Expression and Function of Peroxisome Proliferator–Activated Receptor-γ in Mesangial Cells

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Abstract— Peroxisome proliferator–activated receptor-γ (PPARγ) is a novel nuclear receptor, which enhances insulin-mediated glucose uptake. Ligands to PPARγ are currently used as therapy for type II diabetes. Using Western blot analysis, RNase protection assay, and immunostaining, we identified the presence of PPARγ message and protein in cultured primary rat mesangial cells. Electrophoretic mobility of a labeled PPARγ response element (PPRE) was retarded in the presence of mesangial cell nuclear extract, suggesting that PPARγ is functional in these cells. The addition of unlabeled PPRE efficiently competed away the PPARγ-PPRE protein complex, confirming specificity of binding of the PPARγ to the PPRE. PPARγ ligands rosiglitazone (1 to 10 μmol/L) and troglitazone (1 to 10 μmol/L) inhibited platelet-derived growth factor–induced DNA synthesis, measured as bromodeoxyuridine incorporation (P<0.01). This inhibition was dose dependent. When administered in antidiabetic doses to streptozotocin-induced diabetic rats, troglitazone substantially normalized albumin excretion at 3 months (from 687.1 to 137.6 mg urinary albumin/mg creatinine, P<0.05) but did not affect hyperglycemia or blood pressure in this model. This treatment also decreased glomerular plasminogen activator inhibitor-1 (PAI-1) expression. These data suggest that PPARγ activation may directly attenuate diabetic glomerular disease, possibly by inhibiting mesangial growth, which occurs early in the process of diabetic nephropathy, or by inhibiting PAI-1 expression. PAI-1 inhibits the activation of plasmin and matrix metalloproteinase, which degrade extracellular matrix in the glomerulus. Excess glomerular PAI-1 allows the accumulation of extracellular matrix, leading to glomerulosclerosis. These results have therapeutic implications for diabetic nephropathy as well as for proliferative mesangial diseases of the kidney. (Hypertension. 2001;37[part 2]:722-727.)

Key Words: diabetes mellitus • insulin • mesangium • kidney • albuminuria

The peroxisome proliferator–activated receptors (PPARS) are a subfamily of the nuclear receptor gene superfamily that includes thyroid, retinoic acid receptor, and vitamin D. These receptors are important in regulating a variety of genes involved in lipid metabolism, fatty acid transport, and cellular catabolism or storage. The PPARs consist of 3 isotypes, PPARα, PPARβ, and PPARγ. Through alternate promoter usage and splicing, the human PPARγ gene is organized into 3 isoforms, PPARγ1, PPARγ2, and PPARγ3, which are differentially expressed and regulated in various tissues. Thiazolidinediones (TZDs) are ligands for PPARγ. Rosiglitazone (RSG) and troglitazone (TRO) not only have demonstrated significant utility as insulin-sensitizing agents but also have demonstrated their effectiveness in decreasing albuminuria in patients with early diabetic nephropathy. This effect was not seen with other oral agents despite similar degrees of glucose control. TRO was also reported to decrease albumin excretion in the streptozotocin (STZ)-induced diabetic rat without lowering glucose, whereas RSG lowered albumin excretion in the obese Zucker diabetic rat.

The present investigation demonstrates that PPARγ1 message and protein are expressed in mesangial cells (MCs) and upregulated in the presence of glomerular injury. Ligands to PPARγ inhibit MC growth, and in the STZ rat, TRO not only decreased albumin excretion but also decreased plasminogen activator inhibitor-1 (PAI-1) in the absence of changes in blood pressure or glucose. Therefore, we hypothesize that PPARγ ligands directly affect MC function. Ultimately, PPARγ ligands may prove to be useful in the prevention or treatment of diabetic nephropathy.

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**Methods**

**MC Culture**

Rat glomeruli were isolated from normal adult (200- to 650-g) Sprague-Dawley rats (Charles River, Boston, Mass) by using the sieving technique. Glomerular cores were plated in DMEM containing 0.25% BSA, 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma Chemical Co), 2.5 µg/mL amphotericin B (GIBCO), and 1 µg/mL recombinant human insulin (Sigma). The glomerular cores attached after 10 days. After the fourth or fifth passage, RPMI media containing 20% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma), 2.5 µg/mL amphotericin B (GIBCO), and 1 µg/mL recombinant human insulin (Sigma) facilitated MC growth. MCs were characterized before use by phenotype and immunostaining. These cells have a stellate appearance and stain positively for fibronectin and α-smooth muscle actin (Sigma) and negatively for cytokeratin. The cells were used between passages 6 and 10. Nonconfluent cells were serum-starved (0.5% FBS) for 24 hours before use.

**Western Blot Analysis**

MCs were lysed in lysis buffer (20 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 1 µg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). Protein concentrations were determined by using the DC protein assay (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Life Sciences). The membrane was incubated in 25 mL of blocking buffer (TBS, containing 0.1% Tween 20 and 5% nonfat dry milk) overnight at 4°C, followed by anti-PPARγ antibody (Glaxo Wellcome Research and Development) and anti-RXRα (Santa Cruz Biotechnology) for 3 hours at room temperature, washed with TBS/Tween 20, and incubated with anti-rabbit IgG horseradish peroxidase–conjugated antibody (1:1000, Amersham Life Sciences) for 1 hour at room temperature. The membranes were then washed with TBS/Tween 20, and protein was detected by enhanced chemiluminescence method as per the manufacturer’s instructions (Amersham Pharmacia Biotechnology).

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated by using Trizol reagent (Life Technologies) as per the manufacturer’s instructions. Twenty to 30 µg of total RNA was electrophoresed in 1.2% agarose gels containing formaldehyde, transferred to nylon membranes (MCI), and cross-linked in a Stratalinker (Stratagene). The nylon membranes were hybridized and protein was detected by enhanced chemiluminescence method as per the manufacturer’s instructions.

**RNase Protection Assay**

Partial cDNA probes for PPARγ1, PPARγ2, and RXRα within the pGEM-3 vectors were provided by Dr. Jeffrey Flier (Harvard University, Cambridge, Mass), and cDNA for rat PAI-1 in the pGEM-T Easy vector was obtained from Promega Corp. RNase protection assay (RPA) vectors were linearized with HindIII or EcoRI and labeled by using the RiboQuant in vitro transcription kit (Pharmingen). The RiboQuant RNase protection kit (Pharmingen) was subsequently used. Briefly, [32P]UTP-labeled RNA probes were incubated with 5 to 10 µg of MC total RNA in hybridization solution for 12 to 16 hours at 56°C followed by 45 minutes at 30°C. After ethanol precipitation, samples were resuspended in 7 µL of gel-loading buffer (80% formamide, 1 mmol/L EDTA [pH 8.0], 50 mmol/L Tris borate [pH 8.3], 0.05% bromophenol blue, and 0.05% xylene cyanol) and electrophoresed on a 6% nondenaturing polyacrylamide gel at 200 to 250 V for 3 hours. Protected bands were visualized by autoradiography.

**Immunohistochemistry**

MCs were grown in RPMI-1640 with 20% FBS and 1 µmol/L insulin on culture chamber slides as described above. The cells were fixed with 4% paraformaldehyde for 15 minutes. Blocking solution containing 1% BSA in 1× PBS was added, and the cells were incubated with rabbit anti-PPARγ antibody (1:300 dilution) or anti-RXRα antibody (Biomol Research Laboratories, Inc) at 4°C overnight. Biotinylated secondary antibody (Zymed Laboratories, Inc) was applied for 30 minutes, followed by a 20-minute incubation with streptavidin peroxidase. Peroxidase activity was detected by using aminomethylcarbazole as a chromogen in the liquid AEC kit, as per the manufacturer’s instructions (Zymed Laboratories, Inc). The slides were then counterstained with Mayer’s acid hematoxylin for 3 minutes.

**Electrophoretic Mobility Gel Shift Analysis**

Nuclear extract from MCs was prepared as described. A double-stranded 20-mer (5’TGAAACTAGGTTAAGGTTCA3’) was synthesized according to the sequence of the rat CYP4A1, which has a PPARγ responsive element (PPRE), and radiolabeled with the use of T4 polynucleotide kinase. Ten micrograms of nuclear extract and 25 000 cpm of probe were incubated on ice for 30 minutes in binding buffer (50 mmol/L KCl, 15 mmol/L HEPES [pH 7.9], 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 6 mmol/L MgCl2, 10% glycerol, 2 µg poly(dI-dC), and 10 µg BSA). DNA-protein complexes were separated by electrophoresis on a 6% native polyacrylamide gel in 1× Tris/borate/EDTA buffer at 4°C. The gel was dried and exposed to radiographic film at −80°C. Competition assays were performed by using 100-fold molar excess unlabeled wild-type and mutant oligonucleotides.

**BrdU Incorporation**

MCs were plated on 6-well plates, grown to ~60% to 70% confluence, and serum-starved in 0.5% FBS for 24 hours. The cells were preincubated with RSG (BRL 49653, 1 to 10 µmol/L) and TRO (1 to 10 µmol/L) for 30 minutes and stimulated with human recombinant platelet-derived growth factor (PDGF-BB, 50 ng/mL, Sigma) for 24 hours. Controls were kept in serum-free media. Cells were incubated with bromodeoxyuridine (BrdU), a thymidine analogue. The cells were methanol-fixed and incubated in 1N HCl for 2 hours. Monoclonal mouse anti-BrdU antibody was added, and cells were incubated for 30 minutes at 37°C. The cells were covered with alkaline phosphatase–conjugated sheep anti-mouse IgG antibody for 30 minutes at 27°C and exposed to color substrate (nitro blue tetrazolium). Each step was separated by several washes with 1× PBS. MC nuclei, which incorporated BrdU, appeared brown and were counted in 4 to 6 different fields per well and related to the total cell number. Data were based on different experiments from 4 preparations of MCs.

**STZ-Induced Experimental Diabetes**

Normal (200- to 450-g) Sprague-Dawley rats (Charles River) were given a single intraperitoneal injection of STZ (65 mg/kg) and housed in a 12-hour light cycle. Three days after recovery, the blood glucose was measured by glucose analyzer (Beckman) to document hyperglycemia (~500 to 650 mg/dL). The STZ-induced diabetic animals were divided into treated and untreated groups. Treated animals were fed TRO (2 g/kg, SmithKline Beecham) for 3 months, and blood pressures were measured weekly by the tail-cuff method. On the day before kidney harvest, diabetic and weight-matched control animals were placed in metabolic cages for 24-hour urine collection, and final plasma glucose was determined.

**Plasma Albumin and Urine Creatinine Measurement**

Urine from each animal was used for creatinine and albumin determination. Urine albumin concentration was measured by rat albumin enzyme immunosassay as per the manufacturer’s instructions (Cayman). Creatinine was measured by using the colorimetric method, based on the Jaffe reaction, as per the manufacturer’s instructions.
instructions (Sigma Diagnostics). From these measurements, the ratio of urinary albumin to creatinine was determined.

**Statistical Analysis**

The raw data are expressed as the mean±SEM. Differences between groups were analyzed by using standard statistical analyses and the Student t test. A level of \( P<0.05 \) was considered statistically significant.

**Results**

**PPAR\( \gamma_1 \) and RXR\( \alpha \) Are Present in MC Nuclear Extract**

Protein from MC whole-cell nuclear extract and the glomerular fraction were obtained from normal Sprague-Dawley rats and compared with that from the cytoplasmic extract by Western blot analysis. Nuclear extracts from adipocytes and preadipocytes were used as positive and negative controls, respectively (Figure 1A). The double band observed in adipocyte nuclear extract is presumed to be due to alternative splicing and is always present. PPAR\( \gamma \) protein was absent from MC cytoplasmic extract but was present in MC nuclear extract, whole-cell extract, and glomerular fractions. RXR\( \alpha \) protein, the heterodimeric partner of PPAR\( \gamma \), was present in readily detectable amounts in most samples but only minimally present in cytoplasmic extract. We examined total RNA obtained from MCs in culture, glomerular and nonglomerular fractions of kidney, and the whole kidney, with the use of RPAs to identify PPAR\( \gamma \) mRNA. Figure 1B indicates minimal expression of PPAR\( \gamma_2 \) in any of the samples. However, PPAR\( \gamma \) was present in whole kidney, in glomerular and nonglomerular cortical tissue, and in cultured MCs, as was message for RXR\( \alpha \). PPAR\( \gamma_1 \) is the primary form of the receptor in other tissues outside of adipose tissue. Similar findings were recently demonstrated by Asano et al.\(^1\)

Cultured rat primary MCs were immunostained to confirm the nuclear localization of both PPAR\( \gamma \) and RXR\( \alpha \). As Figure 1C shows, both nuclear transcription factors localize to the nuclei of MCs. There was little or no staining for PPAR\( \gamma \) or RXR\( \alpha \) in the cytoplasm. All figures are representative of at least 3 separate experiments.

**PPAR\( \gamma \) Binds to Its DNA Response Element in Primary MCs**

An electrophoretic mobility shift assay was performed to determine whether the PPAR\( \gamma \) protein detected in the present study is potentially functional. The DNA response element for PPAR\( \gamma \) (PPRE) was used as labeled probe in Figure 2. Nuclear extract from primary MCs was incubated with labeled PPRE and run on an electrophoretic gel. Lane 1

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Analysis of PPAR\( \gamma_1 \), PPAR\( \gamma_2 \), and RXR\( \alpha \) in MCs. A, Western blot analysis was performed by using nuclear extract (NE), whole-cell extract, and cytoplasmic extract from primary MCs as well as glomerular fraction from normal rat kidneys. NEs from differentiated 3T3-L1 adipocytes and undifferentiated 3T3-L1 preadipocytes were used as positive and negative controls, respectively. B, Total RNA from cultured MCs, whole kidney, and glomerular and nonglomerular fractions was used for RPA. C, Immunostaining was performed on cultured primary MCs.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Electrophoretic mobility shift assay. This competition assay was performed by using synthetically labeled oligonucleotide containing PPRE (lane 1) and nuclear extract from primary MCs. All lanes contained labeled probe. Lanes are as follows: 2, band shift in the presence of primary MC nuclear extract; 3, result of positive competition in the presence of excess unlabeled probe; 4, result of labeled mutated PPRE incubated with MC nuclear extract; and 5, result of negative competition in the presence of labeled wild-type PPRE and unlabeled mutated PPRE. This is representative of at least 3 assays.
shows that the mobility of the free probe was not retarded and ran to the bottom of the gel. Lane 2 shows that incubation of labeled PPRE with nuclear protein formed a complex, which retarded its mobility on the gel. In lane 3, the shifted band was efficiently competed away in the presence of unlabeled probe, indicating that protein binding to the labeled PPRE was specific. There was no binding of nuclear protein when labeled mutated PPRE was incubated with nuclear extract (lane 4). Furthermore, there was no competition for binding to the wild-type PPRE in the presence of cold unlabeled mutated PPRE (lane 5) and nuclear protein. These results suggest that the PPARγ protein found in primary MCs binds specifically to the PPRE and, therefore, is potentially functional.

**TZDs Inhibit PDGF-Stimulated Growth of MCs and Ang II–Induced PAI-1 Expression In Vitro**

PDGF-BB, which activates both α and β PDGF receptors, is the most potent mitogen for MC growth. Therefore, PDGF was used to achieve a maximum proliferative response in MCs. Cultured primary MCs were preincubated with RSG (BRL 49653, 1 to 10 μmol/L) or TRO (1 to 10 μmol/L) for 30 minutes, followed by stimulation with PDGF (50 ng/mL) for 24 hours. DNA synthesis was determined by BrdU incorporation. Results are expressed as percentage of PDGF-stimulated cells per treatment compared with control (n = 4). *P < 0.05.

Figure 3. PPARγ ligands inhibit PDGF-stimulated MC proliferation. Primary MCs were serum-starved in 0.5% FBS for 24 hours. Cells were pretreated for 30 minutes with RSG (BRL 49653, 1 to 10 μmol/L) or TRO (1 to 10 μmol/L), followed by stimulation with PDGF (50 ng/mL) for 24 hours. DNA synthesis was determined by BrdU incorporation. Results are expressed as percentage of PDGF-stimulated cells per treatment compared with control (n = 4). *P < 0.05.

**Effect of TRO in STZ-Induced Diabetic Rats**

To determine whether the functional effect of PPARγ activation demonstrated by our in vitro experiments is translated in vivo, STZ-induced diabetic (fasting blood glucose 500 to 650 mg/dL) rats were fed chow or TRO (2 g/kg) plus chow, after the induction of diabetes, for 3 months. Glomerular PPARγ expression was increased 4-fold in kidneys from the diabetic animals compared with normal age-matched animal kidneys (Figure 5A). TRO treatment did not increase glomerular PPARγ expression over the diabetic levels. In contrast, PAI-1 expression was also increased in glomerular cores taken from the diabetic kidneys compared with control kidneys; TRO attenuated PAI-1 expression to normal levels (Figure 5B). TRO also effectively normalized the urine albumin-to-creatinine ratio in the treated animals (Figure 5C). In this animal model, there was no difference between the TRO-treated and untreated animal groups in blood pressure (104 ± 1.9 [n = 7] versus 109 ± 1.8 [n = 7] mm Hg, respectively) or glucose (639 ± 29 [n = 7] versus 654 ± 17 [n = 7] mg/dL, respectively). These results are similar to recently published data.

Discussion

The present study shows that the TZDs, originally intended as insulin sensitizers, also have a direct effect on MCs, which may contribute in part to their action to reduce and even normalize microalbuminuria. Specifically, we have demonstrated that PPARγ1 is expressed in the glomerular fraction of the kidney, is localized to the nuclei of MCs, and could activate a PPRE. TZD activation of MC PPARγ inhibited PDGF-stimulated cell growth and Ang II–induced PAI-1 expression, indicating that PPARγ is functional in MCs.

Because TZDs are now commonly used oral hypoglycemics in type II diabetes, we were specifically interested in their effects on the microvascular complications of diabetes that may extend beyond glucose control. The process of diabetic nephropathy involves extracellular matrix (ECM) expansion, and to some extent, early mesangial proliferation may contribute to the renal pathology. Therefore, we focused on TZD actions that may affect these mechanisms. Because activation of PPARγ has been suggested to inhibit growth in vascular smooth muscle cells, ocular endothelial
Figure 5. TRO effects in STZ-induced diabetic rats. STZ-induced diabetic rats were fed chow (n = 8) or TRO (2 g/kg) plus chow (n = 4) for 3 months and compared with age-matched control rats (n = 7). PPARγ and PAI-1 expression were determined by RPAs using glomerular RNA, and the albumin-to-creatinine ratio was measured. A, Bar graph represents quantitative analysis of at least 3 RPAs of glomerular PPARγ expression. Diabetes increases PPARγ expression. B, Representative RPA of PAI-1 expression in glomerular RNA is shown. Bar graph represents quantitative analysis of at least 3 experiments. N indicates normal. TRO normalized PAI-1 expression. C, Urine albumin-to-creatinine ratio was determined on 24-hour urine collection from study animals. TRO normalized the urine albumin-to-creatinine ratio compared with that of control animals (n = 7). *P < 0.5 vs control.

that some of the effects of TZDs in diabetic nephropathy may be the inhibition of cell growth and/or inflammation associated with macrophage infiltration.

Enhanced MC production and decreased metabolism of fibronectin, laminin, and collagens III and IV ultimately restrict glomerular filtering capacity, leading to overt nephropathy, which progresses to end-stage renal disease. Transforming growth factor-β1 (TGF-β1), a cytokine, has emerged as a key culprit in the development of glomerulosclerosis.30–33 Overproduction of TGF-β1 occurs in diabetic glomeruli as a result of hyperglycemia, Ang II, insulin and insulin-like growth factor-1, increased stretch (due to hypertension), and other factors. TGF-β1 increases the mesangial production of ECM proteins and decreases their metabolism, leading to progressive glomerular ECM accumulation and, ultimately, fibrosis. In cultured MCs, TGF-β1 and other growth factors (Ang II and insulin) stimulate the synthesis and secretion of plasminogen activation inhibitor 1 (PAI-1) which regulates a cascade of events importantly controlling degradation of ECM.34,35 PAI-1 prevents ECM breakdown, inasmuch as monoclonal antibodies against PAI-1 enhance ECM degradation.36

Although we did not see major effects of TZDs on ECM expression, TRO inhibited PAI-1 expression in cultured MCs to which Ang II was added and in glomerular cores of rats made diabetic with STZ. Indeed, PAI-1 expression was upregulated in diabetic glomeruli compared with normal glomeruli, consistent with its potential role to prevent ECM breakdown, leading to ECM accumulation in diabetic nephropathy. The inhibition of PAI-1 expression by TRO treatment was associated with a decrease in albumin excretion in the absence of a change in glucose or blood pressure. Whether the changes in glomerular PAI-1 expression contributed to the normalization of the albumin-to-creatinine ratio remains to be determined, but in a model of renal irradiation fibrosis, inhibition of the renin-angiotensin system decreased renal PAI-1 expression and interstitial fibrosis.36 Moreover, bleomycin-induced pulmonary fibrosis was substantially attenuated in the PAI-1 knockout mouse.37 Thus, PAI-1 has been implicated as an important profibrotic factor. TRO has been reported to decrease circulating PAI-1 levels in patients with diabetes and with polycystic ovarian disease; in both of these clinical situations, plasma PAI-1 levels and activity are elevated.38,39 The increase in PPARγ expression in diabetic glomeruli is of interest and is similar to the upregulation seen in vascular injury.7 The endogenous PPARγ ligand, PGJ2, is a known potent inhibitor of macrophage inflammatory functions,40 and the increase in PPARγ in tissue injury may be an attempt to limit cell growth, inflammation, and the injury response.41 Pharmacological activation of these receptors early after injury may limit disease. The present study shows a direct role for PPARγ ligands in reducing microalbuminuria and implies that these agents may have therapeutic implications in the treatment of diabetic nephropathy. TZDs may potentially alter expression of multiple genes involved in diabetic glomerulosclerosis.
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