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

Baoliang Guo, Daisuke Koya, Motohide Isono, Toshiro Sugimoto, Atsunori Kashiwagi, and Masakazu Haneda

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-Δ12,14-prostaglandin J2 (15d-PGJ2), a natural PPAR-γ ligand, also inhibited TGF-β1–induced fibronectin expression by suppressing AP-1 activation by TGF-β1. 15d-PGJ2 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-PGJ2 on TGF-β1–induced AP-1 reporter activity. ΔPPAR-γ overexpression did not affect the inhibitory effect of 15d-PGJ2 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-PGJ2 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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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 NH2-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-Δ12,14-prostaglandin J2 (15d-PGJ2) (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...
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-PGJ2, 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
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-PGJ2 was purchased from Calbiochem (La Jolla, CA). Pioglitazone was kindly provided by Takeda Chemical (Osaka, Japan). Anti-phospho-ERK, p38 MAPK, and Smad2 were determined by immunoblot analysis with anti-phospho-ERK, anti-phospho-p38, and anti-phospho-Smad2 antibodies, respectively. All results are a representative one of three independent experiments.

**RNA isolation and Northern blot analysis.** 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 reagent (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-β1 for 24 h with or without pretreatment with pioglitazone or 15d-PGJ2 for 30 min. Aliquots of total RNA (1 μg) were reverse transcribed with random hexamers using the Gene-Amp RNA-PCR kit (Perkin-Elmer, Branchburg, NJ), following the manufacturer’s directions. The cycling conditions below. AP-1 consensus: 5'-TATGCTCTCCCTCACGCCAT-3'.

**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 growth factor independence and transformation, and are Mycoplasma-free.

**Cell culture**

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**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. 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-p38 MAPK, and anti-Smad2/3 antibodies, respectively. All results are a representative one of three independent experiments.
and 5'-CTCAGGAGGACAATGCT-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. 1A 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. 1C).

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. 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-32P]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-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.
binding activity by EMSA. As shown in Fig. 3A, 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. 3B). To further evaluate the effect of pioglitazone on the TGF-β1 signaling pathway, we examined the effect of pioglitazone on TGF-β1-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. 3C), while SBE luciferase level was not affected (Fig. 3D).

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. 4A), AP-1 DNA binding activity (Fig. 4B), and AP-1 luciferase activity (Fig. 4C) induced by TGF-β1. However, similar to pioglitazone, PPAR-γ overexpression failed to inhibit TGF-β1-induced phosphorylations of MAPKs and Smad2 (Fig. 4D).

Effect of 15d-PGJ2 on TGF-β1-induced fibronectin mRNA expression. We then examined the effect of 15d-PGJ2, a natural PPAR-γ ligand, on TGF-β1-induced fibronectin expression in mouse mesangial cells. As shown
in Fig. 5A, TGF-β-induced fibronectin mRNA expression was significantly suppressed by 15d-PGJ2 in a dose-dependent manner. The 15d-PGJ2 was also able to inhibit AP-1 DNA-binding activity (Fig. 5B) and AP-1 reporter activity (Fig. 5C) induced by TGF-β1. Furthermore, immunoblot analysis revealed that the pretreatment with 15d-PGJ2 significantly inhibited TGF-β1–induced phosphorylation of ERK1/2 and p38MAPK (Fig. 5D) 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
activity. The ΔPPAR-γ construct lacks 11 amino acids (PLLQEYKDL) in the AF-2 domain at its carboxyl terminus (24). RT-PCR revealed a small amount of PPAR-γ expression in mesangial cells (Fig. 6A, 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. 6A, 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. 6A, lower panel). We found that 15d-PGJ2 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

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-PGJ2 (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-PGJ2 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.
of pioglitazone (Fig. 6B) and incompletely blocked the inhibitory effect of 15d-PGJ_2 on TGF-β1-induced AP-1 luciferase reporter activity (Fig. 6C). However, APPAR-γ overexpression did not affect the inhibitory effect of 15d-PGJ_2 on TGF-β1-induced MAPK activation (Fig. 6D).

**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 region-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 antiinfectious 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–dependent 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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