Peroxisome Proliferator–Activated Receptor-γ Ligands Inhibit TGF-β1–Induced Fibronectin Expression in Glomerular Mesangial Cells

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The thiazolidinedione (TZD) class of antidiabetic drugs, which are ligands for peroxisome proliferator–activated receptor (PPAR)-γ, has been shown to possess potent anti-inflammatory and antineoplastic actions. Here, we show in mesangial cells that PPAR-γ agonists inhibit fibronectin expression by transforming growth factor (TGF)-β1. TGF-β1 enhanced fibronectin mRNA expression, and this enhancement was abrogated by pretreatment with pioglitazone. Electrophoretic mobility shift assay identified that pioglitazone inhibited TGF-β1–induced DNA binding of activator protein-1 (AP-1). Pioglitazone inhibited AP-1 reporter activity but not Smad binding elements reporter activity without affecting TGF-β1–induced activation of mitogen-activated protein kinases (MAPKs) or Smad2. PPAR-γ overexpression inhibited TGF-β1–induced fibronectin expression as well as the activation of AP-1. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a natural PPAR-γ ligand, also inhibited TGF-β1–induced fibronectin expression by suppressing AP-1 activation by TGF-β1. 15d-PGJ_2 inhibited the TGF-β1–induced MAPK activation. Dominant-negative PPAR-γ (ΔPPAR-γ) completely abrogated the inhibitory effect of pioglitazone and incompletely blocked its effect of 15d-PGJ_2 on TGF-β1–induced AP-1 reporter activity. ΔPPAR-γ overexpression did not affect the inhibitory effect of 15d-PGJ_2 on TGF-β1–induced MAPK activation. In conclusion, pioglitazone inhibits TGF-β1–induced fibronectin expression by inhibiting AP-1 activation dependent on PPAR-γ, while 15d-PGJ_2 acts through a dual mechanism independent of and dependent on PPAR-γ activation in mouse mesangial cells. *Diabetes* 53:200–208, 2004

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15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; ΔPPAR; dominant-negative peroxisome proliferator–activated receptor; AP-1, activator protein-1; dCTP, deoxycytidine triphosphate; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; EMSA, electrophoretic mobility shift analysis; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator–activated receptor; PPRE, PPAR-responsive element; SBE, Smad-binding element; TGF, transforming growth factor; TZD, thiazolidinedione.

Diabetic nephropathy is characterized by renal hypertrophy, glomerular and tubular basement thickening, and mesangial matrix expansion with extracellular matrix (ECM) protein accumulation (1). High glucose itself and various growth factors were shown to contribute to the accumulation of ECM proteins in the kidney during diabetes (2). Among them, transforming growth factor (TGF)-β has been proposed as a key cytokine that enhances ECM protein synthesis, leading to the development of diabetic nephropathy (2,3). Indeed, studies (4,5) using neutralizing anti–TGF-β antibodies have provided convincing evidence that inhibiting TGF-β activity could prevent and even reverse the pathological and functional abnormalities in the kidney of diabetic animals.

TGF-β exerts its multiple biologic actions by activating several intracellular signal transduction systems. The Smad family of proteins has been recently identified as a predominant signal transducer of TGF-β (6). Mitogen-activated protein kinases (MAPKs), including the extracellular signal–regulated kinases (ERKs), the c-jun NH_2-terminal kinases, and the p38MAPK (7), have also been proposed to participate in TGF-β–induced ECM protein synthesis in a number of different cell types, including mesangial cells (8–13).

Thiazolidinedione (TZD) compounds, such as rosiglitazone and pioglitazone (14), a new class of antidiabetic agents, act by increasing insulin sensitivity and are widely used for the treatment of type 2 diabetic subjects. The antidiabetic effect of TZDs was shown to be mediated through peroxisome proliferator–activated receptor (PPAR)-γ, a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (15). PPAR-γ forms a heterodimer with retinoid X receptor, and the complex binds to a PPAR-γ–responsive element (PPRE) in the promoter of target genes in response to a variety of endogenous and exogenous ligands (16). PPAR-γ is also activated by a natural ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (17,18). PPAR-γ is expressed at high levels in adipose tissue (17), colon and activated macrophages, and at lower levels in other tissues (14,19–21). Although the antidiabetic effect of TZDs is well recognized, we previously reported (22) a novel action of these agents in that TZDs prevented not only glomerular dysfunction such as hyperfiltration and albuminuria, but

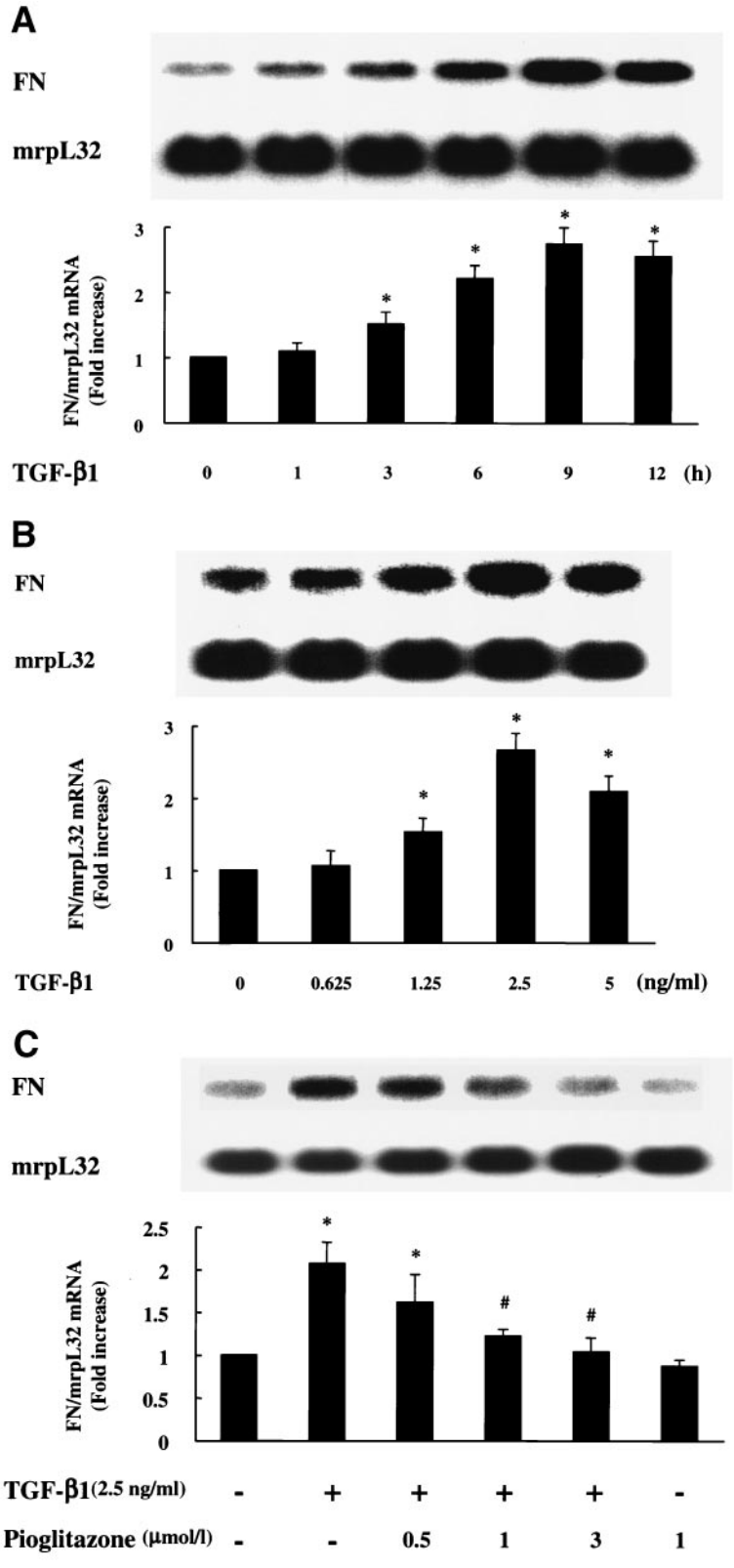

FIG. 1. Effect of pioglitazone on TGF-β–induced fibronectin (FN) mRNA expression in mouse mesangial cells. *A*: Cells were treated with TGF-β1 (2.5 ng/ml) for the indicated periods. Total RNA (20 μg) was subjected to Northern blot analysis and hybridization with [α-^{32}P]dCTP-labeled fibronectin cDNA. The membrane was rehybridized with mitochondrial ribosomal protein L32 (mrpL32) cDNA. *B*: Cells were incubated with TGF-β1 at the indicated concentrations for 9 h, and total RNA was analyzed by Northern blot analysis. *C*: Cells were preincubated with pioglitazone at the indicated doses for 30 min, followed by stimulation with TGF-β1 for 9 h. Quantitative results of three to four independent experiments are shown. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. TGF-β1.

also overexpression of TGF-β and ECM proteins in streptozotocin-induced diabetic rats, a model of type 1 diabetes, without affecting blood glucose levels. Furthermore, we provided evidence that TZDs have an antifibrotic action in diabetes and block high glucose-induced renal dysfunction by possibly inhibiting diacylglycerol-protein kinase C activation. However, the molecular mechanisms of the interaction between TZDs and TGF-β have not yet been elucidated.

Thus, the aim of this study is to investigate in mesangial cells the effect of PPAR-γ agonists, pioglitazone and 15d-PGJ_2, on TGF-β1–induced fibronectin expression, a major component of ECM proteins accumulated in the kidney of diabetic nephropathy. We also evaluated the effect of these

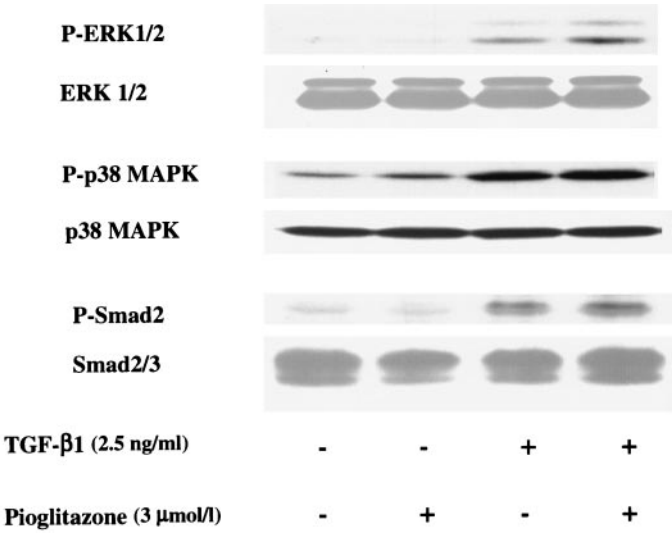

FIG. 2. Effect of pioglitazone on phosphorylation of MAPKs and phosphorylation of Smad2 induced by TGF-β1. Cells were treated in the presence or absence of 3 μmol/l pioglitazone for 30 min, followed by stimulation with TGF-β1 (2.5 ng/ml) for 1 h. Cell lysates were electrophoresed on 12% SDS-PAGE. The activities of ERK, p38 MAPK, and Smad2 were determined by immunoblot analysis with anti-phospho-ERK, anti-phospho-p38, and anti-phospho-Smad2 antibodies, respectively. After stripping, the membranes were reprobed with anti-ERK, anti-p38MAPK, and anti-Smad2/3 antibodies, respectively. All results are a representative one of three independent experiments.

agents on TGF-β1 signaling, such as Smad and MAPK pathway, to elucidate the underlying mechanism of the antifibrotic effect of PPAR-γ agonists.

RESEARCH DESIGN AND METHODS

Human recombinant TGF-β1 was obtained from R&D Systems (Minneapolis, MN). The 15d-PGJ_2 was purchased from Calbiochem (La Jolla, CA). Pioglitazone was kindly provided by Takeda Chemical (Osaka, Japan). Anti-phospho-p42/44 ERK and anti-phospho-p38 MAPK were bought from New England Biolabs (Beverly, MA), and anti-phospho-Smad2 was obtained from Upstate Biotechnology (Lake Placid, NY). The 3×AP-1 (activator protein-1) luciferase reporter construct was purchased from Stratagene (La Jolla, CA). The 4×SBE (Smad-binding element) luciferase reporter plasmid was kindly provided by Dr. C.H. Heldin (Uppsala, Sweden). The consensus oligonucleotide of AP-1 was purchased from Promega (Madison, WI). Anti-ERK, p38MAPK, and anti-Smad2/3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The [α-^{32}P] deoxycytidine triphosphate (dCTP) and [γ-^{32}P] ATP were purchased from New England Nuclear (Boston, MA). The AOx-TK-luciferase reporter construct containing three PPAR-responsive elements (PPREs) and PPAR-γ expression plasmid were generously provided by Dr. C.K. Glass (University of California) (23). The dominant-negative PPAR (ΔPPAR)-γ construct was provided by Dr. K. Kishida (Osaka University, Japan) (24).

Cell culture

Mycoplasma-free. SV40 MES 13 (murine mesangial) cells were purchased from the American Type Culture Collection (Rockville, MD). These cells, derived from glomerular explants of SV40 transgenic mice, exhibit both biochemical and morphologic features of normal mesangial cells in culture (25). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Primary mouse mesangial cells were obtained by culturing glomeruli isolated from kidneys of male BALB/C mice (15–20 g) as previously described (26). Cells were cultured in DMEM with 20% FBS. For experiments, subconfluent cells were starved for 24 h by incubation with DMEM containing 0.4% BSA. Quiescent cells were incubated with TGF-β1 in an experimental medium (DMEM with 0.4% BSA and 20 mmol/l HEPES, pH 7.4) for the indicated time intervals at 37°C. For the experiments with pioglitazone or 15d-PGJ_2, cells were incubated with the indicated concentrations of pioglitazone or 15d-PGJ_2 for 30 min at 37°C before exposure to TGF-β1. After the incubation, cells were harvested for the determination described below.

RNA isolation and Northern blot analysis. Mouse mesangial cells were maintained as described above. After 24 h of incubation in 0.4% BSA DMEM, cells were treated with TGF-β1 in the presence or absence of either pioglitazone or 15d-PGJ_2. Total RNA was isolated from cells with Trizol Reagent (Gibco, Grand Island, NY) following the manufacturer's protocol. Northern blot analysis was performed as previously described (26). Briefly, 20-μg aliquots of RNA were electrophoresed on 1% formaldehyde-agarose gels and transferred onto nylon membranes (Nytran, Schleider & Schuell, Dassel, Germany). After fixation by ultraviolet cross-linking, the membranes were baked for 2 h at 80°C incubation. Then, the membranes were hybridized with [α-^{32}P]dCTP-labeled rat fibronectin cDNA, which was labeled by the Bca BEST labeling kit (Takara, Otsu, Japan), and subjected to autoradiography. The membranes were reprobed with mitochondrial ribosomal protein L32 cDNA as an internal standard (27). Quantification of mRNA levels was performed using NIH Image.

Transient transfections and transcriptional reporter assays. Mouse mesangial cells were grown to 70–80% confluence in DMEM supplemented with 10% FBS on 6-well plates. Transient transfections with the indicated luciferase reporter construct were performed with the LipofectAMINE regent (Invitrogen, Carlsbad, CA). The total amount of transfected DNA was kept constant by using a corresponding empty-vector mock DNA. After 3 h of transfection, cells were recovered with complete medium for 24 h, then were maintained in FBS-free DMEM containing 0.4% BSA for 24 h, followed by stimulation with TGF-β for 24 h with or without pretreatment with pioglitazone or 15d-PGJ_2 for 30 min. After washing twice with cold PBS, cells were harvested in 200 μl of reporter lysis buffer (Promega). Aliquots (20 μl) of extracts were used to measure luciferase activity by the Luciferase Assay System (Promega) and a luminometer (Auto LUMIcounter Nu1422ES; Nition, Tokyo, Japan). Luciferase activities were normalized to the β-galactosidase activity (Promega). Results were expressed as ratio (fold-induction) of normalized luciferase activities.

Western blot analysis. Mouse mesangial cells were grown to subconfluence in DMEM containing 10% FBS. The cells were cultured for 24 h in DMEM containing 0.4% BSA, followed by treatment with TGF-β1 in the presence or absence of pioglitazone or 15d-PGJ_2. After washing twice with cold PBS, cells were lysed in an ice-cold lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, U.K.). Cell lysates were loaded on 12% SDS-polyacrylamide gels. After electrotransferring to polyvinylidine fluoride membranes (Immobilon, Bedford, MA), membranes were probed with specific antibodies against phospho-MAPKs, MAPKs, phospho-Smad2, or Smad2/3. Bound primary antibodies were detected with horseradish peroxidase–labeled anti-mouse, anti-rabbit, or anti-goat secondary antibodies, respectively, and developed with an enhanced chemiluminescence detection system (New England Biolab).

Nuclear extraction and electrophoretic mobility shift analysis. Nuclear extracts were prepared as previously described (26). In brief, cells were lysed with the addition of a hypotonic buffer (10 mmol/l HEPES, pH 7.8, 0.1 mmol/l EDTA, 15 mmol/l KCl, 2 mmol/l MgCl2, 1 mmol/l dithiothreitol, and protease inhibitor cocktail) with 0.8% Nonidet P-40 and microcentrifuged at 6,000*g* for 10 min. Pellets were resuspended with a high-salt buffer (20 mmol/l HEPES, pH 7.8, 420 mmol/l NaCl, 1.5 mmol/l MgCl2, 0.5 mmol/l dithiothreitol, 0.2 mmol/l EDTA, and 25% glycerol), rotated for 30 min at 4°C, and centrifuged at 13,000*g* for 30 min. Supernatants were used as nuclear proteins for electrophoretic mobility shift analysis (EMSA). The nuclear proteins (4 μg) were incubated with 1 μg of poly(dI-dC) in a binding buffer (10 mmol/l Tris, pH 7.5, 50 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, and 5% glycerol) for 30 min and then reacted at room temperature for 20 min with an AP-1 consensus oligonucleotide (Promega), which was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolab). The reaction mixtures were electrophoresed through 4% polyacrylamide gels and were subjected to autoradiography. For supershift analysis, the indicated antibodies were preincubated at 37°C for 30 min before the reactions with the radiolabeled probes. Competition experiments were performed by the addition of an excess amount of the indicated unlabeled double-stranded oligonucleotide to the reaction mixtures. The consensus AP-1 sequences in EMSA are described below. AP-1 consensus: 5′-CGCTTGA**TGAGTCA**GCCGGAA-3′.

RT-PCR. For RT-PCR analysis, total RNA (1 μg) was reverse transcribed with random hexamers using the Gene-Amp RNA-PCR kit (Perkin-Elmer, Branchburg, NJ), following the manufacturer's directions. The cycling conditions were 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. The primers for mouse PPAR-γ are described as follows: forward, 5′-AGTCCTTCCCGCTGACCAAA-3′; reverse 1, 5′-GCTCATGTCTGTCTCTGT-3′ for ΔPPAR-γ; reverse 2, 5′-TCCTTGTAGATCTCCTGG-3′ for PPAR-γ. The primers for ΔPPAR-γ and PPAR-γ amplified a 721-bp fragment and a 753-bp fragment, respectively. The primers for β-actin, used as control, were 5′-TATGCTCTCCCTCACGCCAT-3′

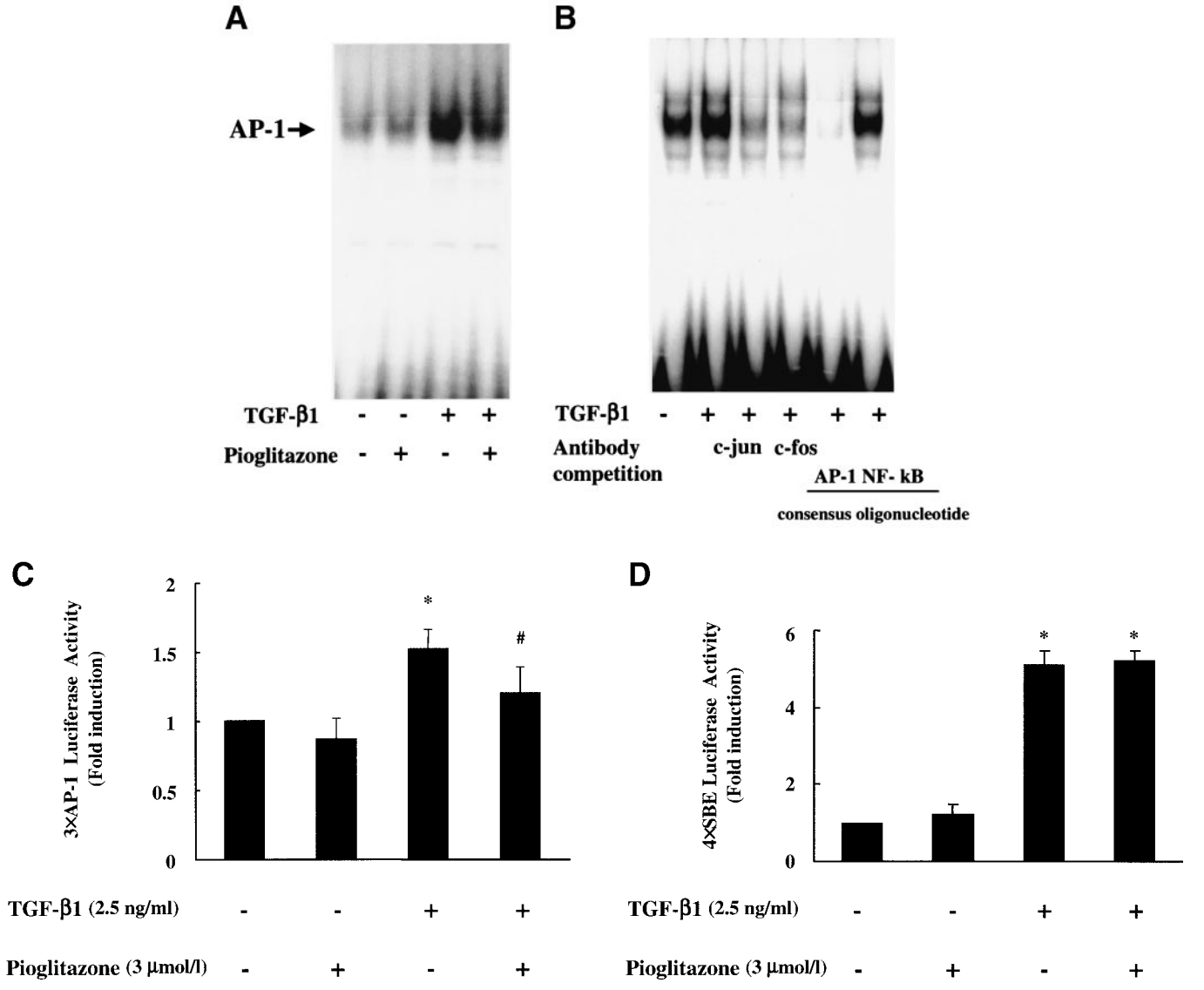

FIG. 3. Effect of pioglitazone on AP-1 activity. *A*: Cells were starved for 24 h, followed by treatment with or without 3 μmol/l pioglitazone for 30 min, and stimulated with 2.5 ng/ml TGF-β1 for 45 min. Nuclear extracts were incubated with [γ-^{32}P]ATP-labeled AP-1 consensus oligonucleotide probe and were subjected to electrophoresis on 4% polyacrylamide gel. The arrow shows the specific binding of AP-1. A representative of one of three independent experiments is shown. *B*: Supershift assays identified the subunit components for AP-1 dimers. Competition experiments demonstrated that the arrow shown is the specific binding of consensus AP-1. *C* and *D*: Cells were transfected with 1 μg 3×AP-1 (*C*) or 4×SBE (*D*) reporter construct plasmid along with 0.2 μg cytomegalovirus-β-galactosidase–containing plasmid as a control of transfection efficiency. After starvation for 24 h, cells were preincubated in the presence or absence of 3 μmol/l pioglitazone for 30 min and stimulated with 2.5 ng/ml TGF-β1 for 24 h. Luciferase activities were measured as described in RESEARCH DESIGN AND METHODS. Luciferase activity was normalized against β-galactosidase activity and was expressed as relative light units. Data are means ± SE of five independent experiments. **P* < 0.05 vs. control; #*P* < 0.05 vs. TGF-β1.

and 5′-CTCAGGAGGAGCAATGATCT-3′, and then amplified a 506-bp fragment. The resulting products were separated on 2% agarose gel and stained with ethidium bromide.

Statistical analysis. Results were expressed as the means ± SE. ANOVA with subsequent Scheffe's test was used to determine significant differences in multiple comparisons. A $P < 0.05$ was considered statistically significant.

RESULTS

Effect of pioglitazone on TGF-β1–induced fibronectin mRNA expression in mouse mesangial cells. TGF-β1 induced fibronectin mRNA expression in a time-dependent (9 h maximum) and dose-dependent manner, with a maximal stimulation at 2.5 ng/ml (Fig. 1*A* and *B*). In subsequent experiments, cells were treated with 2.5 ng/ml TGF-β1 for 9 h. When cells were incubated with indicated concentrations of pioglitazone for 30 min before stimulation with TGF-β1, TGF-β1–induced fibronectin mRNA expression was significantly suppressed by pioglitazone in a dose-dependent manner (Fig. 1*C*). Similar results were obtained in primary mouse mesangial cells (data not shown).

Effect of pioglitazone on TGF-β signaling. Consistent with previous reports (8,28,29), MAPKs and Smad2 were rapidly activated by TGF-β, as early as 15 min, with the peak stimulation at 1 h (data not shown). However, the treatment with pioglitazone failed to affect TGF-β1–induced phosphorylation of ERK1/2, p38 MAPK, and Smad2 (Fig. 2).

Because a previous report (23) suggested that PPAR-γ might exert its effects by interacting with other transcription factors such as AP-1, we examined the AP-1 DNA-

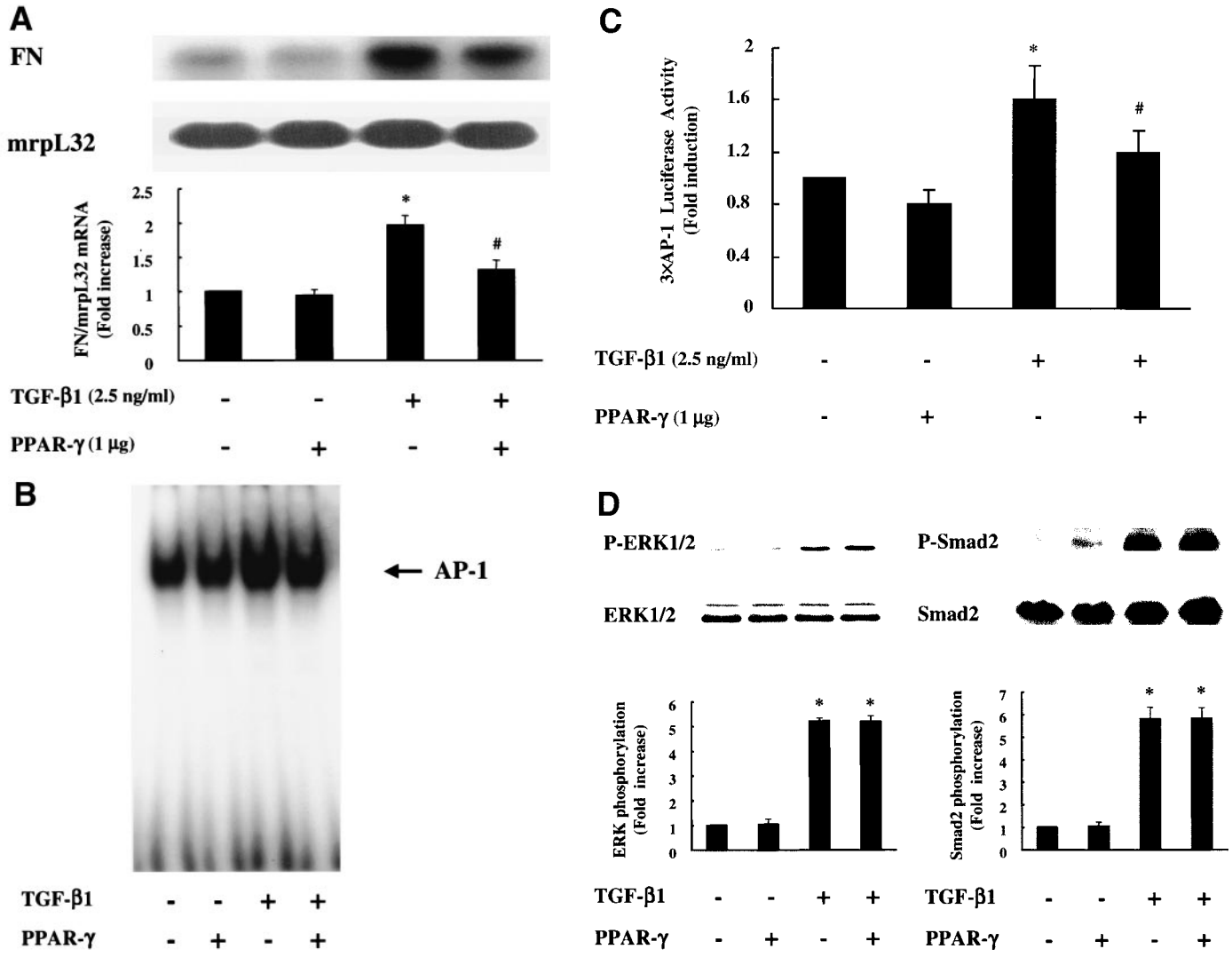

FIG. 4. Effect of PPAR-γ overexpression on TGF-β signal. *A*: Cells were transfected with 1 μg PPAR-γ expression-construct plasmid or pcDNA3 empty-vector plasmid as control. After starvation, cells were incubated with or without TGF-β1 for 9 h. Quantitative results of four independent experiments are shown. Data are means ± SE. **P* < 0.05 vs. control; #*P* < 0.05 vs. TGF-β1. FN, fibronectin; mrpL32, mitochondrial ribosomal protein L32. *B*: Cells were transfected with PPAR-γ expression-construct plasmid or pcDNA empty-vector plasmid, followed by incubation with TGF-β for 45 min. The arrow shows the specific binding of AP-1. A representative one of four independent experiments is shown. *C*: Cells were transfected with 1 μg AP-1 reporter construct plasmid and 1 μg PPAR-γ expression-construct plasmid or pcDNA3 empty-vector plasmid, along with 0.2 μg cytomegalovirus-β-galactosidase–containing plasmid. After recovery and starvation, the cells were incubated with or without TGF-β1 for 24 h. Luciferase activities were measured as described above. Data are means ± SE of three independent experiments. **P* < 0.05 vs. control; #*P* < 0.05 vs. TGF-β1. *D*: Cells were transfected with PPAR-γ expression-construct plasmid or pcDNA empty-vector plasmid. After starvation, cells were incubated with TGF-β1 for 1 h. The activities of ERK and Smad2 were evaluated by immunoblot analysis. Quantitative results of three experiments are shown. **P* < 0.05 vs. control.

binding activity by EMSA. As shown in Fig. 3*A*, the DNA binding activity of AP-1 was obviously increased at 45 min after TGF-β1 stimulation, and this activation was significantly inhibited by pretreating cells with pioglitazone. The specificity of AP-1 DNA binding complex was confirmed by supershift assays (Fig. 3*B*). To further evaluate the effect of pioglitazone on the TGF-β signaling pathway, we examined the effect of pioglitazone on TGF-β–responsive reporters, including 3×AP-1 and 4×SBE luciferase reporters. TGF-β1 significantly stimulated AP-1 and SBE activities, whereas pioglitazone alone did not have any effect. When cells were treated with pioglitazone before stimulation with TGF-β1, AP-1 luciferase reporter activity was significantly inhibited (Fig. 3*C*), while SBE luciferase level was not affected (Fig. 3*D*).

Effect of overexpression of PPAR-γ on TGF-β signaling. To further elucidate the molecular mechanism of PPAR-γ in modulating the TGF-β signaling pathway by pioglitazone, we investigated the effect of transient PPAR-γ overexpression on TGF-β1–induced fibronectin expression. The overexpression of PPAR-γ inhibited fibronectin mRNA expression (Fig. 4*A*), AP-1 DNA-binding activity (Fig. 4*B*), and AP-1 luciferase activity (Fig. 4*C*) induced by TGF-β1. However, similar to pioglitazone, PPAR-γ overexpression failed to inhibit TGF-β1–induced phosphorlyations of MAPKs and Smad2 (Fig. 4*D*).

Effect of 15d-PGJ$_2$ on TGF-β1–induced fibronectin mRNA expression. We then examined the effect of 15d-PGJ$_2$, a natural PPAR-γ ligand, on TGF-β1–induced fibronectin expression in mouse mesangial cells. As shown

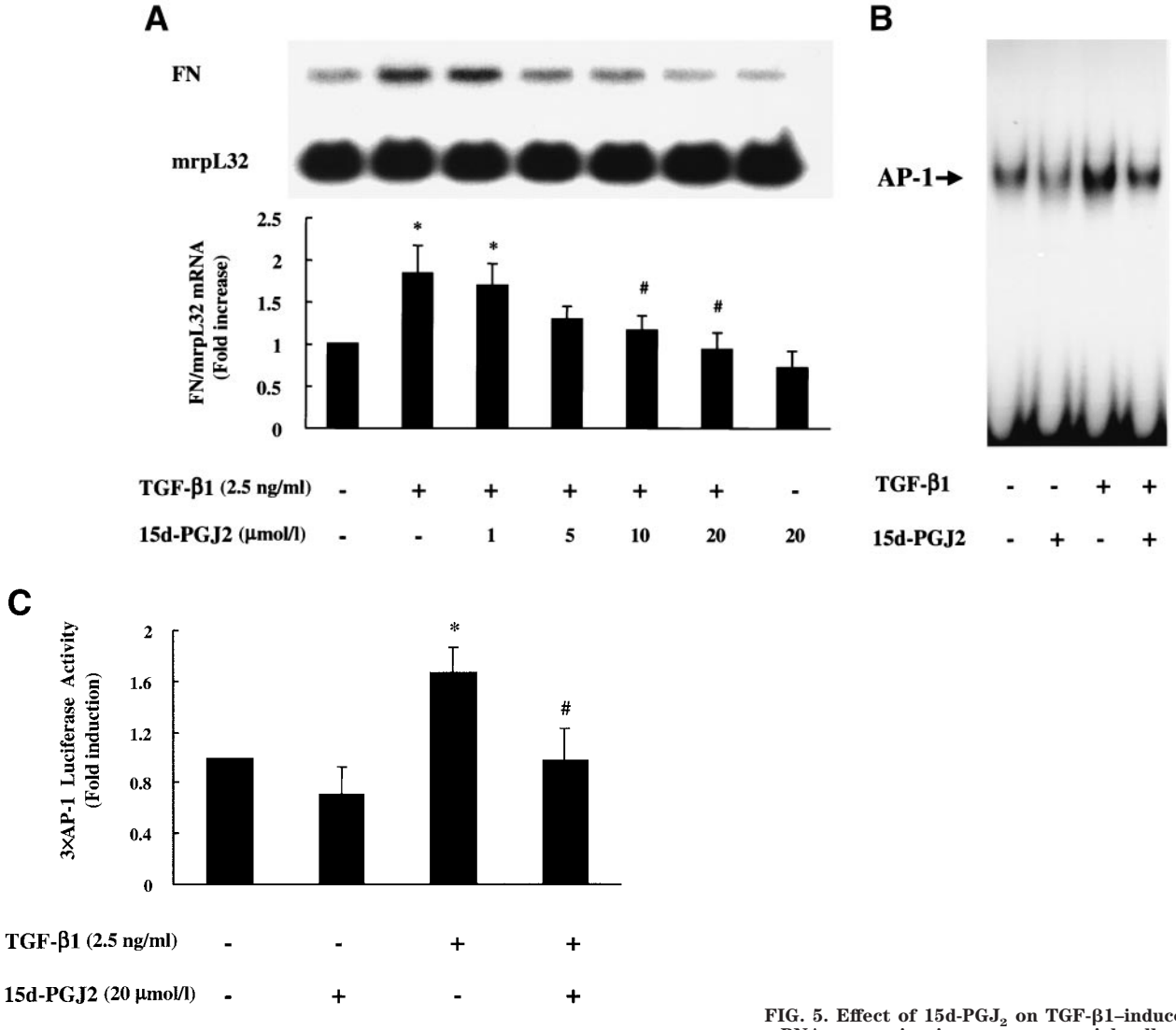

FIG. 5. Effect of 15d-PGJ$_2$ on TGF-β1–induced fibronectin mRNA expression in mouse mesangial cells. *A*: Cells were treated with 15d-PGJ$_2$ at the indicated concentrations for 30 min, followed by stimulation with TGF-β1 for 9 h. Total RNA was subjected to Northern blot analysis. Densitometric analysis of four independent experiments is shown. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. TGF-β1. FN, fibronectin; mrpL32, mitochondrial ribosomal protein L32. *B*: Cells were incubated with or without 15d-PGJ$_2$ for 30 min, followed by stimulation with TGF-β1 for 45 min. Nuclear proteins were subjected to EMSA. The arrow shows the specific binding of AP-1. A representative one of three independent experiments is shown. *C*: Cells were transfected with 1 μg 3×AP-1 reporter construct and 0.2 μg cytomegalovirus-β-galactosidase–containing plasmid. After starvation, cells were preincubated in the presence or absence of 20 μmol/l 15d-PGJ$_2$ for 30 min and stimulated with TGF-β1 for 24 h. Luciferase activities were measured as described above. Data are means ± SE of at least five independent experiments. *P < 0.05 vs. control; #P < 0.05 vs. TGF-β1. *D*: Cells were treated with or without 15d-PGJ$_2$ for 30 min and stimulated with TGF-β1 for 1 h. The activities of ERK and p38MAPK were evaluated by immunoblot analysis. Data are means ± SE of four to five independent experiments. *P < 0.05 vs. control; #P < 0.05 vs. TGF-β1.

in Fig. 5*A*, TGF-β–induced fibronectin mRNA expression was significantly suppressed by 15d-PGJ$_2$ in a dose-dependent manner. The 15d-PGJ$_2$ was also able to inhibit AP-1 DNA-binding activity (Fig. 5*B*) and AP-1 reporter activity (Fig. 5*C*) induced by TGF-β1. Furthermore, immunoblot analysis revealed that the pretreatment with 15d-PGJ$_2$ significantly inhibited TGF-β1–induced phosphorylation of ERK1/2 and p38MAPK (Fig. 5*D*) without affecting the phosphorylation of Smad2 (data not shown).

Effect of ΔPPAR-γ construct on TGF-β1–induced AP-1 reporter activity. We finally examined the effect of the ΔPPAR-γ construct on TGF-β–induced AP-1 reporter

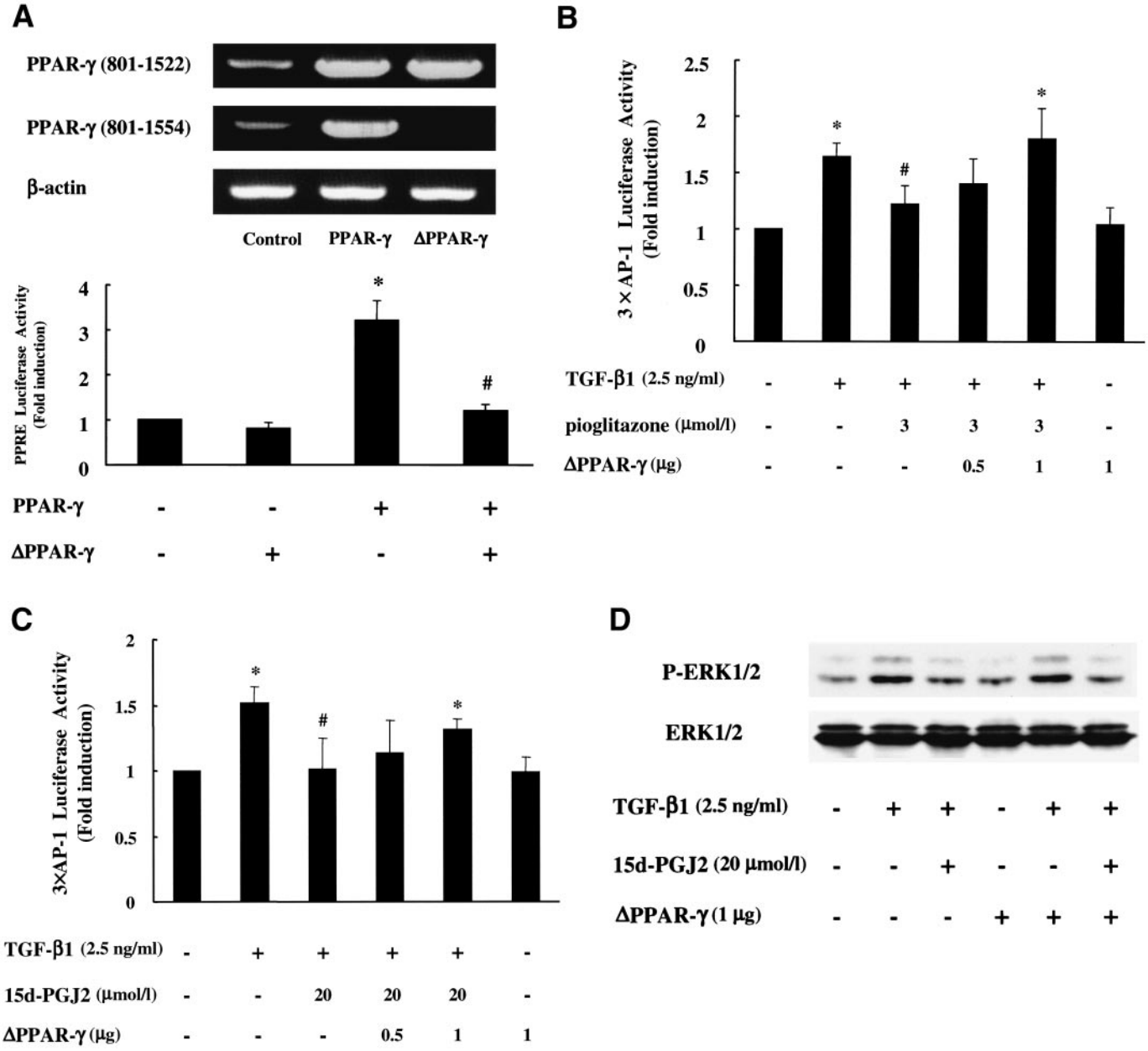

FIG. 6. Effect of ΔPPAR-γ overexpression on TGF-β–induced AP-1 reporter activity. *A*: Cells were transfected with constructs expressing with PPAR-γ, ΔPPAR-γ, or empty-vector plasmid as control. After 24 h, mRNA was extracted, and PPAR-γ and ΔPPAR-γ mRNA expressions were assessed by RT-PCR. A representative one of three similar results is shown (*upper panel*). Cells were cotransfected with PPRE-luc plasmid and either PPAR-γ or/and ΔPPAR-γ expression vector and pcDNA3 empty-vector plasmid along with cytomegalovirus-β-galactosidase plasmid as a control for transfection efficiency. After 24 h, luciferase activity was measured and normalized to the β-galactosidase activity. Results are expressed as relative light units. Data are means ± SE of three independent experiments. *$P < 0.05$ vs. control; #$P < 0.05$ vs. PPAR-γ. *B* and *C*: Cells were transfected with ΔPPAR-γ expression construct and AP-1 reporter construct along with cytomegalovirus-β-galactosidase containing plasmid. After recovery and starvation, cells were incubated with pioglitazone (*B*) or 15d-PGJ_2 (*C*) for 30 min, followed by stimulation with TGF-β1 for 24 h. Luciferase activity was measured and normalized to the β-galactosidase activity. Results are expressed as relative light units. Data are means ± SE of three to six independent experiments. *$P < 0.05$ vs. control; #$P < 0.05$ vs. TGF-β1. *D*: Cells were transfected with ΔPPAR-γ expression construct. After recovery and starvation, cells were incubated with or without 15d-PGJ_2 for 30 min, followed by stimulation with TGF-β1 for 1 h. The activity of ERK was evaluated by immunoblot analysis. Three identical experiments independently gave similar results.

activity. The ΔPPAR-γ construct lacks 11 amino acids (PLLQEIYKDLY) in the AF-2 domain at its carboxyl terminus (24). RT-PCR revealed a small amount of PPAR-γ expression in mesangial cells (Fig. 6*A*, *upper panel*). PPAR-γ and ΔPPAR-γ expressions were strongly induced by transfection with PPAR-γ and ΔPPAR-γ expression plasmids, respectively. ΔPPAR-γ overexpression inhibited endogenous PPAR-γ expression (Fig. 6*A*, *upper panel*). In addition, PPAR-γ overexpression significantly enhanced PPRE luciferase reporter activities, and this enhancement was significantly inhibited by cotransfection with 1 μg ΔPPAR-γ (Fig. 6*A*, *lower panel*). We found that 15d-PGJ_2 and pioglitazone can significantly enhance PPRE luciferase reporter activity, and this enhancement was significantly inhibited with cotransfection of increasing amounts of the ΔPPAR-γ expression construct with a maximum effect at 1 μg ΔPPAR-γ (data not shown). ΔPPAR-γ overexpression completely prevented the inhibitory effect

of pioglitazone (Fig. 6*B*) and incompletely blocked the inhibitory effect of 15d-PGJ_2 on TGF-β1–induced AP-1 luciferase reporter activity (Fig. 6*C*). However, ΔPPAR-γ overexpression did not affect the inhibitory effect of 15d-PGJ_2 on TGF-β1–induced MAPK activation (Fig. 6*D*).

DISCUSSION

In the present study, we demonstrated that PPAR-γ ligands, pioglitazone, and 15d-PGJ_2 can inhibit TGF-β–induced fibronectin expression. The effect of pioglitazone was mediated by inhibiting PPAR-γ–dependent AP-1 activation, while 15d-PGJ_2 exerted its effect through PPAR-γ–independent and –dependent actions in mouse mesangial cells. These observations are consistent with our previous report (22) demonstrating that TZDs not only ameliorated albuminuria in diabetic rats, but also inhibited the overexpression of ECM proteins. Our current study shows a novel anti–TGF-β action of PPAR-γ ligands in addition to our previous reports (22) showing that TZDs exerted an antifibrotic effect through decreasing the diacylglycerol-protein kinase C pathway in diabetic glomeruli or mesangial cells exposed to high glucose.

There is increasing evidence that TGF-β–induced MAPKs activation plays an important role in regulating ECM protein accumulation and degradation in renal mesangial cells, although TGF-β exerts its physiological functions mainly through the Smad family of proteins (9,13). Indeed, we have previously reported (8) that TGF-β–induced ERK activation is required for the induction of fibronectin in mesangial cells by using a specific MAPK/ERK kinase inhibitor, PD98059. In the present study, we have further identified that AP-1 activity is enhanced by TGF-β1 using a consensus AP-1 oligonucleotide as well as an AP-1 luciferase reporter in mesangial cells. AP-1, which is a menagerie of dimeric basic resion-leucine zipper proteins, is enhanced through MAPKs and eventually modulates a wide range of cellular responses, including cell proliferation and differentiation and ECM protein expression (28–30). In the present study, we have extended our previous findings in characterizing the ability of PPAR-γ ligands to suppress TGF-β1–induced fibronectin expression through inhibiting AP-1 activity but not SBE activity. We have also demonstrated that the overexpression of PPAR-γ, similar to pioglitazone, inhibits TGF-β–induced fibronectin expression by suppressing AP-1 reporter activity and AP-1 DNA-binding activity. Others have also reported that TGF-β–induced fibronectin expression occurs in a Smad-independent manner (31,32). Using a loss of function approach, Piek et al. (33) clearly characterized the role of MAPKs on fibronectin expression by finding that TGF-β–induced fibronectin expression in wild-type fibroblasts was similar to that in Smad2 knockout or Smad3 knockout fibroblasts. Therefore, our results suggest that the MAPKs–AP-1 cascade is at least in part responsible for TGF-β–induced fibronectin expression and that PPAR-γ agonists can act as antifibrotic agents in mesangial cells.

The high-affinity ligand for PPAR-γ, 15d-PGJ_2, also inhibits the induction of inflammatory response genes, including nitric oxide synthase and tumor necrosis factor-α in a PPAR-γ–dependent manner (23,34). However, others have shown that 15d-PGJ_2 can regulate the transcription of some target genes in a PPAR-γ–independent manner (26,35). Indeed, we demonstrate that 15d-PGJ_2 is able to abrogate the TGF-β1–induced fibronectin expression by suppressing the activation of AP-1 via the inhibition of ERK and p38 MAPK activation. These results suggest that 15d-PGJ_2 may have other actions in inhibiting TGF-β1–induced AP-1 activity through a PPAR-γ–independent manner, which is in contrast to pioglitazone. This phenomenon was further strengthened by the findings that the overexpression of ΔPPAR-γ completely prevented the inhibitory effect of pioglitazone, incompletely blocked the inhibitory effect of 15d-PGJ_2 on TGF-β1–induced AP-1 reporter activity, and did not affect the inhibitory effect of 15d-PGJ_2 on TGF-β1–induced MAPK activation. Taken together, it seems likely that pioglitazone downregulates TGF-β1–induced fibronectin expression via suppressed AP-1 activity that is possibly mediated by PPAR-γ activation, whereas the inhibitory effects of 15d-PGJ_2 are mediated by both PPAR-γ–dependent and –independent mechanisms. Similarly, Straus et al. (35) found that 15d-PGJ_2 was able to inhibit nuclear factor-κB–dependent gene expression through suppression of the inhibitor of κB kinase in a PPAR-γ–independent manner in addition to PPAR-γ–dependent inhibition of nuclear factor κB. Thus, it is possible that 15d-PGJ_2 inhibits the MAPKs–AP-1 signaling pathway by PPAR-γ–dependent and –independent manners, although the precise mechanism of how 15d-PGJ_2 inhibits MAPKs remains to be clarified.

In summary, our findings that TZDs could inhibit TGF-β signaling cascades via the interaction of the AP-1 complex and PPAR-γ strengthen the beneficial role of TZDs in treating fibrotic kidney disease, including diabetic nephropathy. Moreover, the results showed that 15d-PGJ_2 could inhibit cascades through PPAR-γ–dependent and –independent actions. These findings may provide a new action of PPAR-γ agonists on fibrotic response and a therapeutic implication of PPAR-γ agonists in the prevention of and intervention in diabetic nephropathy.

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