here. The data were normalized by cellular protein.

RNA extraction and Northern blot analysis. Total RNA was extracted from the glomeruli by the guanidium thiocyanate method with phenol-chloroform extraction using TRI-REAGENT (Molecu-

lar Research Center, Cincinnati, OH) (27). Total RNA (20 μ g) was loaded on a 1% agarose gel with 9% formaldehyde, which was separated in Mops buffer (20 mM Mops, 5 mM Na acetate, 0.5 mM EDTA, pH 7.0). The RNA was transferred on nylon membrane (ICN, Aurora, OH). After UV-crosslinking, the membranes were prehybridized and hybridized to 32 P-labeled 0.3 kb EcoRI fragment of pCRII TGF- β 1 cDNA for TGF- β 1 kindly provided by Dr. Ziyadeh

(28), 1.8 kb EcoRI and Sall fragment of clone PE 123 for mouse α 1(IV) collagen by Dr. Kurkinen (29), and 582-bp PCR fragment for rat fibronectin (30) by Dr. Lorenzi, and Dr. Willert in 0.1 M Pipes, 0.2 M NaPO₄, 0.1 M NaCl, 1 mM EDTA, 5% SDS, and 30 μ g/ml salmon sperm DNA at 65°C. Stringent washing was done in 50% of 1 \times SSC, 5% SDS at 65°C for over 1 h. The expression of mRNA was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized using 36B4 as the standard cDNA probe (31).

Immunoblot analysis of PKC α and β 1. Rat glomerular mesangial cells or isolated glomeruli were washed three times with PBS, pH 7.4 and once with the buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 0.3 M sucrose, and 25 μ g/ml leupeptin). PKC proteins in the membranous and cytosolic fractions were partially purified using DEAE cellulose (12, 13) and then protein concentrations were determined according to the method of Bradford (24). Partially purified PKC fractions (30–50 μ g protein/lane for PKC α and 70–100 μ g/lane for PKC β 1) were separated on 8% SDS-PAGE gel under reducing conditions, transferred to PVDF membranes (Schleicher & Schuell, Keene, NH) and blocked overnight with PBS containing 0.1% Tween-20 and 3% BSA. The membrane was incubated with anti-PKC α antibody which was raised against the peptide corresponding to amino acids 313–326 or β 1 antibody which was raised against the peptide corresponding to amino acids 661–671 (Gibco BRL) overnight at 4°C. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp.) according to the manufacturer's instructions. Protein expressions were quantified with a phosphor image densitometer (Molecular Dynamics).

In situ 32 P orthophosphate labeling of PKC α and β 1 in glomeruli. Isolated glomeruli from each group were labeled with 1 mCi/ml of 32 P orthophosphate (New England Nuclear) in HEPES-buffered phosphate-free DME for 3 h at 37°C. After centrifugation at 800 g, the medium was removed. The labeled glomeruli were washed three times with ice-cold phosphate free DME and lysed with 1 ml of RIPA buffer; 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 40 mM NaF, 2 mM Na₃VO₄, 1 mM DTT. The solubilized glomeruli were sonicated and centrifuged at 14,000 g for 20 min at 4°C. Protein concentrations of supernatants were measured (24) and the supernatants were then used to immunoprecipitate PKC's with either anti-PKC α or β 1 antibodies (Gibco BRL) overnight at 4°C. Immunoprecipitates were captured on protein A sepharose (Pharmacia, Uppsala, Sweden), washed five times with RIPA buffer and analyzed by SDS-

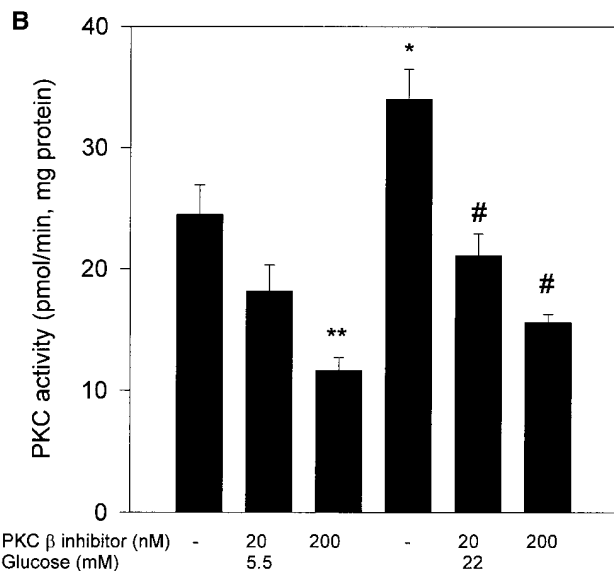
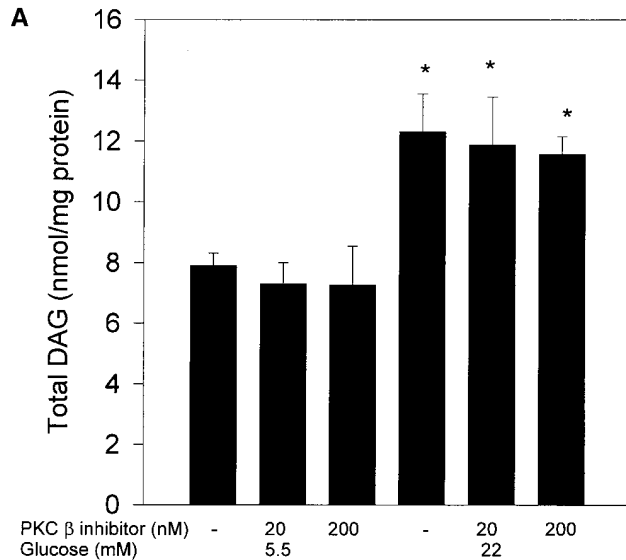


Figure 1. (A) Effect of PKC β inhibitor, LY333531 on high glucose-induced total DAG contents in rat glomerular mesangial cells. After 4 days exposure to 5.5 or 22 mM glucose, total lipids were extracted and total DAG was determined by an enzymatic assay with DAG kinase. The results are derived from six separate experiments and each experiment was performed in triplicate. * $P < 0.01$ vs 5.5 mM. (B) Effect of PKC β inhibitor, LY333531 on high glucose-induced PKC activity in rat glomerular mesangial cells. After 4 d exposure to 5.5 or 22 mM glucose, PKC activity was measured by in situ PKC assay that used a specific PKC substrate, RKRTLRL in digitonin-permeabilized cells. The results are derived from six separate experiments and each experiment was performed in triplicate. * $P < 0.01$ vs 5.5 mM glucose. ** $P < 0.01$ vs 5.5 mM glucose without PKC β inhibitor. # $P < 0.01$ vs 22 mM glucose without PKC β inhibitor.

Table I. Effect of PKC β Inhibitor LY333531 on PKC Activity and AA Release in Cultured Human Mesangial Cells

Culture conditions	In situ PKC activity	AA release (%)
	pmol/mg protein, min	
5.5 mM glucose	103.6 \pm 4.5	1.8 \pm 0.1
5.5 mM glucose + 20 nM PKC β inhibitor	66.2 \pm 1.9*	1.6 \pm 0.1
5.5 mM glucose + 200 nM PKC β inhibitor	28.2 \pm 0.8	1.6 \pm 0.1
22 mM glucose	168.2 \pm 2.8	2.6 \pm 0.1 [‡]
22 mM glucose + 20 nM PKC β inhibitor	75.8 \pm 3.5	1.6 \pm 0.1
22 mM glucose + 200 nM PKC β inhibitor	32.4 \pm 1.2	1.5 \pm 0.1

Data were shown by mean \pm SEM, $n = 5$ for PKC activity, $n = 4$ for AA release. * $P < 0.01$ vs other groups. [‡] $P < 0.01$ vs 5.5 mM glucose. Condition of each group for human mesangial cells were described in the Methods section.

PAGE followed by autoradiography. The ^{32}P labeled PKC's were visualized and quantitated by PhosphorImager (Molecular Dynamics).

Statistical analysis. Results were expressed as mean \pm SEM. Comparison among each group was performed by one-way ANOVA followed by Neuman-Keuls test to evaluate statistical significance between two groups. *P* values of < 0.05 were defined as statistically significant.

Results

Effect of PKC β isoform selective inhibitor LY333531 on glucose-induced activation of DAG levels and PKC. The effect of

PKC β selective inhibitor LY333531 on glucose-induced increase in DAG and PKC levels was determined in culture mesangial cells exposed to either 5.5 or 22 mM of glucose. The concentrations of LY333531 used were 20 and 200 nM. We have previously shown that 20 nM of LY333531 can selectively inhibit protein kinase C β but not PKC α in aortic smooth muscle cells overexpressing PKC β (11). At 200 nM, PKC β selective inhibitor LY333531 inhibited the activation of both PKC α and PKC β 1, β 2 isoforms (11, 19). Total DAG levels were increased in the cultured rat mesangial cells from 7.9 ± 0.4 to 12.3 ± 1.2 nM/mg protein when the glucose concentration in the media was increased from 5.5 to 22 mM (Fig. 1A). The ad-

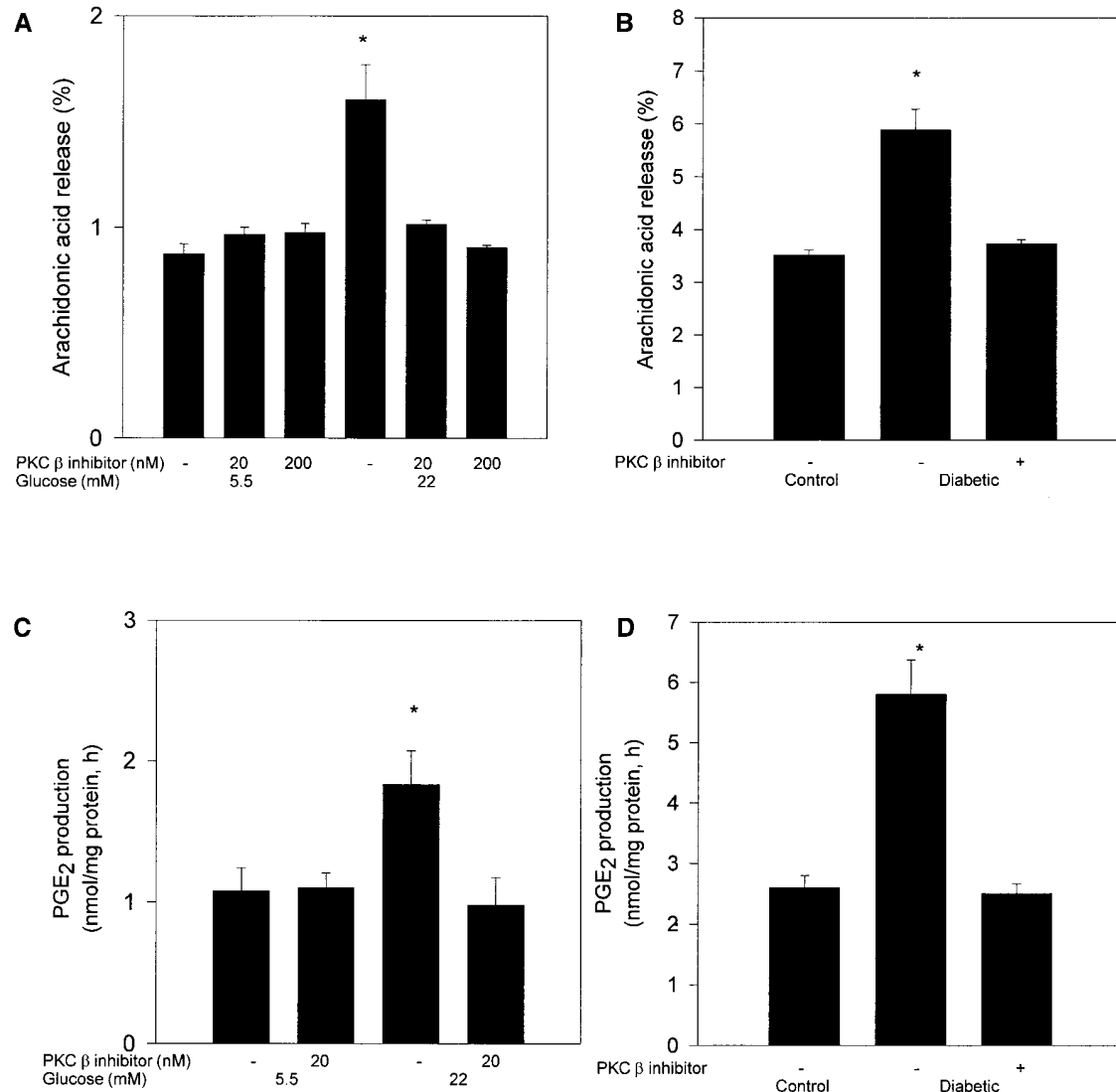


Figure 2. (A) Effect of PKC β inhibitor, LY333531 on high glucose-induced arachidonic acid release. Rat glomerular mesangial cells were labeled with [^3H]arachidonic acid after 3 d exposure to 5.5 or 22 mM glucose. The cells were treated with or without LY333531 for 1 h before measurement of [^3H]arachidonic acid release. The results were derived from three separate experiments and each experiment was performed in triplicate. $*P < 0.05$ vs other columns. (B) Effect of PKC β inhibitor, LY333531 treatment (10 mg/kg body wt) on 4-wk diabetes-induced arachidonic acid release. Isolated glomeruli labeled with [^3H]arachidonic were incubated for 1 h before measurement of [^3H]arachidonic acid release. The results were derived from four separate rats. $*P < 0.05$ vs other columns. (C) Effect of PKC β inhibitor, LY333531 on high glucose-induced PGE₂ production in rat glomerular mesangial cells. Rat glomerular mesangial cells were incubated with 5.5 or 22 mM glucose for 4 d and treated with or without LY333531 for 1 h before the measurement of PGE₂ production. The results were derived from three separate experiments and each experiment was performed in duplicate. $*P < 0.05$ vs other columns. (D) Effect of PKC β inhibitor, LY333531 treatment (10 mg/kg body wt) on 4-wk diabetes-induced PGE₂ production in rat glomeruli. After incubating isolated glomeruli for 1 h, the supernatants were used to determine PGE₂ production. The results were derived from four separate rats. $*P < 0.05$ vs other columns.

dition of PKC β isoform inhibitor LY333531 at 20 or 200 nM did not change the levels of total DAG in the rat mesangial cell (Fig. 1 A). The addition of LY333531 for 1 h did not change the total protein concentrations compared with those cultured in the absence of LY333531.

Increasing glucose concentration in the cultured rat mesangial cells from 5.5 to 22 mM also increased the specific in situ PKC activity from 24.5 ± 2.5 to 34.0 ± 2.5 pmol/mg protein, min in parallel with increases in DAG levels (Fig. 1 B). The addition of 20 nM of PKC β isoform selective inhibitor LY333531 did not significantly lower PKC activity at 5.5 mM of glucose but significantly decreased the PKC activity in cells cultured under 22 mM of glucose to 21.1 ± 1.8 pmol/mg protein per min (Fig. 1 B) reaching levels comparable with that in cells exposed to 5.5 mM glucose alone. The addition of 200 nM of PKC β inhibitor LY333531 significantly decreased the in situ PKC activities in rat mesangial cells cultured either at 5.5 or 22 mM of glucose by 52 and 54%, respectively. Similar to the results of rat mesangial cells, 22 mM of glucose also significantly increased PKC activity in human mesangial cells by 62% compared with 5.5 mM of glucose (Table I). Interestingly, the addition of PKC β inhibitor LY333531 at the concentrations of either 20 nM or 200 nM decreased the levels of in situ PKC activities in both cells cultured under 5.5 and 22 mM of glucose (Table I).

Effect of PKC β isoform selective inhibitor LY333531 on glucose-induced increases of AA release and PGE₂ production. Since the above results showed that PKC β inhibitor LY333531 can inhibit specifically the glucose-induced PKC activity, we have characterized the effect of LY333531 to prevent the increases in release of AA and the production of PGE₂ in the mesangial cells induced by glucose. Fig. 2 A showed that elevating glucose level from 5.5 to 22 mM in the media increased AA release from rat mesangial cells by 82%. The addition of LY333531 at either 20 or 200 nM did not alter AA release in cells cultured at 5.5 mM of glucose but prevented the increase observed at 22 mM of glucose (Fig. 2 A). As shown in Table I, increasing glucose level from 5.5 to 22 mM in human mesangial cells also increased AA release by 47%, but the addition of 20 nM PKC β inhibitor LY333531 normalized the increased AA release in human mesangial cells exposed to 22 mM of glucose. The glomerular AA release was also measured in control, diabetic, and diabetic rats treated with LY333531. Treatment with LY333531 prevented the increase in AA release induced by diabetes (Fig. 2 B).

PGE₂ production by rat mesangial cells was also increased from 1.0 ± 0.1 to 1.8 ± 0.1 nmol/mg protein per h when glucose concentration in the media was elevated from 5.5 to 22 mM (Fig. 2 C). When the activation of PKC was inhibited by the addition of 20 nM of LY333531, the increased PGE₂ production in cells cultured under 22 mM glucose was prevented (Fig. 2 C). However, LY333531 did not change the basal PGE₂ production in mesangial cells cultured under 5 mM of glucose (1.1 ± 0.1 nmol/mg protein per h) compared to basal values of control cells cultured under 5.5 mM glucose. In addition, glomerular PGE₂ production in the diabetic rats was also increased compared with control rats (Fig. 2 D). Treatment with LY333531 prevented the increase in PGE₂ production in diabetic rats (Fig. 2 D).

Effect of PKC β isoform selective inhibitor LY333531 on glucose-induced inhibition of Na⁺-K⁺ ATPase activity. Since we have previously reported that hyperglycemia-induced activation of cytosolic-phospholipase A₂ (cPLA₂) in aortic smooth

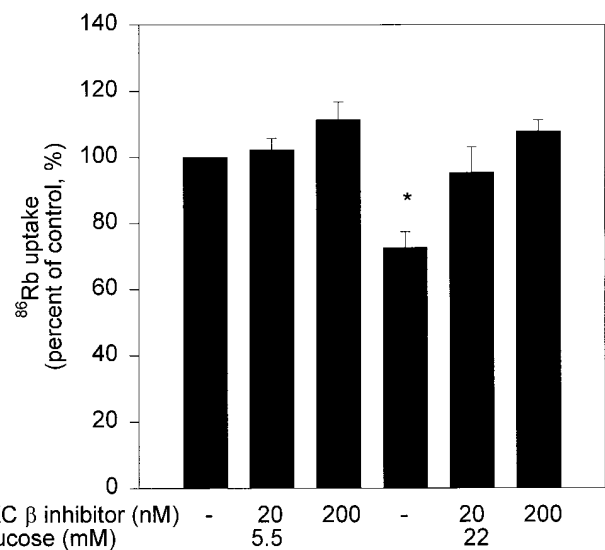


Figure 3. Effect of PKC β inhibitor on glucose-induced inhibition of Na⁺-K⁺ ATPase in rat glomerular mesangial cells. After 4 d exposure to 5.5 or 22 mM glucose, the cells were treated with or without LY333531 for 1 h. ⁸⁶Rb uptake was corrected for ouabain sensitivity and determined after 10 min incubation as described in Methods. The results were derived from three separate experiments and each experiment was performed in triplicate. **P* < 0.05 vs 5.5 mM glucose and 22 mM glucose with 20 or 200 nM PKC β inhibitor.

muscle cells can result in the inhibition of Na⁺-K⁺ ATPase via the overproduction of AA release and PGE₂ (8), the effect of LY333531 to regulate Na⁺-K⁺ ATPase activity in rat mesangial cells exposed to either 5.5 or 22 mM of glucose was examined. After 4 d of incubation, the ouabain-sensitive ⁸⁶Rb uptake, a physiological marker of Na⁺-K⁺ ATPase activity in rat mesangial cells was decreased by 28% in cells exposed to 22 mM glucose as compared to 5.5 mM of glucose (Fig. 3). The addition of either 20 or 200 nM of PKC β inhibitor LY333531 increased and normalized the ouabain-sensitive ⁸⁶Rb uptake by rat mesangial cells cultured under 22 mM of glucose (Fig. 3). The addition of PKC β inhibitor LY333531 at 20 or 200 nM did not alter the Na⁺-K⁺ ATPase activities in cells cultured under 5.5 mM of glucose.

Table II. Physiological Characteristics of Control and Diabetic Rats with or without 10 mg/kg Body Wt PKC β Inhibitor, LY333531 Treatment for 12 wk

Animals	Number	Body wt	Blood glucose	Kidney wt/ body wt
<i>treatment</i>		<i>grams</i>	<i>mg/dl</i>	<i>gram/100 grams</i> [‡]
Control	35	500.5 ± 18.2	102.5 ± 7.5	0.62 ± 0.01
Control + PKC β inhibitor	25	497.5 ± 10.3	98.4 ± 6.2	0.64 ± 0.01
Diabetic	35	266.5 ± 28.9*	434.5 ± 14.9*	1.39 ± 0.10*
Diabetic + PKC β inhibitor	25	252.3 ± 19.1*	464.5 ± 19.7*	1.31 ± 0.07*

**P* < 0.01 vs control and control + PKC β inhibitor; [‡]Data were shown by mean ± SEM and calculated from six rats of each group.

Table III. Physiological Characteristics of Control Rats and Diabetic Rats with or without 10 mg/kg Body Wt PKC β Inhibitor, LY333531 Treatment for 4 wk[‡]

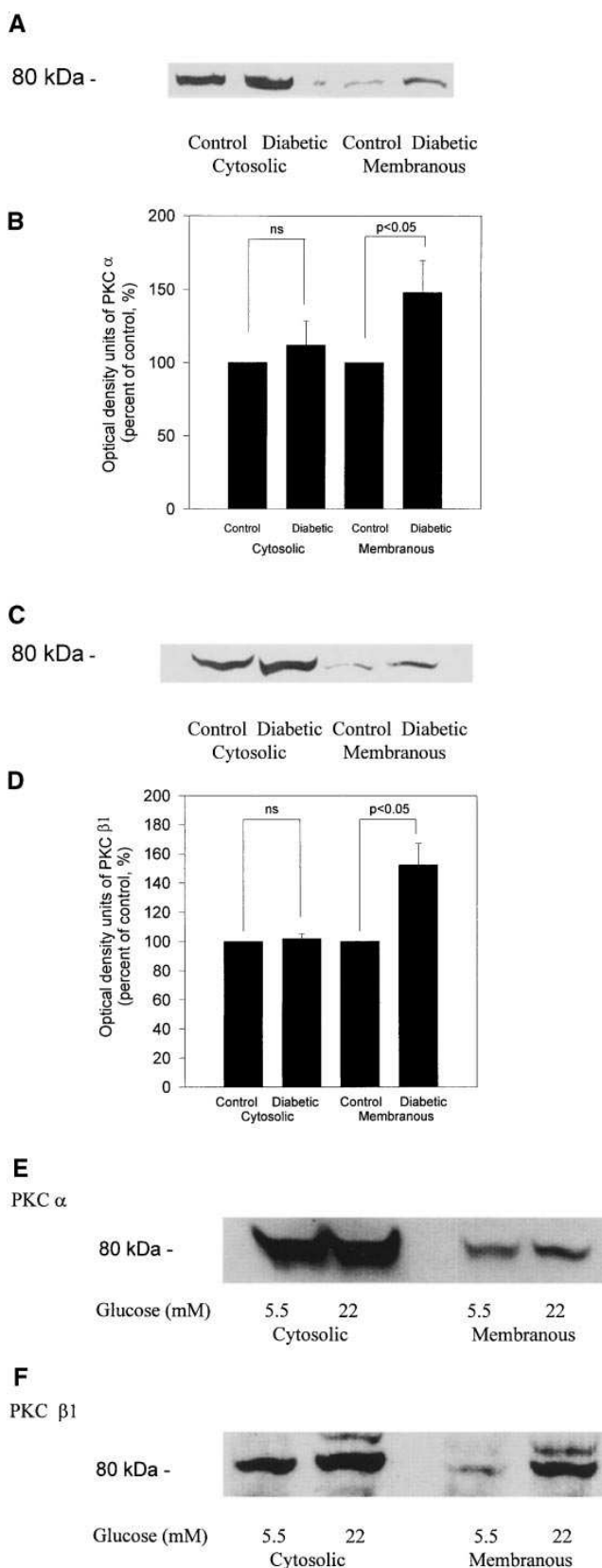
Animals <i>treatment</i>	Number	Body wt <i>grams</i>	Blood glucose <i>mg/dl</i>
Control	8	368.8±9.1	102.5±2.1
Diabetic	8	281.3±12.2*	394.4±14.3*
Diabetic + PKC β inhibitor	8	287.5±8.4*	400.4±18.2*

* $P < 0.01$ vs control rats. [‡]These rats were used for the determination of glomerular AA release and PGE₂ production.

Effect of PKC β isoform selective inhibitor LY333531 treatment on in vivo physiological parameters. Diabetes in the rats was confirmed with glucose measurements of 434.5±14.9 mg/dl in diabetic vs 102.5±7.5 mg/dl in control rats. Treatment of diabetic rats with 10 mg/kg body wt of PKC β selective inhibitor LY333531 in rat chow did not prevent hyperglycemia, changes in weight gain, and increases in kidney wt/body wt ratio in the diabetic animals (Table II). Blood pressure and pulse rate did not change with diabetes or treatment with LY333531 for up to 12 wk (data not shown). The similar trends were observed in the rats with 4 wk of diabetes with or without LY333531 treatment (Table III).

The in vivo effect of diabetes and PKC β selective inhibitor LY333531 on PKC isoform expressions in the glomeruli. We and others have reported that PKC activities in the glomeruli were increased by diabetes and can be inhibited by oral feeding of PKC β inhibitor LY333531 at 10 mg/kg body weight (11, 17). In the present study, we characterized the changes in various PKC isoforms in the glomeruli induced by diabetes by two methods (11, 17). First, the activation of specific PKC isoforms was defined by an increase in the protein expression of specific PKC isoforms in the membranous fraction, an indicator of PKC activation (32). Using immunoblot analysis, PKC α and β 1 isoforms were observed to be increased in the membrane fraction (Fig. 4, A–D) without any alterations in the δ or ϵ isoforms (data not shown). Diabetes increased the amount of PKC α and β 1 by 50% in the membranous fractions isolated from glomeruli as compared to control rat (Fig. 4, A and B for α ; Fig. 4, C and D for β 1). PKC β 2 isoform did not appear to have changed although its content in the glomeruli was barely detectable and was much lower than PKC β 1. The amounts of PKC α and β 1 isoforms in the cytosolic and membranous fractions of rat mesangial cells were also assessed by immunoblot

Figure 4. Immunoblot analysis of glomeruli from control and 12-wk diabetic rats, and rat glomerular mesangial cells exposed to 5 or 22 mM glucose for 4 d with anti-PKC α and β 1 antibody. (A, C, E, and F). PKC in the membranous and cytosolic fractions was partially purified from isolated glomeruli from three rats of control and diabetic rats or rat mesangial cells using DEAE cellulose. 30–50 μ g protein for PKC α and 70–100 μ g for PKC β 1 were loaded in each lane and membranes were blotted with anti-PKC α or β 1 antibodies. Representative results were shown in A for PKC α , in C for PKC β 1 in renal glomeruli, in E for PKC α , and in F for PKC β 1 in rat mesangial cells. (B and D).



A quantification of the relative amounts of membranous and cytosolic PKC α and β 1 of glomeruli in control and diabetic rats was shown in B and D, respectively. The results of glomeruli were derived from three separate experiments and six kidneys were processed for each experiment.

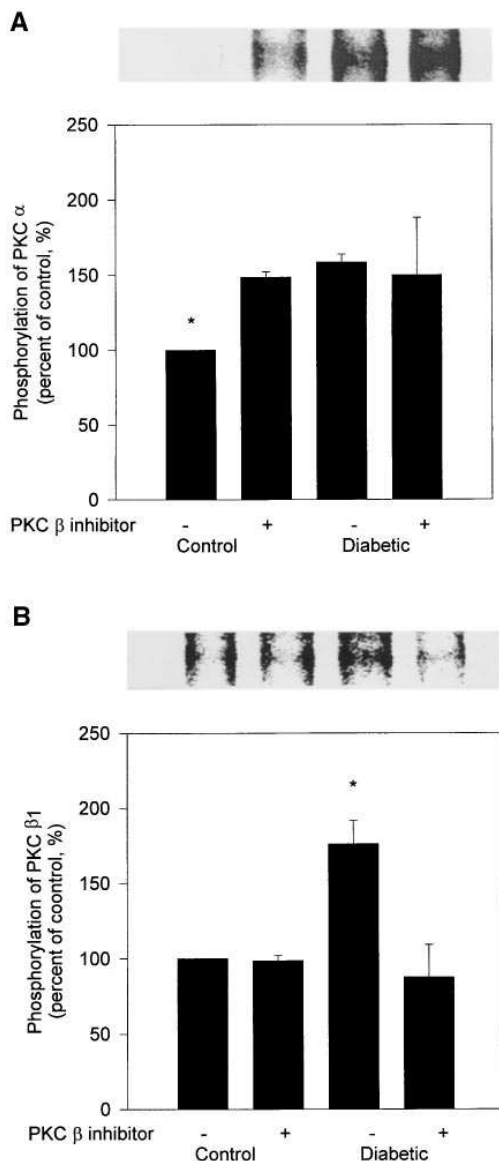


Figure 5. Effect of PKC β inhibitor, LY333531 (10 mg/kg body wt) on diabetes-induced phosphorylation of PKC α and $\beta 1$. Isolated glomeruli from three rats (six kidneys) of each group were labeled with ^{32}P orthophosphate in phosphate free DME. After lysing glomeruli with lysis buffer, the samples were immunoprecipitated with anti-PKC α and $\beta 1$ antibody and analyzed by SDS-PAGE followed by autoradiography. The representative results from three separate experiments and mean \pm SEM of three experiments were shown in *A* for PKC α and in *B* for PKC $\beta 1$ isoforms. * $P < 0.05$ vs other groups.

analysis showing that incubation of mesangial cells with 22 mM glucose increased the amounts of both PKC α and $\beta 1$ isoforms in the membranous fraction (Fig. 4 *E* for PKC α ; and Fig. 4 *F* for PKC $\beta 1$).

The second method used to evaluate the activation of PKC α and $\beta 1$ by diabetes was by determining the amount of phosphorylation of PKC α and $\beta 1$ in the glomeruli from control and diabetic rats. Increases in phosphorylation of PKC isoforms have been shown to correspond to their state of activation (33–37). For this study, we isolated glomeruli from control and diabetic rats with or without treatment with PKC β inhibi-

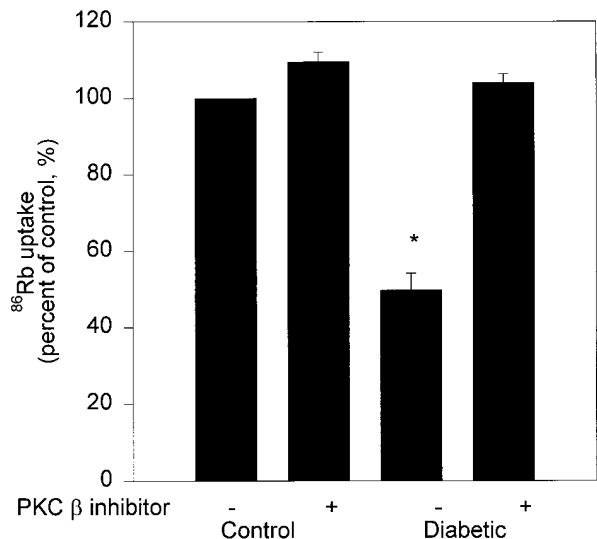


Figure 6. Effect of PKC β inhibitor LY333531 (10 mg/kg body wt) on 12-wk diabetes-induced $\text{Na}^+\text{-K}^+$ ATPase in rat glomeruli. Using glomeruli from four rats of each group, ^{86}Rb uptake was corrected for ouabain sensitivity and determined after 10 min incubation as described in Methods. The results were derived from four separate rats and two kidneys were processed for each experiment. * $P < 0.05$ vs other group.

tor LY333531 and labeled them with ^{32}P orthophosphate. The glomeruli were then processed and immunoprecipitated with either PKC α or $\beta 1$ antibodies as described in the Methods section. The results as shown in Fig. 5, *A* and *B* indicated that diabetes increased phosphorylation of both PKC α and $\beta 1$ by 60 and 75%, respectively. Oral treatment of rats with LY333531 at 10 mg/kg body wt prevented the increase in phosphorylation only of PKC $\beta 1$ isoform but not PKC α isoform, providing the first in vivo evidence that LY333531 can specifically inhibit PKC $\beta 1$ isoform in glomeruli from diabetic animals.

Effect of diabetes and PKC β selective inhibitor LY333531 on $\text{Na}^+\text{-K}^+$ ATPase activity in glomeruli of diabetic rats. As described in the previous section, elevating glucose concentration in the media from 5.5 to 22 mM inhibited $\text{Na}^+\text{-K}^+$ ATPase activity as measured by ^{86}Rb uptake into rat and human mesangial cells which was prevented by LY333531, indicating that glucose was mediating its effect via PKC β isoform activation. To substantiate this finding in vivo, ouabain-sensitive ^{86}Rb uptake was quantitated using isolated glomeruli from control and diabetic rats. After having diabetes for 12 wk, ouabain-sensitive ^{86}Rb uptake in the glomeruli of diabetic rats was decreased by $> 50\%$ compared with control rats (Fig. 6). Oral feeding of PKC β selective inhibitor LY333531 to nondiabetic controls did not significantly change the rate of ^{86}Rb uptake. In contrast, when diabetic rats were fed with LY333531, ouabain-sensitive ^{86}Rb uptake returned to normal at 104% of control (Fig. 6).

Effect of PKC β selective inhibitor LY333531 on the mRNA expression of TGF- $\beta 1$ and extracellular matrix components in glomeruli of control and diabetic rats. Increases in the expression of TGF- $\beta 1$ and extracellular matrix components in the glomeruli of diabetes are associated with the development of basement membrane thickening and mesangial expansion, characteristic of chronic and pathological findings in the glom-

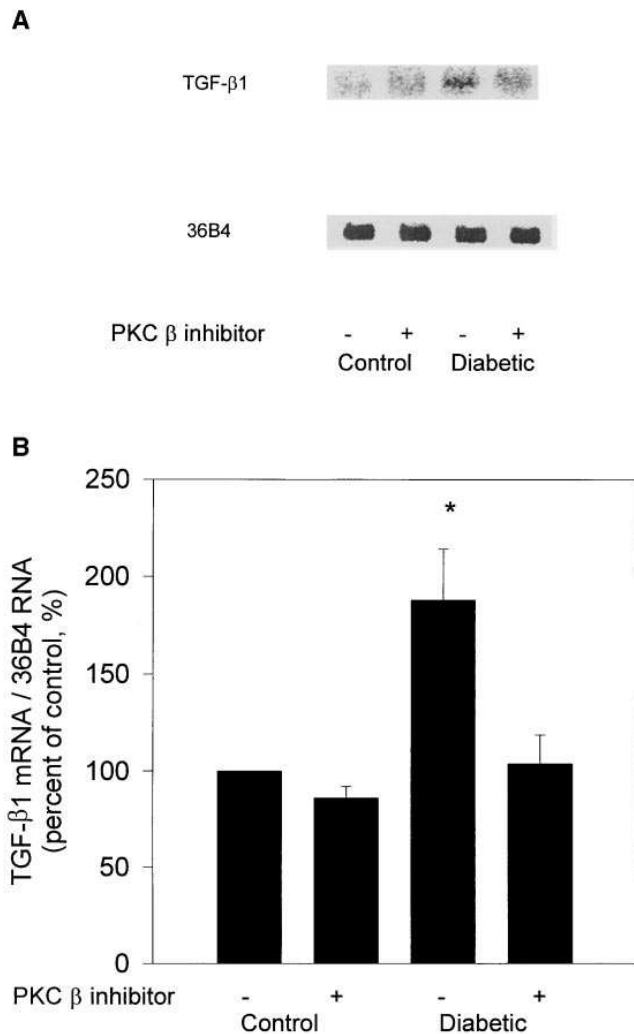


Figure 7. Effect of PKC β inhibitor, LY333531 treatment (10 mg/kg body wt) on 12-wk diabetes-induced fibronectin mRNA expression in glomeruli. (A) A representative Northern blot of glomerular RNA probed with PCR fragment for rat fibronectin in upper panel and 36B4 RNA in lower panel. (B) Summary of quantification of fibronectin/36B4 message ratios. The results were derived from six rats of each group. Four kidneys were processed for each experiment. * $P < 0.05$ vs control and diabetic with PKC β inhibitor treatment.

eruli of diabetic nephropathy (38, 39). The effects of diabetes and treatment with LY333531 at 10 mg/kg body wt on the gene expressions of TGF- β 1, fibronectin, and α 1(IV) collagen were examined in the glomeruli of control and diabetic rats. Glomerular TGF- β 1 mRNA level was increased by $88 \pm 26\%$ in diabetic rats compared with that in control rats (Fig. 7). Oral treatment with PKC β selective inhibitor LY333531 in control rats did not alter TGF- β 1 mRNA expression significantly. However, when LY333531 was given to diabetic rats for 12 wk, the increases in TGF- β 1 mRNA level were prevented (Fig. 7).

The mRNA expressions of extracellular matrix components, fibronectin, and α 1(IV) collagen were also quantitated in the glomeruli. Similar to the expression of TGF- β 1, both fibronectin and α 1(IV) collagen mRNA expressions were increased in diabetic rats by 85 and 104%, respectively (Figs. 8 and 9). Again treatment with PKC β inhibitor LY333531 orally

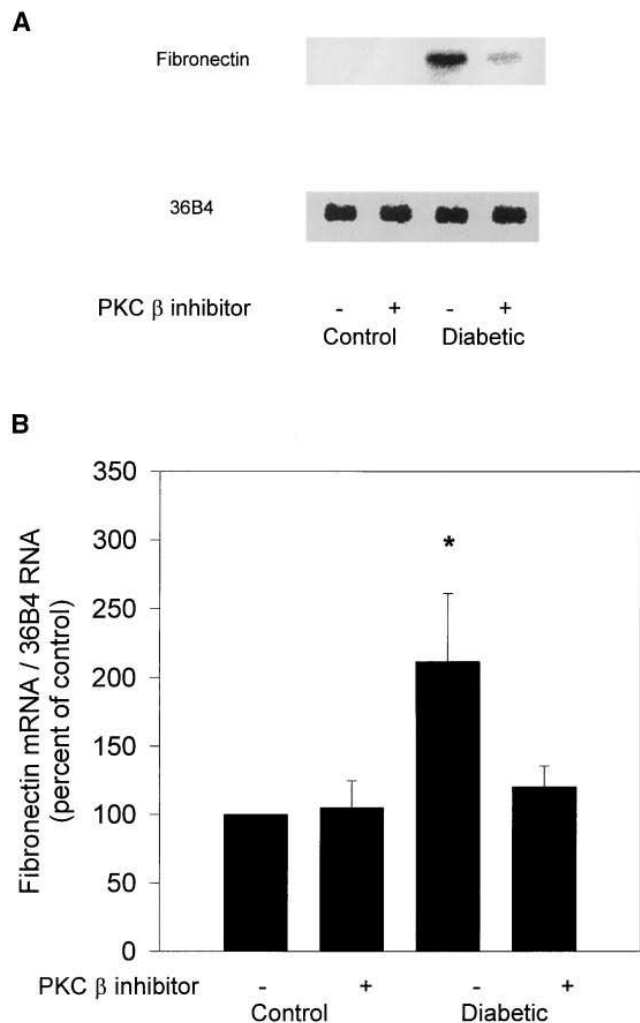


Figure 8. Effect of PKC β inhibitor, LY333531 treatment (10 mg/kg body wt) on 12-wk diabetes-induced fibronectin mRNA expression in glomeruli. (A) A representative Northern blot of glomerular RNA probed with PCR fragment for rat fibronectin in upper panel and 36B4 RNA in lower panel. (B) Summary of quantification of fibronectin/36B4 message ratios. The results were derived from six rats of each group. Four kidneys were processed for each experiment. * $P < 0.05$ vs control and diabetic with PKC β inhibitor treatment.

did not change their expression in the control animals whereas oral application of LY333531 in the chow at 10 mg/kg body wt prevented the increases in mRNA expression of both fibronectin and α 1(IV) collagen in diabetic rats (Figs. 8 and 9).

Discussion

The mechanisms by which hyperglycemia cause adverse effects in the vasculature of diabetic patients are probably multiple since alterations of glucose metabolism and its metabolites can affect the flux and regulation of numerous cellular metabolic pathways (3–7). One such important cellular pathway which has been shown to be consistently activated is the DAG–PKC signal transduction system (7). Consistent with previous reports from our laboratory and others, the levels of total DAG and activated fraction of PKC were increased by

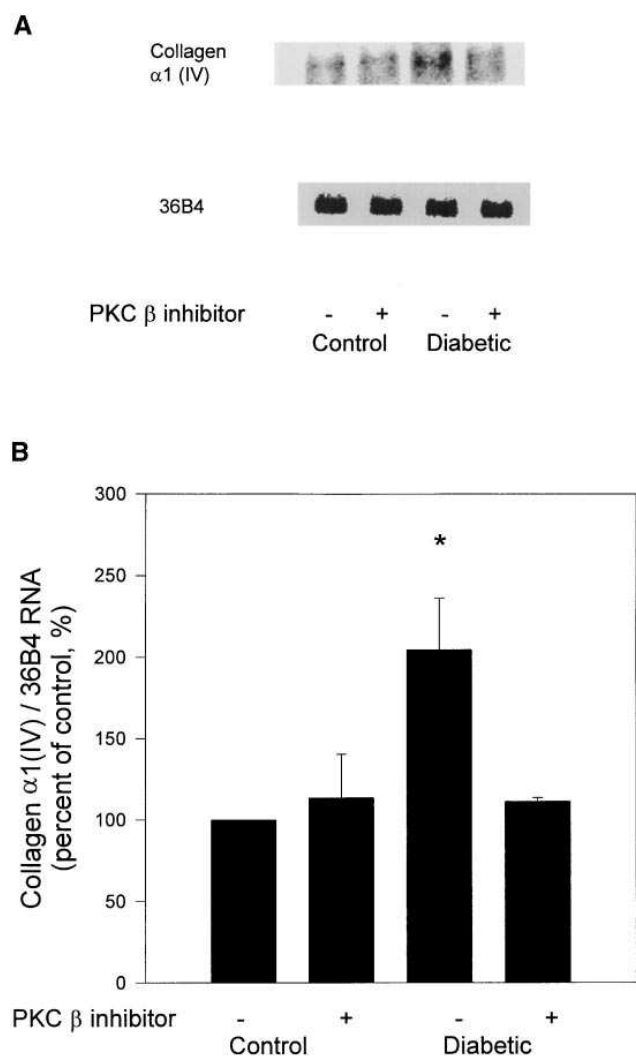


Figure 9. Effect of PKC β inhibitor, LY333531 treatment (10 mg/kg body wt) on 12-wk diabetes-induced type IV collagen mRNA expression in glomeruli. (A) A representative Northern blot of glomerular RNA probed with cDNA for mouse $\alpha 1$ (IV) collagen in upper panel and 36B4 RNA in lower panel. (B) Summary of quantification of $\alpha 1$ (IV) collagen/36B4 message ratios. The results were derived from six rats of each group. Four kidneys were processed for each experiment. * $P < 0.05$ vs control and diabetic with PKC β inhibitor treatment.

elevating glucose levels in the cultured mesangial cells and in the renal glomeruli of diabetic rats (11, 14, 17, 18). The activation of the various PKC isoforms was evaluated by their phosphorylation levels and by the changes of their protein levels in the membranous fractions. The use of the PKC protein ratio of membranous to cytosolic fraction by itself may not be adequate since it is possible that some of the activated PKC could have been dissociated from the membrane fraction during the isolation procedures (25). Increases in serine and threonine phosphorylation of PKC isoforms have been shown to occur after PKC has been activated by DAG and Ca^{2+} (33–36). Thus, quantitation of the phosphorylation levels of PKC isoform provided a complementary method to determine whether the various specific isoforms are activated (33–36). The assess-

ments of isoform activation and inhibition were performed on the renal glomeruli and on cultured mesangial cells to evaluate the in vivo and in vitro actions of PKC β isoform inhibitor LY333531 in the renal glomerular cells which was not done previously (11). Both the immunoblotting and phosphorylation assays suggested that PKC isoform α and $\beta 1$ were activated. The lack of decrease in the PKC band from the cytosolic fractions for these isoforms could be due to the insensitivity of the assay to detect the small changes (Fig. 5). The finding of both α and $\beta 1$ PKC isoforms to be activated in the glomeruli are similar to the findings in the retina where multiple PKC isoforms including PKC α and $\beta 2$ were activated by diabetes (13). However, the finding of PKC $\beta 1$ to be activated in the glomeruli differed from the retina, heart, or the aorta which exhibited predominately PKC $\beta 2$ isoform activations (12, 13). One possible explanation for this difference between vascular tissues and glomeruli is that the expression of PKC $\beta 1$ appears to be much greater than PKC $\beta 2$ in the renal glomeruli. This possibility was supported by the results of immunoblot analysis which also showed increased expression of PKC α and $\beta 1$ isoforms in the membranous fraction of rat mesangial cells exposed to elevating glucose concentrations.

The use of ^{32}P orthophosphate-labeling study provided the direct in vivo evidence that PKC isoform α and $\beta 1$ were activated in the glomeruli of diabetic animals. This method allowed us to determine for the first time whether the inhibitory effect of LY333531 was specific to PKC β isoforms in vivo. In a previous report, we have reported that the inhibitory effect of LY333531 was reversible and did not prevent the translocation of PKC, and was a competitive inhibitor at the ATP binding site in in vitro studies (11, 19). Therefore, the use of translocation assay would not be able to detect the activation states of PKC or the effectiveness of LY333531 since translocation induced by DAG preceded the binding of ATP in the activation sequence of PKC (37). In contrast, quantitation of the phosphorylation level of the PKC isoforms will directly determine the activation status of the various PKC isoforms (33–36). Our present results clearly indicated that LY333531 used at 10 mg/kg body wt specifically inhibited PKC $\beta 1$ isoform activations, thus providing in vivo evidence that LY333531 can selectively inhibit PKC $\beta 1$ similar to its in vitro inhibitory effect on PKC activity in addition to our previous report showing its inhibitory effect on PKC $\beta 2$ (11, 19). The use of LY333531 affected only PKC activity but not DAG levels in mesangial cells, suggesting that PKC activity may not affect phospholipase C (PLC) activity. Alternatively, the source of the increased DAG is due to de novo synthesis and not due to activation of PLC activity as shown previously (9, 17). It is also interesting to note that the inhibition of PKC $\beta 1$ isoform phosphorylation by LY333531 has consistently caused a parallel increase in basal PKC α phosphorylation level. The mechanism of this increase in PKC α phosphorylation is not clear. Further studies are needed to characterize the cellular consequences of this change in PKC α isoform phosphorylation by LY333531.

Functionally, our previous report suggested that oral treatment of LY333531 can ameliorate the early hemodynamic dysfunctions in the glomeruli of diabetic rats suggesting that PKC activation may potentially have a pathophysiologic role in the development of diabetic vascular complications (11). However, the hemodynamic changes were very early parameters which may not translate into chronic and pathological findings of diabetic nephropathy (11). In the present study, we have

characterized the effect of PKC β inhibitor LY333531 on a host of changes both in cultured mesangial cells and in glomeruli of diabetic rats. Hyperglycemia's effect to activate cPLA₂ has been reported previously by us in the aortic smooth muscle cells exposed to elevated level of glucose (8). Furthermore, PLA₂ appeared to be activated in the renal glomeruli of diabetic rats as reported by Craven et al. (9). The result of the activation of PLA₂ can lead to an increase in the amount of PGE₂ and arachidonic acid release which have been reported to be increased in vivo in the glomeruli of diabetic rats (9, 40, 41). These findings suggest that activation of PLA₂ may be responsible for the increased production of the prostanoids in the glomeruli of diabetic rats.

The possible consequence of increases in the prostanoids could be the vasodilatation of afferent arterioles that leads to increased glomerular filtration rate of diabetic animals (11). These effects of hyperglycemia were also studied in cultured human mesangial cells since results derived from human may not be similar to those from rodent cells. The results of human mesangial cells were very consistent with our previous findings that hyperglycemia can increase PKC activity and PLA₂ activity in a similar manner as those in smooth muscle cells (8) and rat mesangial cells (10). These results have provided the first direct comparative study of rodent and human mesangial cells with respect to glucose-induced activation of PKC and its possible cellular effects.

The consequences of PKC activation specifically the β 1 isoform were evaluated by measuring PLA₂ activity and the inhibition of Na⁺-K⁺ ATPase in the renal glomeruli. Although the inhibition of Na⁺-K⁺ ATPase by hyperglycemia or diabetes has been reported in multiple vascular and neuronal tissues (4, 8), our present finding in the renal glomeruli and cultured mesangial cells provided direct evidence that the activation of PLA₂ and the inhibition of Na⁺-K⁺ ATPase both in vitro and in vivo was due to activation of PKC β isoforms. The possible consequences of Na⁺-K⁺ ATPase inhibition in the renal glomeruli could be multiple including the maintenance of the cellular integrity, vascular and epithelial cell permeability, and sodium/hydrogen transport (42, 43). Abnormalities in many of these cellular functions have been reported in the diabetic vascular complications.

One of the hallmarks of diabetic nephropathy is characterized by progressive mesangial expansion, eventually leading to a decrease in filtration surface area and a reduction in glomerular filtration rate (38, 39). Mesangial expansion is due to an accumulation of ECM components, caused by either increased production or impaired degradation. A substantial amount of data suggest that the increased ECM synthesis by mesangial cells and other vascular cells was induced by elevated levels of glucose (44–48). Studies have suggested that TGF- β expression stimulated by hyperglycemia may contribute to the expansion of the mesangium since TGF- β is known to stimulate ECM production (3, 49–53). In this study, we have shown that the activation of PKC β correlated with an increased mRNA expression of TGF- β 1 and ECM components in glomeruli of diabetic rats. Moreover, treatment with PKC β inhibitor LY333531 normalized the gene expression of TGF- β 1, fibronectin and α 1(IV) collagen. Our results did not demonstrate that increased TGF- β mRNA expression directly caused enhanced gene expression of fibronectin and α 1(IV) collagen. However, others have reported that TGF- β stimulated ECM accumulation such as fibronectin, laminin, and type IV col-

lagen in renal cells (52, 54). In addition, neutralization of TGF- β by anti-TGF- β antibody attenuated the increase in mRNA levels for fibronectin and α 1(IV) collagen in kidney of diabetic mice and cultured mesangial cells exposed to high glucose (53, 55), suggesting that TGF- β expression was directly responsible in the mesangial matrix expansion.

Using PKC β isoform specific inhibitor (LY333531), we have provided evidence that the activation of PKC β isoform by hyperglycemia could be involved in the mediation of abnormal mesangial and glomerular functions such as mRNA overexpression of TGF- β and ECM components and overproduction of prostanoids. Our previous studies have shown that PKC β 2 isoform was preferentially activated by the increase in de novo DAG synthesis in cultured vascular cells exposed to elevated levels of glucose and various vascular tissues from diabetic animals (12, 13). In retina, aorta, and the heart, PKC β isoform was predominately increased although PKC α was also increased in the retina to a lesser extent (13). Thus, PKC activation in the glomeruli differed from other vascular tissues in two ways. First, PKC β 1 isoform was activated and not PKC β 2 isoform. Second, membranous protein levels of both PKC α and β 1 were equally increased. The selectivity of PKC β 1 over β 2 was likely due to a greater expression of PKC β 1 isoform in the glomeruli than other tissues. It is not surprising that PKC α isoform was detected to be activated since Kikkawa et al. has made similar observations in rat mesangial cells exposed to high concentrations of glucose (16). Since PKC β isoform inhibitor LY333531 (10 mg/kg body wt) was able to prevent diabetes-induced changes in Na⁺-K⁺ ATPase activity, and gene expressions of TGF- β 1 and ECM, it is likely that only PKC β isoform was responsible. This conclusion was substantiated by the measurements of the phosphorylation levels of PKC α and β 1 in the renal glomeruli directly. In addition, at an oral dose of 10 mg/kg body wt, the plasma level of LY333531 was 19 nM which was threefold greater than the IC₅₀ for PKC β 1 at 6 nM but 10-fold less than IC₅₀ of PKC α at 360 nM (11, 19).

In summary, these results provided substantial evidence that the activation of PKC particularly PKC β 1 isoform by hyperglycemia and diabetes can alter glomerular cell functions and gene expressions that are related to the chronic pathological changes of diabetic nephropathy. The availability of an orally effective PKC β selective inhibitor has provided an important insight into the specific biological actions of PKC β isoform and suggested the usefulness of PKC β specific inhibitor in the treatment of diabetic complications.

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