

# Thiazolidinedione Compounds Ameliorate Glomerular Dysfunction Independent of Their Insulin-Sensitizing Action in Diabetic Rats

Keiji Isshiki, Masakazu Haneda, Daisuke Koya, Shiro Maeda, Toshiro Sugimoto, and Ryuichi Kikkawa

**Thiazolidinedione (TZD) compounds are widely used as oral hypoglycemic agents. Herein, we provide evidence showing that troglitazone, one of the TZD compounds, is able to prevent glomerular dysfunction in diabetic rats through a novel mechanism independent of its insulin-sensitizing action. We examined the effect of troglitazone on functional and biochemical parameters of glomeruli in streptozotocin-induced diabetic rats. Troglitazone was able to prevent not only diabetic glomerular hyperfiltration and albuminuria, but an increase in mRNA expression of extracellular matrix proteins and transforming growth factor- $\beta$ 1 in glomeruli of diabetic rats, without changing blood glucose levels. Biochemically, an increase in diacylglycerol (DAG) contents and the activation of the protein kinase C (PKC)-extracellular signal-regulated kinase (ERK) pathway in glomeruli of diabetic rats were abrogated by troglitazone. The activation of DAG-PKC-ERK pathways in vitro in mesangial cells cultured under high glucose conditions was also inhibited by troglitazone. Troglitazone enhanced the activities of DAG kinase, which could metabolize DAG to phosphatidic acid, in both glomeruli of diabetic rats and mesangial cells cultured under high glucose conditions. Surprisingly, pioglitazone, another TZD compound without  $\alpha$ -tocopherol moiety in its structure, also prevented the activation of the DAG-PKC pathway and activated DAG kinase in mesangial cells cultured under high glucose conditions. These results may identify the TZDs as possible new therapeutic agents for diabetic nephropathy that prevent glomerular dysfunction through the inhibition of the DAG-PKC-ERK pathway. *Diabetes* 49:1022-1032, 2000**

**T**hiazolidinediones (TZDs), novel insulin-sensitizing agents, have been shown to attenuate hyperinsulinemia and hyperglycemia in insulin-resistant diabetic animals and human subjects with type 2 diabetes (1,2). These agents are currently in clinical trials and have been widely used as oral antidiabetic agents in subjects with type 2 diabetes in the U.S. and other countries. Although it is generally believed that TZDs exert insulin-sensitizing activity through the activation of peroxisome proliferator-activated receptor (PPAR)- $\gamma$ , a member of the PPAR nuclear receptor superfamily (3), the exact mechanism of their action remains to be clarified (4-6). Besides its insulin-sensitizing activity, troglitazone, one of the TZD compounds, was found to inhibit high glucose-induced proliferation and migration of vascular smooth muscle cells by inhibiting the activation of protein kinase C (PKC) (7); to inhibit platelet aggregation (8); to reduce balloon injury-induced intimal hyperplasia in rat artery (9); and to suppress both insulin- and angiotensin II-induced increase of DNA synthesis by inhibiting the activation of extracellular signal-regulated kinase (ERK) (10,11). Troglitazone was also shown to ameliorate albuminuria and peripheral neuropathy in streptozotocin (STZ)-induced diabetic rats, regardless of blood glucose levels (12,13). These results indicate that troglitazone has various actions other than its insulin-sensitizing activity both in vivo and in vitro.

Interestingly, troglitazone has  $\alpha$ -tocopherol moiety, the chromane ring, as well as TZD moiety in its structure.  $\alpha$ -Tocopherol was shown to be capable of preventing the activation of the diacylglycerol (DAG)-PKC pathway and to improve early glomerular dysfunction, such as glomerular hyperfiltration, in diabetic rats (14). We and others have recently proposed that the activation of the DAG-PKC pathway, followed by the activation of ERK in mesangial cells cultured under high glucose conditions or in glomeruli from diabetic rats, could be a putative mediator by which hyperglycemia causes mesangial and glomerular dysfunction (14-21). Indeed, we found that inhibition of PKC by calphostin C or inhibition of ERK by PD098059, a mitogen-activated protein kinase kinase inhibitor, could normalize diabetes-induced cellular alterations, such as the activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) (20). Furthermore, LY333531, a specific PKC- $\beta$  inhibitor, was also shown to improve glomerular dysfunction in diabetic rats (17,22). These results support the hypothesis that the DAG-PKC-

From the Third Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan.

Address correspondence and reprint requests to Masakazu Haneda, MD, The Third Department of Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan. E-mail: haneda@belle.shiga-med.ac.jp.

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ANP, atrial natriuretic peptide; BSA, bovine serum albumin; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; CRE, cyclic AMP-responsive element; DAG, diacylglycerol; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FEalb, fractional excretion of albumin; FF, filtration fraction; GFR, glomerular filtration rate; PA, phosphatidic acid; PAH, para-aminohippurate; PBS, phosphate-buffered saline; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; RPF, renal plasma flow; STZ, streptozotocin; TBS, Tris-buffered saline; TGF, transforming growth factor; TZD, thiazolidinedione.

ERK–signaling pathway plays an important role in the pathogenesis of diabetic nephropathy.

From these observations, we hypothesized that troglitazone could ameliorate early and late glomerular dysfunction by inhibiting the DAG-PKC-ERK pathway in diabetic rats. To prove this hypothesis, we examined the effect of troglitazone on glomerular dysfunction, such as glomerular hyperfiltration, albuminuria, and an increase in mRNA expression of extracellular matrix (ECM) proteins and transforming growth factor (TGF)- $\beta$ 1, in addition to its effect on the DAG-PKC-ERK pathway in glomeruli of insulin-deficient diabetic rats. We also evaluated the effect of troglitazone on the DAG-PKC-ERK pathway *in vitro* in rat mesangial cells cultured under high glucose conditions. Furthermore, to confirm the specificity of the action of troglitazone, we also examined the effect of pioglitazone (23), another TZD compound without  $\alpha$ -tocopherol moiety in its structure, on the DAG-PKC-ERK pathway in mesangial cells exposed to high concentrations of glucose.

## RESEARCH DESIGN AND METHODS

**Materials.** Anti-active ERK antibody was purchased from Promega (Madison, WI). Anti-ERK2 antibody was bought from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine myelin basic protein was bought from Sigma (St. Louis, MO). Rat fibronectin cDNA was provided by Dr. J.E. Schwarzbauer (24). Mouse  $\alpha$ 1 type IV collagen cDNA was provided by Dr. K.M. Kurkinen (25). Mouse TGF- $\beta$ 1 cDNA was provided by Dr. F.N. Ziyadeh (26). [ $\gamma$ - $^{32}$ P]ATP and [ $\alpha$ - $^{32}$ P]CTP were bought from New England Nuclear (Boston, MA). Troglitazone was provided by Sankyo (Tokyo). Pioglitazone was provided by Takeda (Osaka, Japan). All other reagents were of chemical grade and were purchased from standard suppliers.

**Experimental protocol.** Male Sprague-Dawley rats weighing 220–260 g were made diabetic by a single intravenous injection of STZ (50 mg/kg body wt) in 0.1 mol/l citrate buffer (pH 4.5). Rats receiving an injection of citrate buffer were used as controls. The levels of blood glucose were determined 2 days after the injection of STZ or vehicle, and rats with blood glucose levels >16.7 mmol/l were used as diabetic rats. These rats were divided into 4 groups: control rats, control rats treated with troglitazone (0.2% mixed in standard diet), diabetic rats, and diabetic rats treated with troglitazone (0.2% mixed in standard diet). All rats were allowed free access to food and water. Either 4 weeks (4-week group) or 12 weeks (12-week group) after the injection in rats of STZ or vehicle, the following studies were performed. Renal clearance studies were performed in rats from the 4-week group. Renal glomeruli were isolated from rats in the 4-week group by sieving with stainless steel and nylon meshes, as described previously (27) for the measurement of total DAG contents and the activities of PKC, ERK, and DAG kinase. Rats in the 12-week group were placed in metabolic cages, and 24-h urine samples were collected for the measurement of urinary albumin excretion rates. Glomeruli were then isolated for the determination of mRNA and protein expression of ECM proteins and TGF- $\beta$ 1. All experiments were approved by the Shiga University of Medical Science Animal Care Committees.

Mesangial cells were obtained from a culture of glomeruli isolated from male Sprague-Dawley rats weighing 100–150 g in RPMI-1640 medium (glucose 11.1 mmol/l) containing 20% fetal bovine serum (FBS) and antibiotics. Cultured cells were identified as mesangial cells, as previously described (27). Subconfluent cells from the 4th to 9th passages were exposed to the experimental RPMI-1640 medium (glucose free) containing 0.4% FBS and antibiotics supplemented with either 5.6 or 27.8 mmol/l glucose. The medium was changed daily, and the cells were harvested after 5 days. In some experiments, cells were incubated with 3  $\mu$ mol/l troglitazone or 0.3  $\mu$ mol/l pioglitazone for 5 days.

**Renal clearance studies and the measurement of urinary albumin excretion rates.** Renal clearance studies were performed in rats in the 4-week group as previously described (28). Rats were anesthetized by an intraperitoneal injection of thiopental sodium (50 mg/kg) and placed on a temperature-regulated table. A catheter was inserted in the left carotid artery for blood sampling. A catheter was also inserted in the left jugular vein for continuous infusion of inulin and para-aminohippurate (PAH) solution. The urinary bladder was catheterized for urine sampling, and urine was collected into tubes of known weight. Inulin (1%) and PAH (2.5%) in saline were infused at a rate of 3.6 ml/h for 20 min as a priming load and then infused continuously with a syringe pump at a rate of 1.2 ml/h. After a 60-min equilibration period, 2 consecutive timed

30-min clearance studies were performed. Blood samples (0.25 ml) were collected at the midpoint of each clearance period for the measurement of plasma concentrations of inulin and PAH, and the blood loss was immediately replaced by the same volume of saline. Inulin and PAH in plasma and urine were measured with a cysteine-tryptophan reaction (29) and a slightly modified version of Brun's method (30), respectively. Urine samples were treated with concentrated HCl for 10 min at 70°C to hydrolyse glycosylated PAH (30).

The concentrations of albumin in 24-h urine samples of rats in the 12-week group were measured using an enzyme-linked immunosorbent assay kit (Nephra; Exocell, Philadelphia) according to the manufacturer's instructions. The concentrations of serum albumin, serum creatinine, and urinary creatinine were measured using a dye-binding method for albumin, and an enzymic reaction-rate assay was used to measure creatinine concentrations (VetScan; Abaxis, Sunnyvale, CA).

**mRNA and protein expression of ECM proteins and TGF- $\beta$  in rat glomeruli.** For the Northern blot analysis of ECM proteins and TGF- $\beta$ , total RNA was isolated from glomeruli of rats in the 12-week group by using a commercial preparation based on guanidinium and phenol extraction (TRIzol Reagent; Gibco BRL, Grand Island, NY). Total RNA (12  $\mu$ g per lane) was electrophoretically separated on formaldehyde 1.0% agarose gel and transferred onto a nylon membrane (Nytran 0.45  $\mu$ m; Schleicher & Schuell, Dassel, Germany). After immobilizing the RNA by heating a membrane for 2 h at 80°C, hybridization was performed in a buffer (0.5 mol/l NaPO<sub>4</sub>, pH 7.0, 1% bovine serum albumin [BSA], 7% SDS, and 1 mmol/l EDTA) containing rat fibronectin, mouse  $\alpha$ 1 type IV collagen, or mouse TGF- $\beta$ 1 cDNA labeled with [ $\alpha$ - $^{32}$ P]CTP by a random primer method (BcaBEST; Takara, Shiga, Japan) at 65°C for 16 h. Radioactivity of the corresponding bands was measured quantitatively by a phosphoimage analyzer (Molecular Analyst; Bio-Rad Laboratories, Hercules, CA). After radioactive probes were stripped off the membrane, the membrane was rehybridized with a radioactive probe of acidic ribosomal phosphoprotein PO (36B4) as an internal standard (31).

The expression of ECM proteins was further examined by immunoblot analysis as previously described (32). Glomeruli isolated from rats in the 12-week group were homogenized in 0.5 ml ice-cold buffer (30 mmol/l Tris-HCl, pH 7.5, 10 mmol/l EGTA, 5 mmol/l EDTA, 1 mmol/l dithiothreitol [DTT], and 250 mmol/l sucrose). After sonication at 4°C for 10 s, glomerular homogenates were centrifuged at 12,000g at 4°C for 20 min, and supernatants were used for assay. After boiling for 5 min, samples (10  $\mu$ g protein/lane) were electrophoresed on an SDS-PAGE (4–20% acrylamide gradient gel [Tefco, Tokyo]), as described by Laemmli (33), and transferred to a polyvinylidene difluoride (PVDF) filter (Immobilon; Millipore, Bedford, MA) for 5 h at 100 V using the Mini-Transblot Cell apparatus (Bio-Rad). For blocking, the filter was incubated in 5% nonfat milk in a buffer containing 10 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl (Tris-buffered saline [TBS]), and 0.1% Tween-20 at 4°C overnight. The filter was incubated with anti-rat fibronectin antibody (1:300 dilution) (Chemicon International, Temecula, CA) or anti-rat type IV collagen antibody (1:100 dilution) (Progen Biotechnik, Heidelberg, Germany) in the dilution buffer (TBS containing 5% BSA and 0.1% Tween-20) overnight at 4°C. The filter was washed 3 times with TBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG second antibody at room temperature for 1 h. The bands were detected using the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, U.K.). A density of the corresponding bands was measured quantitatively using NIH Image software (<http://rsb.info.nih.gov/nih-image>). Protein was measured using a Bio-Rad protein assay kit.

**Determination of total DAG contents.** Total DAG contents of glomeruli isolated from rats in the 4-week group and of cultured mesangial cells in 100-mm dishes were determined with a radioenzymatic assay kit (Amersham, Arlington Heights, IL) that used DAG kinase (Calbiochem, San Diego, CA), which quantitatively converts DAG to [ $^{32}$ P]-phosphatidic acid (PA) in the presence of [ $\gamma$ - $^{32}$ P]ATP. In brief, total glomerular and cellular lipids were extracted twice according to the methods of Bligh and Dyer (34), and total DAG was measured according to the 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, and water (10:4:3:2:1). PA was visualized by autoradiography and identified by comigration with radiolabeled PA derived from DAG standard from 31.25 to 2,000 pmol/l. Radioactivity of the corresponding spots was measured quantitatively by a phosphoimage analyzer. The values of total DAG contents were normalized by protein contents of glomeruli and cells measured as described by Bradford (35).

**Measurement of the activities of PKC.** The activities of PKC in glomeruli and cultured mesangial cells were determined using an *in situ* PKC assay modified by Koya et al. (14). Glomeruli isolated from rats in the 4-week group were rinsed twice with 2 ml RPMI-1640 containing 20 mmol/l HEPES (pH 7.4)

and once with a salt solution (137 mmol/l NaCl, 5.4 mmol/l KCl, 0.3 mmol/l sodium phosphate, 0.4 mmol/l potassium phosphate, 5.5 mmol/l glucose, 10 mmol/l MgCl<sub>2</sub>, 25 mmol/l β-glycerophosphate, 5 mmol/l EGTA, 2.5 mmol/l CaCl<sub>2</sub>, and 20 mmol/l HEPEs). Subconfluent mesangial cells were washed twice with Hanks' balanced salt solution. Samples were incubated with the salt solution for 15 min at 37°C in the presence or absence of 100 μmol/l PKC-specific peptide substrate, RTLRRL, after the addition of 5 mg/ml digitonin (final concentration 50 μg/ml) and 1 mmol/l ATP (final concentration 100 μmol/l) mixed with [<sup>32</sup>P]ATP (<1,500 cpm/pmol). The reaction was stopped by 5% trichloroacetic acid (final concentration). Aliquots of the reaction were spotted on 2.5 × 2.5 cm phosphocellulose paper (Whatman P81; Whatman, Maidstone, U.K.) and were washed in 3 changes of 75 mmol/l phosphoric acid and 1 change of 75 mmol/l sodium phosphate (pH 7.5). The radioactivity of phosphorylated substrate was determined by liquid scintillation counting. Protein content of each sample was measured by Bradford's method (35).

**Measurement of the activities of ERK.** For the immunoblot analysis of active ERK, glomeruli isolated from rats in the 4-week group were lysed in 0.5 ml ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1% Nonidet-P, 140 mmol/l NaCl, 1 mmol/l sodium orthovanadate, 50 mmol/l NaF, 1 mmol/l EGTA, 50 U/ml aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 1 μg/ml leupeptin). Cultured mesangial cells in 100-mm dishes were harvested in 0.5 ml cell lysis buffer (25 mmol/l Tris-HCl, pH 7.4, 25 mmol/l NaCl, 80 mmol/l β-glycerophosphate, 1 mmol/l sodium orthovanadate, 1 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 1 mmol/l EGTA, 1 mmol/l PMSF, and 10 μg/ml leupeptin). After sonication at 4°C for 10 s, glomerular and cellular homogenates were centrifuged at 12,000g at 4°C for 30 min, and supernatants were used for assay. After boiling for 5 min, samples (25 μg protein measured by a Bio-Rad protein assay kit) were electrophoresed on an SDS-PAGE (10% acrylamide gel), as described by Laemmli (33), and transferred to a PVDF filter for 1 h at 200 mA using Horizblot AE6677P (Atto, Tokyo). For blocking, the filter was incubated in phosphate-buffered saline (PBS) containing 5% nonfat milk and 0.1% Tween-20 at 4°C overnight. The filter was incubated with anti-active ERK antibody (1:10,000 dilution) in the dilution buffer (PBS containing 5% BSA and 0.1% Tween-20) at room temperature for 3 h. The filter was washed 3 times with PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG second antibody at room temperature for 1 h. The bands were detected using an ECL system. The filter was reprobed with anti-ERK2 antibody after stripping. The activities of ERK in cultured mesangial cells were also measured by the immune complex kinase assay as previously described (36).

**Measurement of the activities of DAG kinase.** Isolated glomeruli or cultured mesangial cells in 100-mm dishes were lysed in a buffer containing 25 mmol/l Tris-HCl, pH 7.4, 0.25 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 20 μg/ml aprotinin, 1 mmol/l PMSF, and 20 μg/ml leupeptin and were sonicated and homogenized on a Dounce homogenizer. After a low-speed centrifugation (550g for 10 min), the supernatant was used for the assay of total DAG kinase activity. The DAG kinase activity was measured by the octyl-glucoside mixed micelle assay (14). In brief, the reaction was initiated by the addition of enzyme (10–20 μg total glomerular lysates) in a mixture containing 50 mmol/l MOPS (pH 7.2), 50 mmol/l octyl-glucoside, 100 mmol/l NaCl, 1 mmol/l DTT, 20 mmol/l NaF, 2.1 mmol/l CaCl<sub>2</sub>, 0.8 mmol/l EDTA, 2 mmol/l EGTA, 10 mmol/l MgCl<sub>2</sub>, 6.7 mmol/l phosphatidylserine, and 1 mmol/l [<sup>32</sup>P]ATP (10,000 cpm/pmol) in the presence of 1 mmol/l 1,2-didecanoyl-sn-glycerol (Sigma) and was continued for 10 min at 30°C. Lipids were extracted from the mixture, and PA separated on thin-layer plates of silica gel was counted by a phosphoimage analyzer. The values of DAG kinase activity were normalized by protein content of sample measured by Bradford's method (35).

**Statistical analysis.** Results were expressed as means ± SD. Comparisons between 2 groups were analyzed by Student's unpaired *t* test. Comparisons among 3 or more groups were analyzed by one-way analysis of variance, followed by either Scheffé's test or Bonferroni/Dunn's multiple comparison test to evaluate statistical difference between 2 groups.

## RESULTS

**Effect of troglitazone on renal hemodynamics and albuminuria.** An increase in the glomerular filtration rate (GFR), also known as glomerular hyperfiltration, is one of the important functional abnormalities in diabetic kidneys (37). To clarify whether troglitazone could prevent glomerular hyperfiltration in diabetic rats, we evaluated GFR and renal plasma flow (RPF) by measuring the renal clearance of inulin and PAH, respectively. Four weeks after STZ injection, the lev-

TABLE 1

Characteristics of rats treated with troglitazone for 4 weeks

	<i>n</i>	Body weight (g)	Kidney mass (g/100 g body wt)	Blood glucose (mmol/l)
Control	19	388.2 ± 31.2	0.63 ± 0.05	7.5 ± 1.4
Control + troglitazone	19	380.6 ± 26.0	0.63 ± 0.04	7.7 ± 1.1
Diabetic	19	270.9 ± 27.3*	1.12 ± 0.09*	23.8 ± 3.6*
Diabetic + troglitazone	20	270.3 ± 32.0*	1.09 ± 0.12*	23.9 ± 2.7*

Data are means ± SD. \**P* < 0.01 vs. control or control + troglitazone.

els of blood glucose were significantly higher in diabetic rats than in control rats (Table 1). Troglitazone failed to decrease the blood glucose levels in both control and diabetic rats (Table 1). Body mass was smaller and kidney mass was greater in diabetic rats than in control rats, regardless of the treatment with troglitazone (Table 1). The serum concentrations of troglitazone in diabetic rats (0.91 ± 0.57 μg/ml) were almost equivalent to those in human subjects being treated with troglitazone (38). As shown in Fig. 1A, GFR was significantly higher in diabetic rats than in control rats. This increase in GFR in diabetic rats was prevented by the treatment with troglitazone, whereas GFR in control rats was not affected by troglitazone (Fig. 1A). RPF did not differ among the 4 study groups (Fig. 1B). As a result, the filtration fraction (FF) was significantly elevated in diabetic rats compared with control rats, and this elevation of FF was again normalized by troglitazone (Fig. 1C).

To confirm further the beneficial effect of troglitazone on renal dysfunction in diabetes, we measured urinary albumin excretion rates in rats from the 12-week group. The levels of blood glucose were significantly higher in diabetic rats than in control rats (Table 2). Troglitazone again failed to decrease the concentrations of blood glucose in both diabetic and control rats (Table 2). Urinary albumin excretion rates in diabetic rats (4.6 ± 3.3 mg/day) were significantly higher than those in control rats (1.0 ± 0.7 mg/day) (Fig. 2). Similar to its effect on abnormal renal hemodynamics in diabetic rats, troglitazone ameliorated the increase in urinary albumin excretion rates in diabetic rats (Fig. 2). Fractional excretion of albumin (FEalb) was calculated from albumin clearance and creatinine clearance in rats from the 12-week group. FEalb in diabetic rats was significantly higher than that in control rats. Similar to its effect on urinary albumin excretion rates, troglitazone inhibited the increase in FEalb of diabetic rats partially but significantly (control 1.9 ± 1.5 × 10<sup>-5</sup>, control + troglitazone 1.5 ± 0.5 × 10<sup>-5</sup>, diabetic 8.0 ± 2.1 × 10<sup>-5</sup>, diabetic + troglitazone 4.3 ± 1.0 × 10<sup>-5</sup>, diabetic rats vs. the other 3 groups *P* < 0.01, *n* = 4).

**Effect of troglitazone on mRNA and protein expression of ECM proteins and TGF-β1 in glomeruli.** The most important histological character of diabetic nephropathy is the expansion of glomerular mesangium composed of various ECM proteins (39). Thus, we examined the effect of troglitazone on mRNA expression of ECM proteins and TGF-β1, which was proposed to play an important role in the overproduction of ECM proteins in diabetes (40) and in glomeruli of diabetic rats. The expression of both fibronectin (Fig. 3A) and α1

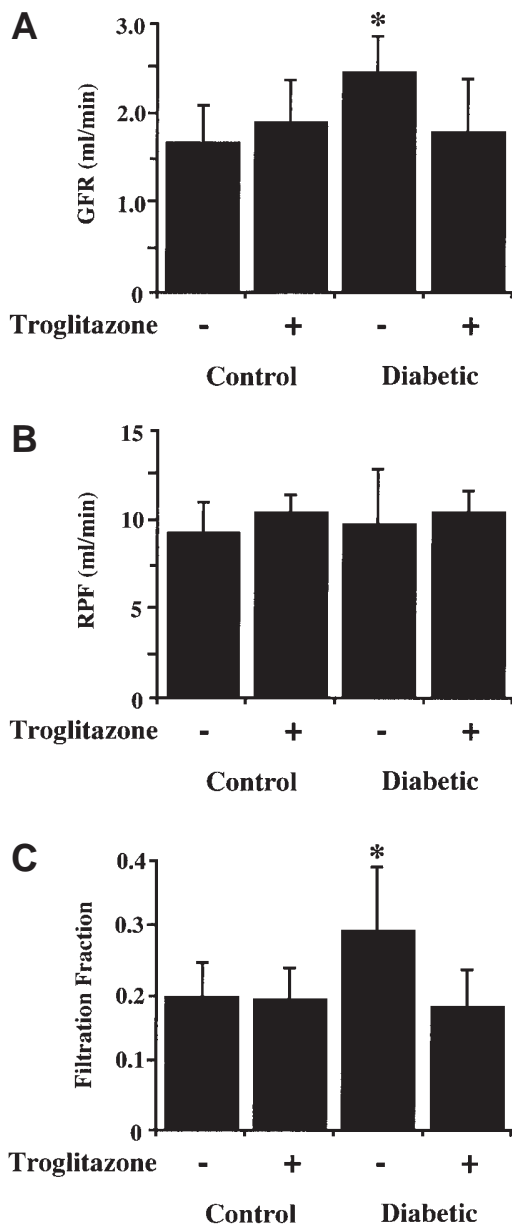


FIG. 1. Effect of troglitazone on GFR (A), RPF (B), and FF (C) in control and diabetic rats. In the 4-week group, GFR and RPF were determined by measuring renal clearance of inulin and PAH, respectively. FF was calculated by a standard formula, the GFR-to-RPF ratio. Data are expressed as means  $\pm$  SD ( $n = 7-8$ , \* $P < 0.05$  vs. the other groups).

TABLE 2  
Characteristics of rats treated with troglitazone for 12 weeks

	<i>n</i>	Body weight (g)	Kidney mass (g/100 g body wt)	Blood glucose (mmol/l)
Control	22	498.0 $\pm$ 42.6	0.62 $\pm$ 0.08	6.3 $\pm$ 1.0
Control + troglitazone	16	517.5 $\pm$ 33.7	0.58 $\pm$ 0.04	6.4 $\pm$ 1.1
Diabetic	22	268.0 $\pm$ 63.3*	1.38 $\pm$ 0.17*	23.5 $\pm$ 3.3*
Diabetic + troglitazone	16	268.0 $\pm$ 58.6*	1.38 $\pm$ 0.30*	22.2 $\pm$ 3.5*

Data are means  $\pm$  SD. \* $P < 0.01$  vs. control or control + troglitazone.

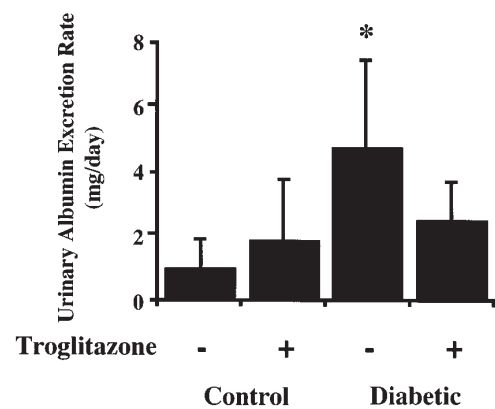


FIG. 2. Effect of troglitazone on urinary albumin excretion rates in control and diabetic rats. In the 12-week group, 24-h urine samples were collected. The concentrations of urinary albumin were determined by an enzyme-linked immunosorbent assay. Data are expressed as means  $\pm$  SD ( $n = 10-16$ , \* $P < 0.05$  vs. the other groups).

type IV collagen (Fig. 3B) mRNA was significantly enhanced in diabetic rats by 85.0 and 54.0%, respectively. Treatment with troglitazone prevented the increase in mRNA expression of both fibronectin and  $\alpha 1$  type IV collagen in diabetic rats, whereas troglitazone did not change their expression in control rats (Fig. 3A and B). Like the expression of ECM proteins, the expression of TGF- $\beta 1$  mRNA was significantly enhanced in glomeruli of diabetic rats compared with that in control rats, and this enhancement in diabetic rats was again prevented by troglitazone (Fig. 3C). Moreover, a significant increase in protein expression of both fibronectin and type IV collagen was also observed in glomeruli of diabetic rats in the 12-week group by immunoblot analysis (Fig. 3D and E). Troglitazone was again able to improve the overexpression of ECM proteins in the glomeruli of diabetic rats, whereas troglitazone did not significantly affect protein expression of fibronectin and type IV collagen in control rats (Fig. 3D and E).

**Effect of troglitazone on the activation of the DAG-PKC-ERK pathway and the mechanism of the inhibitory effect of troglitazone on the DAG-PKC-ERK pathway in glomeruli of diabetic rats.** Because the activation of the DAG-PKC-ERK pathway in diabetes was suggested to be responsible for glomerular dysfunction in diabetic rats (14,17), we next examined whether troglitazone could prevent an increase in DAG content and the activation of the PKC-ERK pathway in vivo in glomeruli from diabetic rats. Four weeks after STZ injection, total DAG contents in glomeruli from diabetic rats were significantly increased compared with those of control rats (Fig. 4A). Troglitazone was able to prevent this increase in DAG contents in glomeruli of diabetic rats without affecting DAG contents in control rats (Fig. 4A). The activities of PKC were also enhanced in glomeruli of diabetic rats, and this activation of PKC was also prevented by the treatment with troglitazone (Fig. 4B). Consistent with the activation of DAG-PKC signaling, the activities of ERK determined by the immunoblot analysis with anti-phosphorylated (active) ERK antibody were enhanced in glomeruli of diabetic rats, and troglitazone was able to prevent this activation of ERK in diabetic glomeruli (Fig. 4C).

The results previously described suggest that troglitazone is able to prevent glomerular dysfunction in diabetic rats by

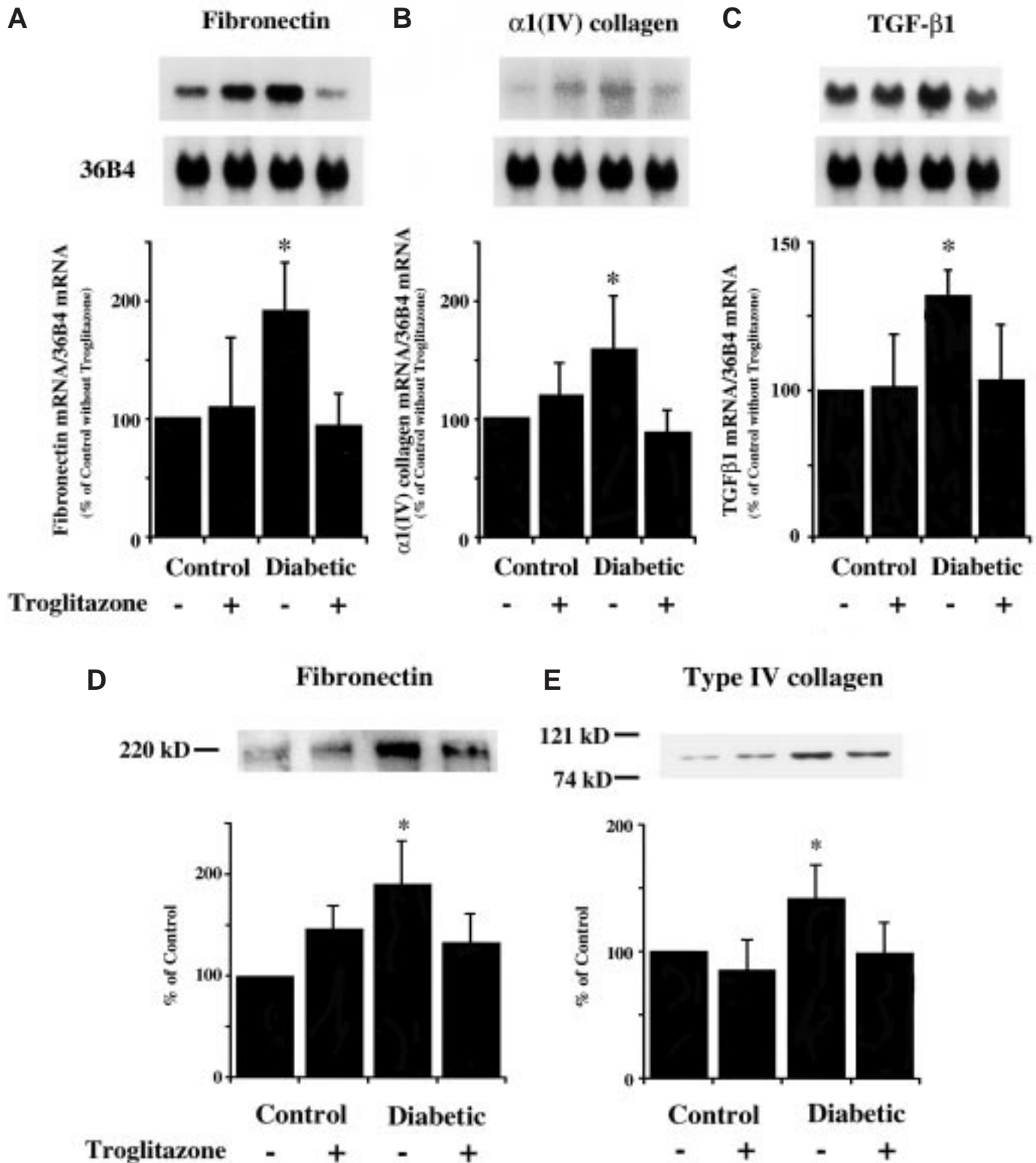
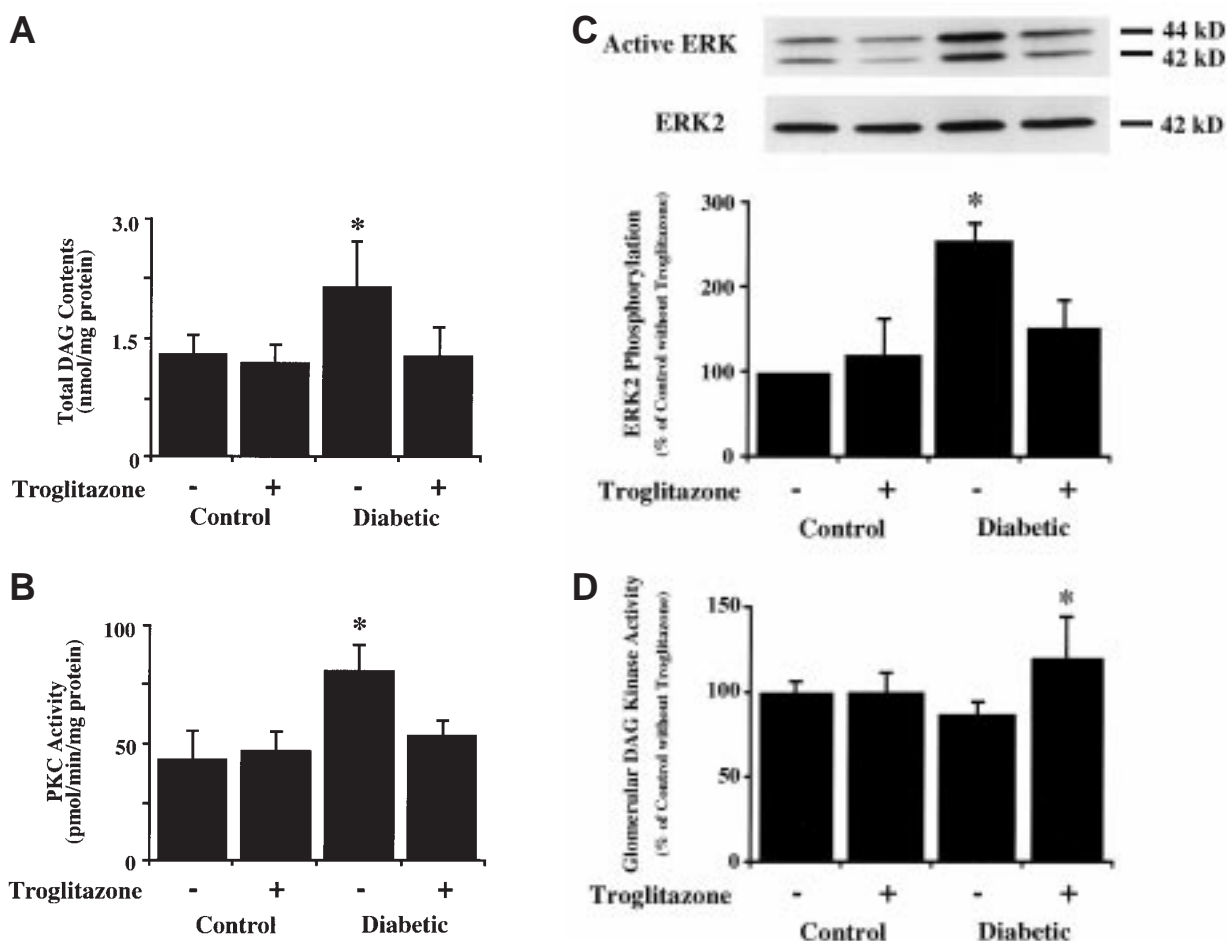


FIG. 3. Effect of troglitazone on mRNA expression of fibronectin (A),  $\alpha 1$  type IV collagen (B), and TGF- $\beta 1$  (C) and on protein expression of fibronectin (D) and type IV collagen (E) in rat glomeruli. Glomeruli were isolated from rats in the 12-week group. A-C: Representative results of Northern blot analysis for glomerular mRNA expression for fibronectin,  $\alpha 1$  type IV collagen, TGF- $\beta 1$ , and 36B4 are shown in the upper panel. Radioactivity of the corresponding bands was measured quantitatively by a phosphoimage analyzer, and the ratio to 36B4 mRNA is shown in the lower panel. Data are expressed as means  $\pm$  SD ( $n = 5-8$ ). The results were derived from 10-16 rats; 4 kidneys from 2 rats were processed for each experiment (\* $P < 0.05$  vs. other groups). D and E: Representative results of immunoblot analysis in glomerular protein expression for fibronectin and type IV collagen are shown in the upper panel. Glomeruli isolated from each rat were processed for immunoblot analysis. Densitometric quantification of the corresponding bands was performed using NIH Image software, and the ratio to control is shown in the lower panel. Data are expressed as means  $\pm$  SD ( $n = 6$ ). The results were derived from 6 rats, and 2 kidneys from a rat were processed for each experiment (\* $P < 0.05$  vs. the other groups).

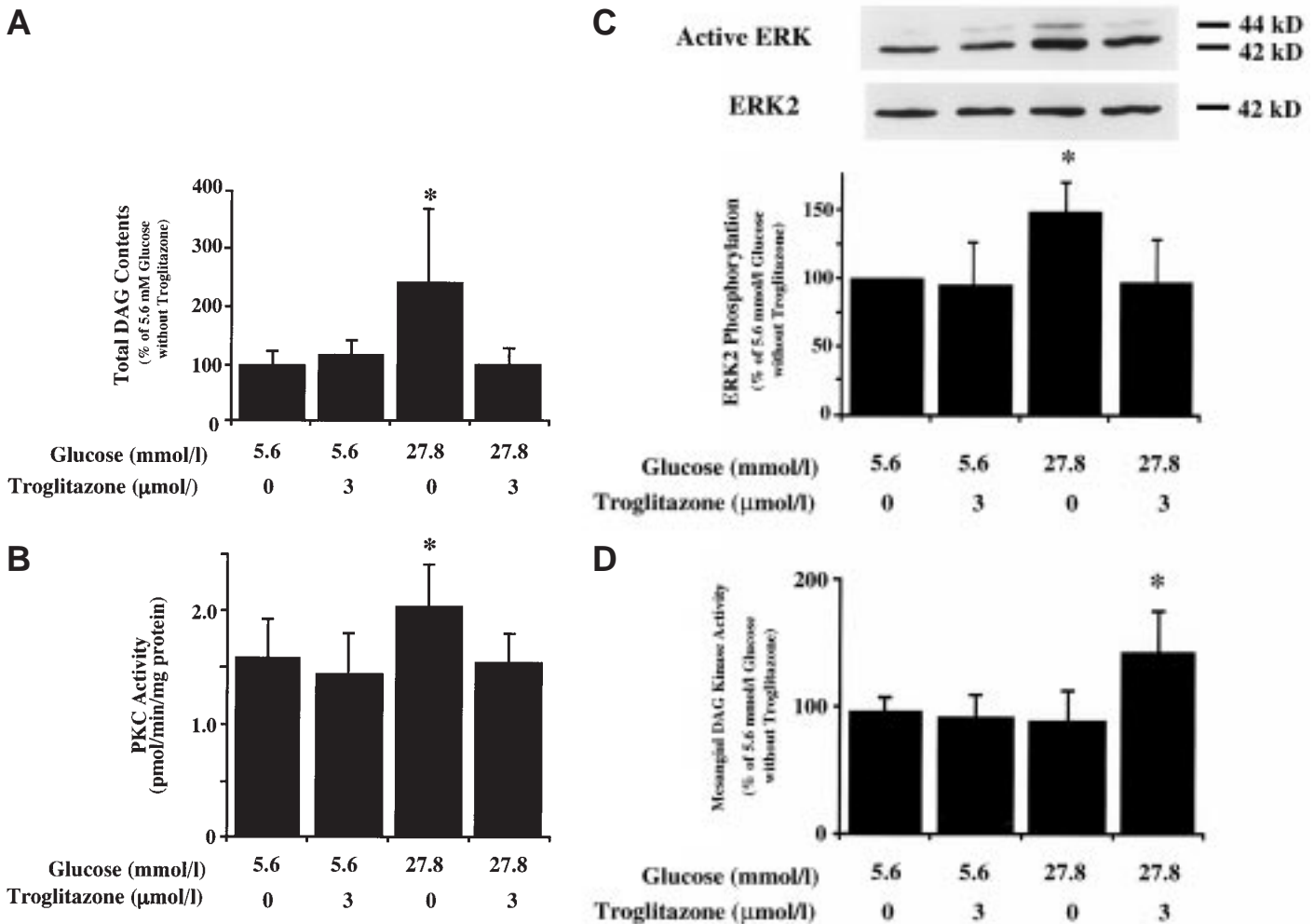


**FIG. 4.** Effect of troglitazone on the DAG-PKC-ERK pathway in rat glomeruli. Glomeruli were isolated from rats in the 4-week group. **A:** After the extraction of total glomerular lipids, total DAG contents were measured by a radioenzymatic assay using DAG kinase. Data are expressed as means  $\pm$  SD ( $n = 5$ ,  $*P < 0.05$  vs. other groups). **B:** The activities of PKC were measured by an in situ PKC assay using a specific PKC substrate, RTLRL, in digitonin-permeabilized glomeruli. Data are expressed as means  $\pm$  SD ( $n = 6-8$ ,  $*P < 0.001$  vs. other groups). **C:** The activities of ERK in glomeruli were determined by measuring the phosphorylation of ERK using immunoblot analysis with anti-active ERK antibody. The glomerular lysates (25  $\mu$ g protein/lane) were electrophoresed on a 10% SDS-PAGE and were transferred to a PVDF membrane. The filter was probed with an anti-active ERK antibody and then reprobbed with an anti-ERK2 antibody. A representative result of immunoblot analysis is shown in the upper panel. Densitometric quantification of phosphorylated ERK2 was performed using NIH Image software, and the ratio of phosphorylated ERK2 to total ERK2 is shown in the lower panel. Data are expressed as means  $\pm$  SD ( $n = 4$ ,  $*P < 0.05$  vs. the other 3 groups). **D:** The activities of DAG kinase were determined by octyl-glucoside-mixed micelle assay after the extraction of total glomerular lipids. Data are expressed as means  $\pm$  SD ( $n = 6-7$ ,  $*P < 0.05$  vs. the untreated diabetic rats).

preventing diabetes-induced activation of the PKC-ERK pathway through the inhibition of an increase in total DAG contents. One possible cellular mechanism of the inhibitory effect of troglitazone on DAG contents is the activation of DAG kinase, which is an important modulator of intracellular DAG by metabolizing DAG to PA (41). Thus, we measured the activities of DAG kinase in glomeruli of diabetic rats and examined the effect of troglitazone on DAG kinase. As shown in Fig. 4D, troglitazone significantly enhanced the activities of DAG kinase in glomeruli of diabetic rats.

**Effect of the TZDs, troglitazone and pioglitazone, on the DAG-PKC-ERK pathway in vitro in mesangial cells cultured under high glucose conditions.** We next examined whether troglitazone could prevent an increase in DAG content and the activation of the PKC-ERK pathway in mesangial cells cultured under high glucose conditions. Total DAG contents in mesangial cells cultured under high glucose conditions (27.8 mmol/l) were significantly increased

compared with those in cells cultured under normal glucose conditions (5.6 mmol/l) (Fig. 5A). Troglitazone (3  $\mu$ mol/l) was able to prevent this increase in mesangial cells cultured under high glucose conditions without affecting DAG contents in cells cultured under normal glucose conditions (Fig. 5A). Consistent with our previous study (19,20), the activities of PKC were significantly enhanced in mesangial cells exposed to high glucose conditions compared with those in cells exposed to normal glucose conditions. Troglitazone was able to prevent the activation of PKC in cells under high glucose conditions (Fig. 5B) in a dose-dependent manner with a maximal effect at 3  $\mu$ mol/l (data not shown). Similar to the activities of PKC, the activities of ERK measured by an immune complex kinase assay were significantly enhanced in cells exposed to high concentrations of glucose (glucose 5.6 mmol/l [ $1.14 \pm 0.24$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  protein] vs. glucose 27.8 mmol/l [ $1.42 \pm 0.37$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  protein],  $P < 0.05$ ,  $n = 22$ ). The activation of ERK in cells under high glucose con-



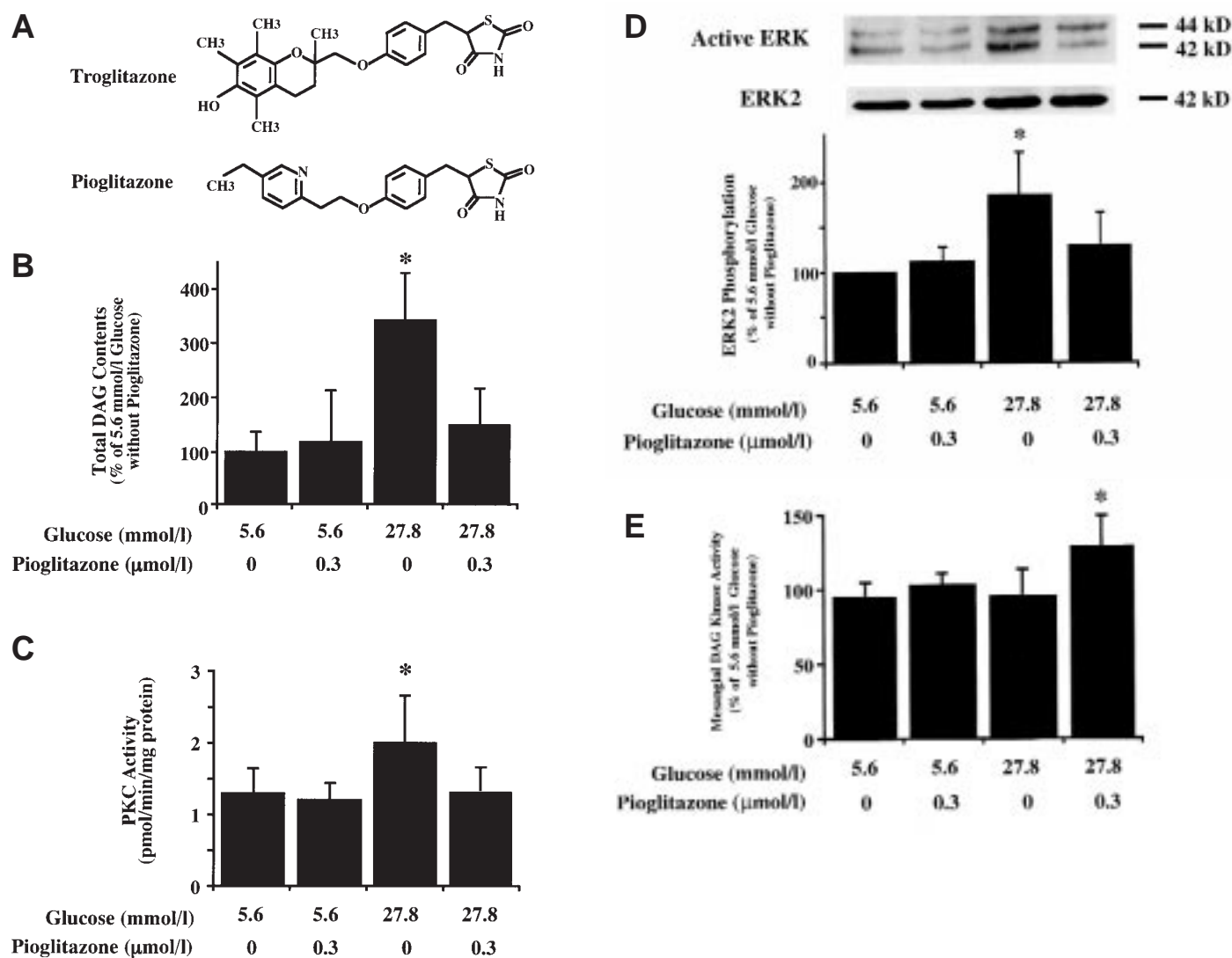
**FIG. 5.** Effect of troglitazone on the activation of the DAG-PKC-ERK pathway in vitro in mesangial cells cultured under high glucose conditions. Subconfluent cells were exposed to either high (27.8 mmol/l) or normal (5.6 mmol/l) concentrations of glucose for 5 days with or without 3  $\mu$ mol/l troglitazone. **A:** Total DAG contents in mesangial cells were measured by a radioenzymatic assay using DAG kinase. Data are expressed as means  $\pm$  SD ( $n = 3-7$ , \* $P < 0.05$  vs. the other groups). **B:** The activities of PKC were measured by an in situ PKC assay. Each experiment was performed in triplicate. Data are expressed as means  $\pm$  SD ( $n = 4-11$ , \* $P < 0.05$  vs. the other groups). **C:** The activities of ERK were evaluated by measuring the phosphorylation of ERK. The cell lysates (25  $\mu$ g protein) were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane. After blocking, the filter was incubated with anti-active ERK antibody. The filter was then reprobated with anti-ERK2 antibody. A representative result of immunoblot analysis of phosphorylated ERK and total ERK2 is shown in the upper panel. Densitometric quantification of phosphorylated ERK2 was performed using NIH Image software, and the ratio of phosphorylated ERK2 to total ERK2 is shown in the lower panel. Data are expressed as means  $\pm$  SD ( $n = 5-6$ , \* $P < 0.05$  vs. the other groups). **D:** The activities of DAG kinase activity were measured by the same methods used in Fig. 4. Data are expressed as means  $\pm$  SD ( $n = 5-6$ , \* $P < 0.05$  vs. cells without troglitazone).

ditions was also prevented by treating the cells with troglitazone ( $1.11 \pm 0.27 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein,  $n = 12$ ). The activities of ERK were evaluated further by immunoblot analysis using antibodies against active ERK. The phosphorylation of ERK was enhanced in cells cultured under high glucose conditions, and troglitazone was again able to prevent this enhancement of the phosphorylation of ERK by glucose (Fig. 5C). We next examined whether troglitazone could stimulate the activities of DAG kinase in mesangial cells cultured also under high glucose conditions. As shown in Fig. 5D, troglitazone significantly enhanced the activities of DAG kinase in mesangial cells cultured under high glucose conditions similar to those in glomeruli of diabetic rats. We finally examined whether pioglitazone, another TZD compound without  $\alpha$ -tocopherol moiety in its structure (Fig. 6A), could prevent high glucose-induced activation of the DAG-PKC-ERK pathway in vitro. Surprisingly, an increase in DAG

contents in mesangial cells cultured under high glucose conditions was prevented by treating the cells with 0.3  $\mu$ mol/l pioglitazone (Fig. 6B). Pioglitazone (0.3  $\mu$ mol/l) was also able to prevent the activation of both PKC and ERK under high glucose conditions (Fig. 6C and D). This inhibitory effect of pioglitazone on PKC was observed in a dose-dependent manner, with a maximal effect at 0.3  $\mu$ mol/l (data not shown). Like troglitazone, pioglitazone enhanced the activities of DAG kinase in mesangial cells cultured under high glucose conditions (Fig. 6E).

## DISCUSSION

In the present study, we clearly demonstrated that troglitazone, one of the insulin-sensitizing agents, was able to prevent glomerular dysfunction, such as glomerular hyperfiltration, albuminuria, and an enhancement of mRNA and protein expression of TGF- $\beta$  and ECM proteins, in diabetic rats with-



**FIG. 6.** Effect of pioglitazone on the activation of the DAG-PKC-ERK pathway in vitro in mesangial cells cultured under high glucose conditions. Subconfluent cells were exposed to either high (27.8 mmol/l) or normal (5.6 mmol/l) concentrations of glucose for 5 days with or without 0.3  $\mu\text{mol/l}$  pioglitazone. **A:** Structures of troglitazone and pioglitazone. **B:** Total DAG contents in mesangial cells were measured by a radioenzymatic assay using DAG kinase. Data are expressed as means  $\pm$  SD ( $n = 3$ ,  $*P < 0.05$  vs. the other groups). **C:** The activities of PKC were measured by an in situ PKC assay. Each experiment was performed in triplicate. Data are expressed as means  $\pm$  SD ( $n = 8-12$ ,  $*P < 0.05$  vs. the other groups). **D:** The activities of ERK were evaluated by measuring the phosphorylation of ERK. Data are expressed as means  $\pm$  SD ( $n = 5-7$ ,  $*P < 0.05$  vs. the other groups). **E:** The activities of DAG kinase activity were measured. Data are expressed as means  $\pm$  SD ( $n = 3-4$ ,  $*P < 0.05$  vs. cells without pioglitazone).

out changing blood glucose levels. This action of troglitazone seems to be independent of its insulin-sensitizing activity and to be based on the inhibition of the activation of the DAG-PKC-ERK pathway that is shown both in vivo in glomeruli from diabetic rats and in vitro in mesangial cells cultured under high glucose conditions. Furthermore, we found that pioglitazone, another TZD compound, was also able to inhibit the activation of the DAG-PKC-ERK pathway in mesangial cells cultured under high glucose conditions. These findings provide the first evidence that TZD compounds, such as troglitazone and pioglitazone, have a novel mechanism of action and thus might be useful in the prevention or treatment of diabetic nephropathy.

First, troglitazone prevented glomerular hyperfiltration. Glomerular hyperfiltration found in the early phase of type 1 diabetes has been postulated to be related to the subsequent

development of diabetic nephropathy (37). Multiple factors have been reported to be involved in the development of glomerular hyperfiltration (42), some of which might be associated with the activation of the PKC-ERK pathway. An increase in the production of vasodilatory prostanoids, such as prostaglandin  $E_2$  ( $\text{PGE}_2$ ), in diabetic glomeruli is one of the factors related to the development of glomerular hyperfiltration (43). The production of  $\text{PGE}_2$  could be enhanced through the activation of  $\text{cPLA}_2$  by the activated PKC-ERK pathway (17,20,44). An elevation of plasma levels of atrial natriuretic peptides (ANPs) in diabetic rats has also been proposed as one of the factors causing glomerular hyperfiltration, because glomerular hyperfiltration in diabetic rats was found to be prevented by the administration of ANP antibodies (45) or ANP receptor antagonists (28). The overproduction of ANPs may be also due to the activation of the PKC-



ERK pathway in heart, because the transcription rate of ANPs in cultured atrial cells was shown to be enhanced by the activation of PKC (46). Finally, reduced contractile responsiveness of mesangial cells to vasoconstrictive peptides, such as angiotensin II, might be related to the development of glomerular hyperfiltration (47). A reduction of both the production of inositol 1,4,5-trisphosphate and the increase in intracellular  $Ca^{2+}$  in response to angiotensin II was found in mesangial cells cultured under high glucose conditions (48,49); this reduction was shown to be prevented by an inhibition of PKC (49,50). These results strongly suggest that the activation of the PKC-ERK pathway in diabetes is responsible for the development of glomerular hyperfiltration and that troglitazone is able to prevent glomerular hyperfiltration by inhibiting the activation of the PKC-ERK pathway. Because our present study and that of others (12) found that troglitazone abrogated an increase in urinary excretion of albumin in STZ-induced diabetic rats, it can be concluded that troglitazone is able to ameliorate early functional disturbance of glomeruli in diabetic rats.

Diabetic nephropathy is characterized histologically by an expansion of glomerular mesangium, which is composed of ECM proteins produced by mesangial cells (51). Because a contribution of TGF- $\beta$ 1 to the overproduction of ECM proteins in diabetic glomeruli has been proposed (40,52,53), we examined whether troglitazone could also ameliorate these later glomerular abnormalities. In the present study, we demonstrated that troglitazone was able to prevent an increase in the mRNA expression of TGF- $\beta$ 1 and ECM proteins. Because the expression of TGF- $\beta$ 1 was found to be regulated by the PKC-ERK pathway, possibly through an increase in the DNA-binding activity of AP-1 (17,54–56), the inhibitory effect of troglitazone on TGF- $\beta$ 1 expression could result from an inhibition of the activation of the PKC-ERK pathway in diabetes. An increase in ECM production, especially an increase in fibronectin, might be induced by AP-1 complexes formed through the activation of the PKC-ERK pathway. This hypothesis is possibly supported by the fact that the AP-1 complexes can increase the transcriptional activation of the fibronectin gene through its binding to cyclic AMP-responsive elements (CREs) in its promoter region of the fibronectin gene (57–60). Troglitazone might normalize the increase in expression of the fibronectin gene directly through the inhibition of binding AP-1 to the CRE site in its promoter by inhibiting the PKC-ERK pathway or indirectly by inhibiting the excessive expression of TGF- $\beta$ 1. Thus, the present results clearly indicate that troglitazone is able to prevent both early and late glomerular abnormalities typically seen in diabetic nephropathy.

The mechanisms of the beneficial effect of troglitazone on glomerular dysfunction in diabetes were examined both in vivo and in vitro. Troglitazone prevented an increase in DAG contents and the activation of the PKC-ERK pathway and enhanced the activities of DAG kinase, which could convert DAG to PA, both in vivo in glomeruli of diabetic rats and in vitro in mesangial cells cultured under high glucose conditions. These effects of troglitazone are similar to those of  $\alpha$ -tocopherol (14), but troglitazone has its specific TZD moiety as well as the  $\alpha$ -tocopherol moiety, the chromane ring, in its structure. Thus, the action of troglitazone in the present study might depend on its TZD moiety. This possibility is supported by the following evidence. First, the concentrations

of troglitazone that we used in vitro (3  $\mu$ mol/l) were much smaller than those of  $\alpha$ -tocopherol (50  $\mu$ mol/l), which was used as an antioxidant in the previous studies (61). Second, the activation of DAG kinase by troglitazone was observed only in glomeruli of diabetic rats or in mesangial cells cultured under high glucose conditions, whereas  $\alpha$ -tocopherol was shown to activate DAG kinase in glomeruli of both control and diabetic rats (14).  $\alpha$ -Tocopherol was also found to enhance basal DAG kinase activities in human endothelial cells (62). Finally, pioglitazone, a TZD compound without  $\alpha$ -tocopherol moiety in its structure, was also able to activate DAG kinase and to prevent the activation of the DAG-PKC-ERK pathway in mesangial cells cultured under high glucose conditions. These results indicate that the TZD moiety of both drugs, but not the  $\alpha$ -tocopherol moiety of troglitazone, is responsible for the activation of DAG kinase in the present study. We found the existence of PPAR- $\gamma$  mRNA in mesangial cells by reverse transcription-polymerase chain reaction (data not shown). Because TZDs are the potent agonists for PPAR- $\gamma$ , and because mesangial cells do express PPAR- $\gamma$ , it might be possible that high levels of glucose have permissive effects on PPAR- $\gamma$ , which will appear only in the presence of TZDs, resulting in the enhancement of DAG kinase activity. A further study is necessary to clarify the regulation of DAG kinase activity and its relationship to TZDs.

In conclusion, troglitazone was able to prevent the development of glomerular dysfunction in STZ-induced diabetic rats by inhibiting the activation of the DAG-PKC-ERK pathway, independent of insulin-sensitizing activities. In addition, another TZD compound, pioglitazone, was also able to normalize the DAG-PKC-ERK pathway in mesangial cells cultured under high glucose conditions. Thus, TZDs might be useful for the prevention of the development and progression of diabetic nephropathy in subjects with type 2 diabetes, by improving metabolic control, and subjects with both types 1 and 2 diabetes, by preventing the activation of the DAG-PKC-ERK pathway. Human trials are necessary to prove this hypothesis.

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