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Peroxisome proliferator-activated receptor-γ agonist troglitazone protects against nondiabetic glomerulosclerosis in rats

Li-Jun Ma, Carmelita Marcantoni, MacRae F. Linton, Sergio Fazio, and Agnes B. Fogo

Department of Pathology and Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA

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Background. Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors with beneficial effects in diabetes mediated by improved insulin sensitivity and lipid metabolism, but potential adverse effects in atherosclerosis by promoting in vitro foam cell formation. We explored whether a PPARγ agonist, troglitazone (TGL), affects sclerosis by mechanisms unrelated to insulin and lipid effects in a model of nondiabetic glomerulosclerosis.

Methods. Adult male Sprague Dawley rats underwent 5/6 nephrectomy and were treated for 12 weeks as follows: control (CONT), no further treatment; triple antihypertensive therapy (TRX); and TGL or TGL + TRX. Functional, morphological, and molecular analyses were performed.

Results. Systolic blood pressure (SBP) was increased in CONT and TGL groups (161 ± 1 and 160 ± 3 mm Hg), but not in TGL + TRX and TRX (120 ± 3 vs. 126 ± 1 mm Hg, $P < 0.0001$ vs. non-TRX). Serum triglyceride and cholesterol levels in all groups remained normal except for slightly higher serum cholesterol levels in TRX group. TGL groups had reduced proteinuria, serum creatinine, and glomerulosclerosis versus CONT, in contrast to no significant effect with TRX alone (sclerosis index, 0 to 4+ scale: CONT 1.99 ± 0.42, TGL 0.85 ± 0.12, TGL + TRX 0.56 ± 0.14, TRX 1.30 ± 0.21; TGL, $P < 0.05$; TGL + TRX, $P = 0.01$ vs. CONT). Glomerular cell proliferation, assessed by proliferating cell nuclear antigen (PCNA), was decreased after treatment with TGL or TGL + TRX, in parallel with decreases in glomerular p21 mRNA and p27 protein compared with CONT and TRX (PCNA + cells/glomerulus: CONT 2.04 ± 0.64, TGL 0.84 ± 0.21, TGL + TRX 0.30 ± 0.07, TRX 1.38 ± 0.37; TGL, $P < 0.05$, TGL + TRX, $P < 0.01$ vs. CONT). Glomerular plasminogen activator inhibitor-1 (PAI-1) immunostaining was decreased in TGL or TGL + TRX groups (0 to 4+ scale, CONT 2.42 ± 0.32, TGL 1.40 ± 0.24, TGL + TRX 1.24 ± 0.17, TRX 2.53 ± 0.24; TGL or TGL + TRX vs. CONT, $P < 0.05$), with a parallel decrease in PAI-1 mRNA by in situ hybridization. Glomerular and tubular transforming growth factor-β (TGF-β) mRNA expression was decreased with TGL treatment. Glomerular macrophages, present in CONT and TRX rats, did not express PPARγ, in contrast to PPARγ + macrophages in control carotid artery plaque. PPARγ was expressed in resident cells.

Conclusions. Our results demonstrate in vivo that the PPARγ ligand TGL ameliorates the progression of glomerulosclerosis in a nondiabetic model. Macrophages show phenotypic diversity in glomerular versus vascular sclerosis, with macrophage PPARγ expression in only the latter. PPARγ beneficial effects are independent of insulin/glucose effects and are associated with regulation of glomerular cell proliferation, hypertrophy, and decreased PAI-1 and TGF-β expression.

Key words: cell turnover, plasminogen activator inhibitor-1, macrophage, p21, p27.

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Insulin resistance has been highlighted as a common causal factor for hypertension, diabetes mellitus, and obesity and is the underlying defect in a distinct clinical entity defined as metabolic syndrome X [1, 2]. Treatment of this insulin resistance has great therapeutic potential for the control of type II diabetes. Troglitazone (TGL) was the first marketed example of a novel therapeutic class of antidiabetic agents, the thiazolidinediones (TZDs), for the treatment of type 2 diabetes. As a class, TZDs (TGL, rosiglitazone, and pioglitazone) have been shown to exert antihyperglycemic and antihyperinsulinemic actions in laboratory animal models of insulin resistance and diabetes [3–7] and in humans [8–10]. Clinically, these actions result in a reduction in fasting hyperglycemia without an increase in plasma insulin levels. In addition to their antihyperglycemic effect, TZDs also reduce circulating levels of triglycerides (TGs) and free fatty acids (FFAs) [7].

Recent evidence indicates that the peroxisome proliferator-activated receptor-γ (PPARγ) is the molecular target of the TZDs [11, 12]. PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcriptional factors that also includes receptors for steroid hormones, thyroid hormone, retinoids, and vitamin D [13]. PPARγ can also be activated by naturally occurring

arachidonic acid metabolites derived from the cyclooxygenase pathway, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-deoxy PGJ2) [14, 15]. Certain nonsteroidal anti-inflammatory drugs (NSAIDs) are also identified as PPARγ ligands [16]. Following activation, PPARγ heterodimerizes with retinoic X receptor (RXR) and binds to peroxisome proliferator response elements (PPREs) in the promoter region of target genes, thereby regulating their transcription [17].

Recent advances in the understanding of the biology of the PPARs suggest a major role for PPARγ in the regulation of adipocyte differentiation and the expression of adipocyte-specific genes and of key regulatory genes involved in carbohydrate and lipid metabolism [18–20]. Although adipose tissues have been recognized as a principal site of expression of PPARγ, these receptors are also expressed at low levels in many other tissues and cell types and may play important roles in nonadipose sites [20, 21].

One of the earliest steps in the formation of atherosclerotic lesions is the conversion of macrophages to cholesterol-engorged foam cells [22]. The formation of foam cells is characterized by dramatic changes in the cells' lipid metabolism, including increased expression of scavenger receptors and uptake of oxidized low-density lipoprotein (LDL). In vitro, PPARγ promoted monocyte to foam cell transformation and uptake of oxidized LDL through transcriptional induction of scavenger receptors CD36 [23, 24]. Furthermore, in vivo PPARγ was expressed at high levels in foam cells of human atherosclerosis lesions [25]. These observations suggested that PPARγ has potential adverse effects in atherosclerosis.

Peroxisome proliferator-activated receptor-γ activation also can regulate other mediators of vascular lesions, such as plasminogen activator inhibitor-1 (PAI-1). Variable response of PAI-1 to PPARγ activation was observed in in vitro studies in endothelial cells [26, 27]. Recent studies in mesangial cells in culture showed that PAI-1 mRNA induction was decreased by PPARγ agonist (communication with S. Nicholas).

In vivo, beneficial effects of PPARγ were demonstrated in diabetic nephropathy and in the WHHL rabbit model of atherosclerosis [28, 29]. Rosiglitazone, a PPAR agonist, ameliorated development of injury in the Zucker fatty rat model of type 2 diabetes. This effect was associated with reduced plasma glucose, total cholesterol, and TG levels [28]. The protective metabolic effects could, however, mask potentially adverse effects on vascular injury suggested by the previously mentioned studies. We thus hypothesized that analogous to the manifold actions of angiotensin now recognized beyond vasoconstriction, PPARγ could have multiple effects beyond lipid metabolism and insulin sensitization due to its effects on transcription factors. Our results indeed show that PPARγ agonist protects against development of glomerulosclerosis in a model of nondiabetic vascular injury. Furthermore, we have linked this protective effect in sclerosis to decreased macrophage infiltration, decreased PAI-1 and transforming growth factor-β (TGF-β), and regulation of glomerular cell growth.

METHODS

Experimental design and animals

Adult male Sprague Dawley rats (250 to 300 g; Charles River, Nashville, TN, USA) were studied. Rats were housed under normal conditions with a 12-hour light/dark cycle at 70°F with 40% humidity and 12 air exchanges per hour and received standard rat powdered diet (Purina Rodent "5001" meal, 23.4% protein, 4.5% fat, 6.0% fiber, 0.40% sodium; Tusculum Feed Center, Nashville, TN, USA) and water. Rats underwent 5/6 nephrectomy (Nx) by right Nx and ligation of two or three main branches of the left renal artery by silk ligature to remove approximately 5/6 renal mass. The surgery was performed under anesthesia with sodium pentobarbital (50 mg/kg body wt, IP). The Nx rats were then divided into four groups as follows: The control group (CONT, $N = 7$) received normal rat chow powder as described previously in this article. The triple-therapy group received antihypertensive drugs [TRX, $N = 7$, reserpine 5 mg/L drinking water (DW), hydralazine 80 mg/L DW, and hydrochlorothiazide 25 mg/L DW]. This dose, although normalizing systemic blood pressure in previous studies in this model, does not protect against glomerulosclerosis [30, 31]. The TGL group received TGL mixed with powdered chow (TGL, 500 mg/kg/day body wt, $N = 7$; kindly provided by Parke Davis, Morris Plains, NJ, USA). The dose of TGL was chosen based on this dose correcting abnormal carbohydrate and lipid metabolism in rats and mice with diabetes [7, 32, 33]. In addition, these studies have shown that this dose results in a plasma concentration of TGL comparable with that achieved in clinical studies. The appropriate amount of TGL in chow in this study was regularly adjusted on the basis of body weight. The last group received a combination of TGL and antihypertensive therapy as discussed previously in this article (TGL + TRX, $N = 7$). Animals were monitored (discussed later in this article) and sacrificed at 12 weeks. Kidneys were harvested for analysis of morphologic and molecular parameters.

Analysis of kidney function

Systolic blood pressure (SBP) and 24-hour urinary protein were assessed at weeks 4, 8, and 12. SBP was measured using tail-cuff plethysmography in unanesthetized prewarmed trained rats at ambient temperature of 29°C. Animals were placed in metabolic cages for 24 hours for urine collection, and urine protein was measured by Bio-Rad Protein Assay Kit (Bio-Rad Labora-

tories, Hercules, CA, USA). Serum creatinine was measured by Vitros CREA slides (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY, USA).

Structural analyses

Kidney tissue from rats was immersion fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) solution and routinely processed, and 4 μm sections were stained with periodic acid-Schiff and Masson's trichrome. Immunohistochemistry and in situ hybridization studies were performed (discussed later in this article).

A semiquantitative score [sclerosis index (SI)] was used to evaluate the degree of glomerular sclerosis. The severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0 represents no lesion; 1+ represents sclerosis of <25% of the glomerulus, while 2+, 3+, and 4+ represent sclerosis of 25 to 50%, 50 to 75%, and >75% of the glomerulus, respectively. A whole kidney average SI was obtained by averaging scores from all glomeruli on one section. Tubulointerstitial fibrosis was evaluated qualitatively on Masson's trichrome-stained section. All sections were examined without knowledge of the treatment protocol.

Serum cholesterol and triglyceride analysis

Serum cholesterol levels were determined using Raichem reagent no. 80035 (Raichem, San Diego, CA, USA), adapted for a microtiter plate assay [34]. Briefly, 100 μL of a 1:100 dilution of serum were mixed with 100 μL of reagent and incubated at 37°C for 10 minutes in a microtiter plate. The 490 nm absorbance was read on a Molecular Devices microplate reader (Molecular Devices, Menlo Park, CA, USA). Serum TG levels were determined using Sigma kit no. 339 (Sigma, St. Louis, MO, USA) similarly adapted for microtiter plate assay.

Immunostaining of PCNA, p27, and PAI-1

Four micrometer sections from tissue fixed overnight at 4°C with paraformaldehyde were dewaxed, dehydrated, treated by 3% hydrogen peroxidase for 10 minutes, and exposed to Power Block (BioGenex Laboratories, San Ramon, CA, USA) for 45 minutes. Proliferating cells were identified in rat kidney by proliferating cell nuclear antigen (PCNA) cyclin polypeptide immunohistochemistry. For PCNA staining, monoclonal mouse anti-human PCNA (Dako Corporation, Carpinteria, CA, USA) was applied and incubated for 30 minutes at room temperature followed by rabbit anti-mouse antibody (Dako) for 30 minutes. For detection of p27, goat anti-rat p27 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added, incubated for 60 minutes, followed by rabbit anti-goat antibody (Dako) for 30 minutes. For PAI-1 staining, sections were incubated with rabbit anti-rat PAI-1 antibody (American Diagnostica Inc., Greenwich, CT, USA) overnight [35], biotinylated goat anti-rabbit Ig (BioGenex Laboratories) for 45 minutes, followed by peroxidase-conjugated streptavidin for 45 minutes. After rinsing with PBS, diaminobenzidene was added as a chromagen. Slides were counterstained with hematoxylin. The number of PCNA or p27-positive nuclei per glomerulus was counted. PAI-1 glomerular expression was evaluated by a semiquantitative score. Scores of 0 to 4 represent negative, trace, <10%, 10 to 25%, and >25% staining, respectively, in each glomerulus. Control slides treated with nonspecific antisera instead of primary antibody showed no staining. All sections were examined without the knowledge of the treatment protocol.

Reverse transcriptase-polymerase chain reaction

Rat PPARγ cDNA fragment comprising part of the A/B and DNA binding domains was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) using 10 μg of total RNA from 5/6 Nx rat kidney tissue. The specific primers were directed against the rat sequences for PPARγ: 5′-GAG ATG GAA TTC TGG CCC ACC AAC TTC GG-3′ (sense) and 5′-TAT CAT AAA TAA GCT TCA ATC GGA TGG TTC-3′ (antisense). Total RNA was extracted by RNAzol™ B method (Tel-Test, Inc., Friendswood, TX, USA) and reverse transcribed to single-stranded cDNAs using Superscript™ II reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA) and 100 μmol/L oligo dT according to the manufacturer's instructions (GIBCO BRL). The cDNA was then amplified using PPARγ-selective primers. PCR reactions were carried out in 10 mmol/L TrisCl, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, and 1 μmol/L primers at 95°C for one minute, 55°C for two minutes, and 72°C for one minute for 35 cycles in a thermal cycler followed by an extension step at 72°C for seven minutes. After amplification, a 403 bp long PPARγ cDNA was sequenced and subcloned in PCRII vector (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA of human p27 was made from 10 μg of total RNA from normal human kidney tissue. The reverse transcription process followed the same protocol as described previously in this article. The gene-specific primers for human p27 were 5′-CCT CTT CGG CCC GGT GGA C-3′ (sense, in the coding region from nucleotide 93 to 111) and 5′-TCT GCT CCA CAG AAC CGG C-3′ (antisense, in the coding region from nucleotide 541 to 559) [36]. PCR reactions were carried out at 95°C for one minute, 62°C for two minutes, and 72°C for one minute for 35 cycles in a thermal cycler followed by an extension step at 72°C for seven minutes. After amplification, a 467 bp long p27 cDNA was sequenced and subcloned in PCRII vector (Invitrogen). A 420 bp length cDNA fragment specific for human p21 in the coding region was amplified by RT-PCR and subcloned into a vector

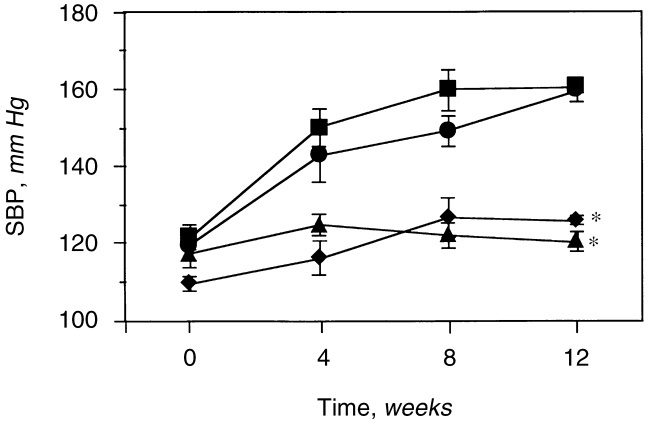

Fig. 1. Systolic blood pressure (SBP) changes after 5/6 nephrectomy (Nx). SBP in the control (CONT; ■) and troglitazone (TGL; ●) groups increased over 12 weeks, contrasting normal SBP in groups receiving triple antihypertensive drug therapy (TRX; ◆) or TGL + TRX (▲).

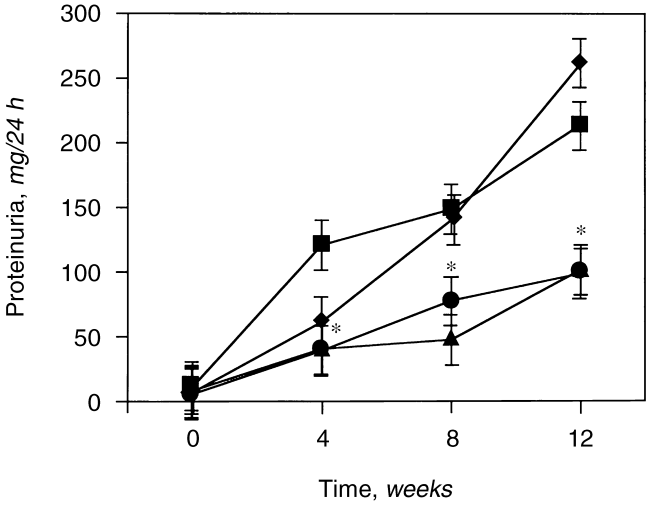

Fig. 2. Urine protein excretion after 5/6 Nx. Urine protein excretion increased in CONT (■) and TRX (◆) groups. TGL (●) alone or TGL + TRX (▲) treatment for 12 weeks significantly reduced proteinuria (*$P <$ 0.01, TGL or TGL + TRX vs. CONT).

[pBlueScript SK(−); Stratagene, La Jolla, CA, USA] [37].

In situ hybridization

[^{35}S]-labeled sense and antisense riboprobes for PAI-1 and TGF-β were prepared as previously described [38, 39]. [^{35}S]-labeled sense and antisense riboprobes for PPARγ and p27 were prepared by transcription of the pCR™ II plasmid (Invitrogen), and [^{35}S]-labeled sense and antisense riboprobes for p21 were prepared by transcription of the pBlueScript SK(−) plasmid [37] with insertion of each responding cDNA fragment by SP6 or T7 RNA polymerase (Ambion, Austin, TX, USA). In situ hybridization was performed as previously described [36]. Briefly, sections were treated with proteinase K and triethanolamine/acetic anhydride, and hybridization was done at 50°C. Sections were washed in buffer with 5 × standard saline citrate (SSC) and 20 mmol/L β-mercaptoethanol at 50°C for 15 minutes, then washed in 2 × SSC, 50% formamide at 68°C for 20 minutes, in TEN twice at 37°C for 10 minutes, treated with RNase at 37°C for 30 minutes, washed in TEN at 37°C for 10 minutes, and incubated twice in 2 × SSC and 0.1 × SSC at 68°C for 15 minutes. Sections were then dehydrated in ethanol and air dried, dipped in photographic emulsion, and exposed at 4°C for 10 days. The sections were developed with D-19 developer (Eastman Kodak, Rochester, NY, USA) and counterstained with toluidine blue. Negative control in situ hybridizations were done with sense probes.

Double immunostaining

Peroxisome proliferator-activated receptor-γ immunostaining was performed with primary mouse monoclonal antibodies to human PPARγ (Santa Cruz Biotechnology, Inc.), followed by peroxidase conjugated rabbit anti-mouse IgG (Dako) and peroxidase-conjugated swine anti-rabbit IgG (Dako) as secondary and tertiary antibodies and finally developed with diaminobenzidine (DAB) as chromagen. Macrophages were detected with mouse monoclonal antibody ED1 (1:50; Dako), followed by biotinylated goat anti-mouse IgG (Innogenex, San Ramon, CA, USA) for 30 minutes and alkaline phosphatase-streptavidin conjugate (Innogenex) for 30 minutes. Sections were developed with fast red TR/Naphtol AS-MX (Sigma) for five minutes and then counterstained and coverslipped. Negative control with nonspecific serum from the species of primary antibody instead of specific antibody showed no staining. Transitional epithelial cells of the pelvis were used as positive controls for PPARγ staining. Human carotid artery plaques were also double stained with antibody to CD68 (Dako) and antibody to PPAR-γ.

Statistical analysis

Results are expressed as mean ± SEM. Statistical difference was assessed by a single-factor variance (analysis of variance, ANOVA) followed by unpaired *t* test as appropriate. Nonparametric data were compared by Mann–Whitney *U*-test. A P value < 0.05 was considered to be significant.

RESULTS

Body and renal functional changes

Body weights of rats in each group ($N = 7$ each group) increased over time, although treated rats gained less than control (at 12 weeks after 5/6 Nx, CONT 407 ± 20, TGL 372 ± 12, TGL ± TRX 327 ± 8, TRX 358 ± 10 g, CONT vs. treated groups $P \leq 0.05$). SBP was increased

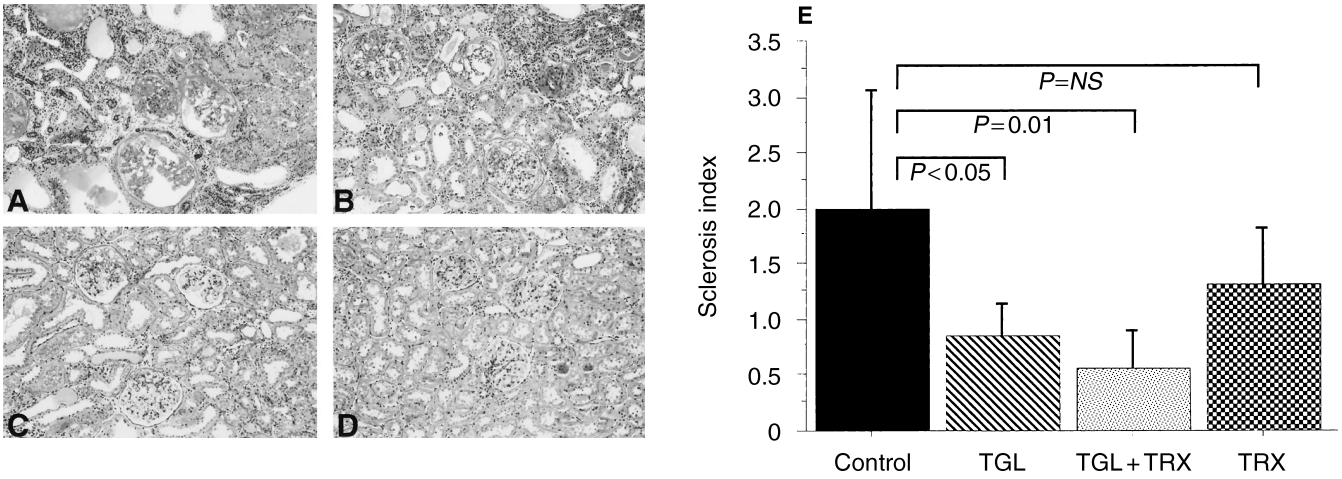

Fig. 3. Renal injury at 12 weeks after 5/6 Nx. TGL alone or TGL + TRX treatment for 12 weeks significantly decreased glomerulosclerosis versus control (*E*). Glomerulosclerosis, tubular atrophy, and interstitial fibrosis were present in CONT (*A*) and TRX (*B*) rats; treatment with TGL (*C*) or TGL + TRX (*D*) for 12 weeks decreased glomerulosclerosis (PAS stain, ×100).

significantly in untreated control rats by four weeks after 5/6 Nx (150 ± 5 vs. baseline 122 ± 3 mm Hg, $P < 0.0001$) and continued to increase slightly until 12 weeks (Fig. 1). Interestingly, TGL did not reduce SBP (at 12 weeks 160 ± 3 vs. CONT 161 ± 1 mm Hg, P = NS). SBP in TRX and TGL + TRX groups remained normal (TRX 126 ± 1, TGL + TRX 120 ± 3 mm Hg, $P < 0.0001$ vs. CONT or TGL, respectively; Fig. 1). Urinary protein excretion was dramatically increased in CONT rats by four weeks after 5/6 Nx (120.9 ± 15.3 vs. baseline 12.0 ± 1.4 mg/24 h, $P < 0.0001$) and continued to increase (at 12 weeks 213.4 ± 29.1 mg/24 h, $P < 0.0001$ compared with baseline). TRX did not affect the course of proteinuria (P = NS vs. CONT). In contrast, TGL alone or in combination with TRX significantly reduced proteinuria at 4, 8, and 12 weeks (Fig. 2). At 12 weeks after 5/6 Nx, serum creatinine was increased in CONT and TRX groups; TGL and TGL + TRX treatment decreased serum creatinine levels compared with CONT (serum creatinine: CONT 3.0 ± 0.7, TGL 1.6 ± 0.2, TGL + TRX 1.0 ± 0.04, TRX 2.0 ± 0.4 mg/dL; TGL or TGL + TRX vs. CONT, $P < 0.05$).

Renal morphological changes

In untreated CONT rats and TRX rats, glomerulosclerosis, tubular atrophy, dilation, and interstitial fibrosis were present. TRX, as expected, had no significant effect on glomerulosclerosis (SI, 0 to 4 scale: CONT 1.99 ± 0.42 vs. TRX 1.30 ± 0.21, P = NS) [30, 31]. Both TGL alone and TGL + TRX significantly ameliorated the development of glomerulosclerosis compared with control (CONT 1.99 ± 0.42 vs. TGL 0.85 ± 0.12, $P < 0.05$, vs. TGL + TRX 0.56 ± 0.14, $P = 0.01$; Fig. 3 A, B). Tubulointerstitial fibrosis was reduced in parallel with glomerulosclerosis after treatment with TGL.

Lipid levels

At 12 weeks after 5/6 Nx, serum TG and cholesterol levels in all groups were comparable and remained in the normal range except for a significantly higher serum cholesterol level in the TRX group (serum TG, CONT 89 ± 9, TGL 134.1 ± 10.4, TGL ± TRX 110.5 ± 17.8, TRX 112.5 ± 31.6 mg/dL, P = NS; serum cholesterol, CONT 122.9 ± 5.4, TGL 130.1 ± 6.7, TGL ± TRX 129.4 ± 13.1, TRX 161.5 ± 14.6 mg/dL, TRX vs. CONT, $P \leq 0.05$).

Cellular proliferation

Immunostaining for PCNA was negative in the normal rat glomerulus (data not shown). In CONT rats, PCNA-positive cells were present in glomeruli and tubules. Glomerular visceral and parietal epithelial cells, mesangial, and endothelial cells showed occasional PCNA staining. Glomerular PCNA positivity was dramatically decreased after treatment with TGL or TGL + TRX, but not after TRX alone (Fig. 4).

p21 and p27 expression

Glomerular p21 mRNA expression, primarily in visceral epithelial cells, was increased in CONT (Fig. 5A) and TRX rats (data not shown) compared with baseline levels in TGL (Fig. 5B). Glomerular p27 mRNA was expressed at a diffuse, low level, similar in all groups (Fig. 5 C, D). p27 protein expression was up-regulated in glomeruli in CONT (Fig. 5E) and TRX rats (data not shown); treatment with TGL (Fig. 5F) or TGL + TRX (data not shown) decreased the p27 protein expression.

PAI-1 mRNA and protein expression

In situ hybridization study revealed high-level PAI-1 mRNA expression in CONT and TRX kidneys (Fig. 6 A, B). Strong PAI-1 mRNA signals were detected

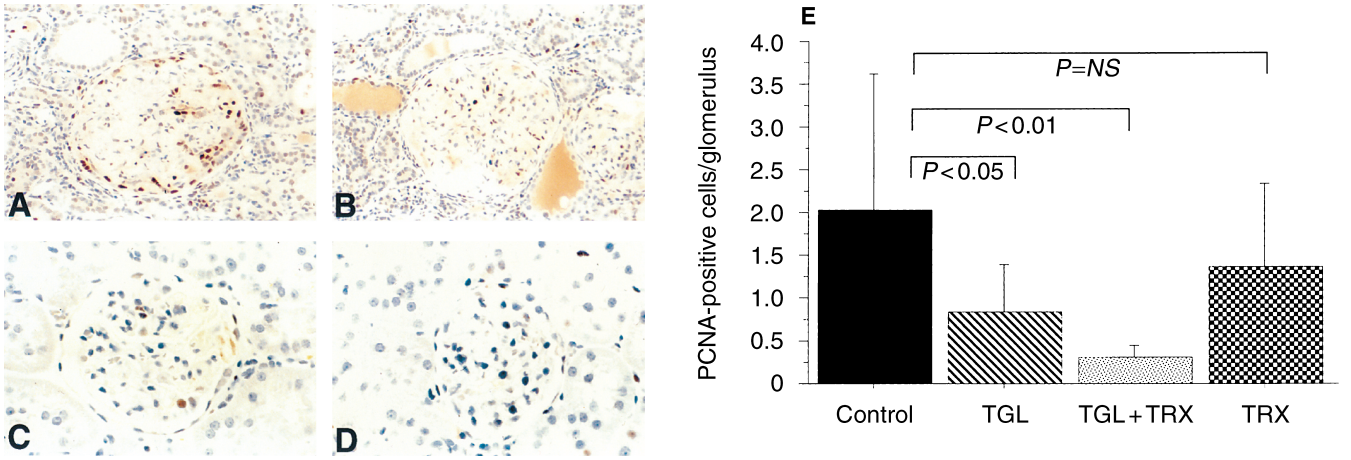

Fig. 4. Glomerular cell proliferation. TGL alone or TGL + TRX treatment for 12 weeks significantly decreased glomerular cell proliferation versus control (*E*). Staining for PCNA was not detected in the normal rat glomerulus (data not shown). There was a dramatic increase in PCNA staining in the glomeruli in CONT (*A*) and TRX-treated rats (*B*) at 12 weeks after 5/6 Nx. TGL alone (*C*) or TGL + TRX treatment (*D*) markedly reduced the PCNA-positive cells in glomeruli.

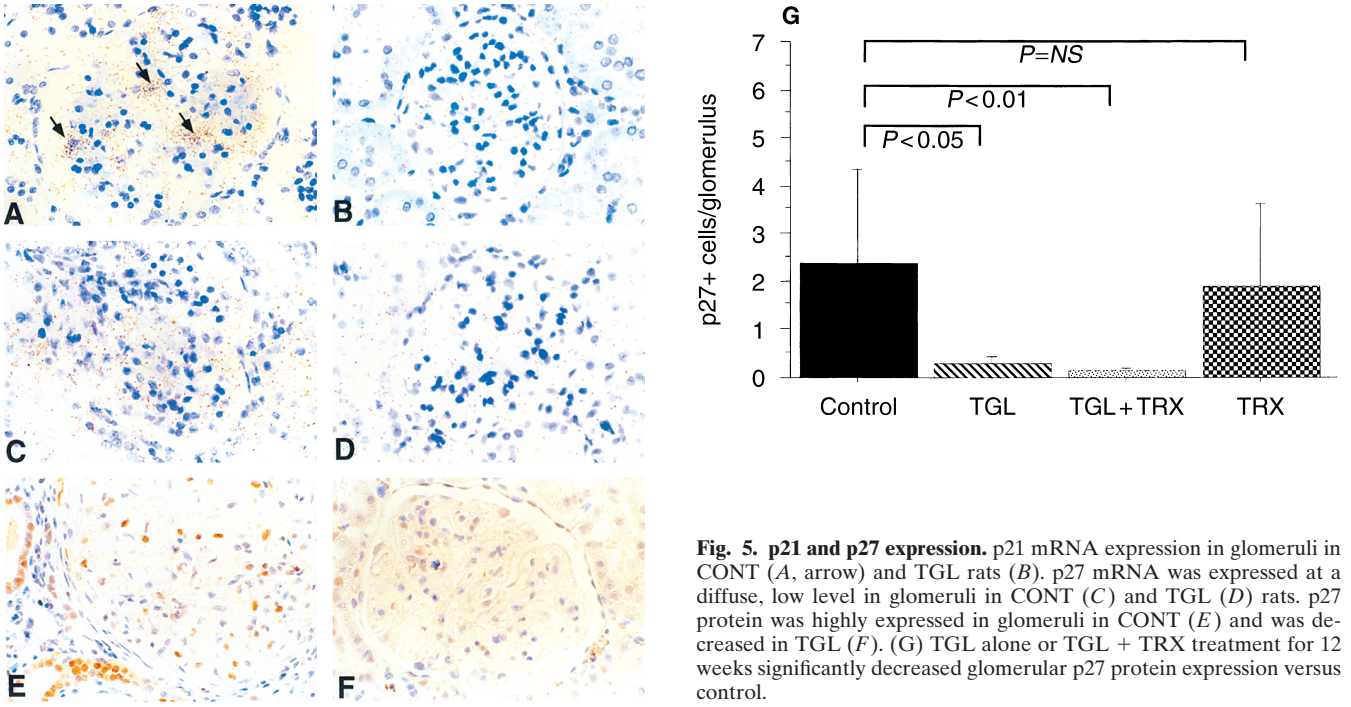

Fig. 5. p21 and p27 expression. p21 mRNA expression in glomeruli in CONT (*A*, arrow) and TGL rats (*B*). p27 mRNA was expressed at a diffuse, low level in glomeruli in CONT (*C*) and TGL (*D*) rats. p27 protein was highly expressed in glomeruli in CONT (*E*) and was decreased in TGL (*F*). (G) TGL alone or TGL + TRX treatment for 12 weeks significantly decreased glomerular p27 protein expression versus control.

primarily in sclerotic glomeruli and some tubules. Glomerular visceral and parietal epithelial, mesangial, and endothelial cells showed signal. PAI-1 mRNA expression was markedly reduced in TGL (Fig. 6C) and TGL + TRX-treated (Fig. 6D) rats compared with untreated CONT rats. In situ hybridizations with sense probes showed no signal. PAI-1 protein expression in TGL and TGL + TRX groups was also decreased compared with CONT (PAI-1 scoring: CONT 2.42 ± 0.32, TRX 2.53 ± 0.24, TGL 1.40 ± 0.24, TGL + TRX 1.24 ± 0.17; TGL or TGL + TRX vs. CONT, $P < 0.05$ and $P < 0.01$, respectively; Fig. 7E). PAI-1 protein expression showed the same pattern as mRNA (Fig. 7 A-D).

TGF-β1 mRNA expression

In situ hybridization showed up-regulated TGF-β mRNA in untreated CONT rat kidneys (both glomeruli and tubules). The TGF-β signal appeared attenuated after TGL treatment for 12 weeks (Fig. 8). Sense probes showed no specific signal.

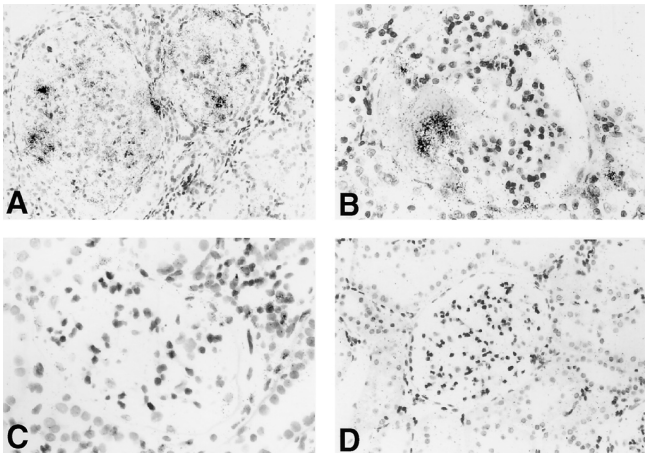

Fig. 6. Plasminogen activator inhibitor-1 (PAI-1) mRNA expression. In situ hybridization for PAI-1 mRNA showed high expression in glomeruli in CONT (*A*) and in TRX-treated rats (*B*), with signals detected primarily in sclerotic glomeruli and some cortical tubules. PAI-1 mRNA expression was markedly diminished in rats treated with TGL alone for 12 weeks (*C*) with trace signal in parietal epithelial cells. No PAI-1 mRNA was expressed in glomeruli in TGL + TRX-treated rats (*D*).

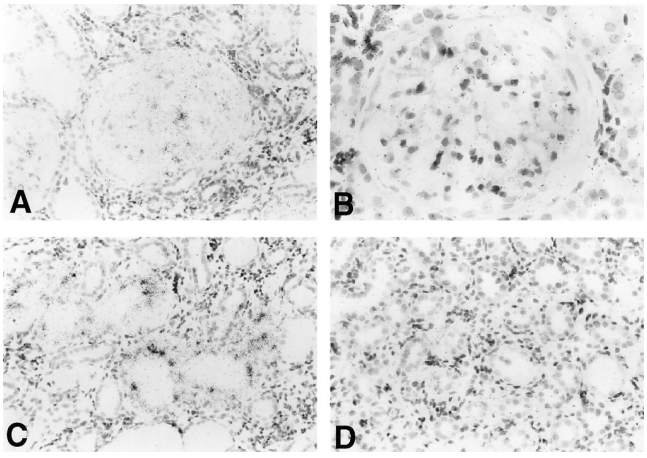

Fig. 8. Transforming growth factor-β (TGF-β) mRNA expression by in situ hybridization. TGF-β mRNA was up-regulated in glomerular cells (*A*) and tubular epithelial cells (*C*) in CONT. TGF-β mRNA expression was attenuated after treatment with TGL for 12 weeks (*B* and *D*).

Modulation of glomerular macrophages

At 12 weeks after 5/6 Nx, macrophages were present in glomeruli of CONT and TRX-treated rats (macrophages/glomerulus: CONT 3.84 ± 1.24, TRX 2.60 ± 1.07, P = NS; Fig. 9). Treatment with TGL alone or TGL + TRX significantly reduced glomerular infiltrating macrophages (TGL 0.30 ± 0.28, TGL + TRX 0.05 ± 0.03, TGL or TGL + TRX vs. CONT, $P < 0.05$ and $P < 0.01$, respectively; Fig. 9).

PPARγ expression

Immunohistochemistry of rat tissue sections demonstrated that PPARγ was intensely expressed in the transitional epithelium of the ureter in all groups as previously described (Fig. 10A) [21]. This staining thus served as a positive internal control. PPARγ was also expressed diffusely in tubules in all rats. Double immunostaining for both macrophages and PPARγ demonstrated that glomerular infiltrating macrophages did not express PPARγ, although resident glomerular cells (including visceral, parietal epithelial cells, and mesangial cells) expressed PPARγ in CONT rats (Fig. 10C). Glomerular visceral epithelial cells and tubular epithelial cells in CONT rats were PPARγ positive, and these cell types were also PCNA positive. Neither PPARγ mRNA nor protein was detected in glomeruli of TGL or TGL + TRX-treated rats. Tubular PPARγ expression was also reduced in rats treated with TGL. TRX-treated rats expressed low levels of PPARγ. In TGL or TGL + TRX rats, there were no macrophages in glomeruli (Fig. 10E). In contrast, macrophages in human carotid artery plaque, stained as control, were identified by CD68 positivity and were also PPARγ positive. In situ hybridization of rat kidneys showed PPARγ mRNA expression with the same distribution pattern as PPARγ protein (Fig. 10 B, D, F).

DISCUSSION

Progressive deterioration of the kidney is common to many renal diseases. The structural injury that leads to this progressive loss of function consists of glomerulosclerosis and tubulointerstitial fibrosis and atrophy. Many intervention strategies have been explored to slow down or even reverse the progression of glomerulosclerosis [40, 41]. In this study, to our knowledge for the first time, we have demonstrated that PPARγ agonist alone can ameliorate the progression of glomerulosclerosis in a nondiabetic, hypertensive glomerulosclerosis.

Many studies have shown efficacy of TZD class agents in diabetic vascular and renal lesions, linked to improved glucose and lipid metabolism [7, 28, 32, 33]. However, so far, studies have not examined PPARγ agonist effects on nondiabetic glomerulosclerosis. The in vitro evidence showing promotion of foamy macrophage transformation by TGL suggested that this class of drugs might be a dual-edged sword with underlying adverse effects to promote atherosclerosis. Our current study shows the efficacy of PPARγ agonist to ameliorate the progression of glomerulosclerosis. The effect is independent of insulin effects and could only be partially due to lipid effects, as TGL treatment eliminated the significant but small increase in cholesterol observed in the TRX group, and plasma TG levels remained normal in all groups of rats. Although blood pressure is an important factor associated with progression [42], in this study, we observed renoprotection with TGL that was independent of effects

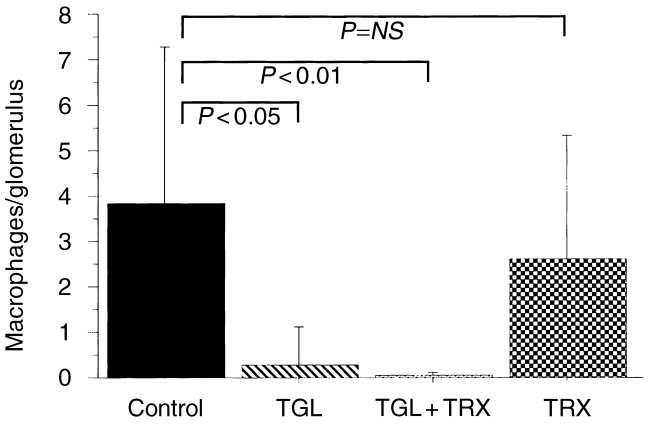

Fig. 9. Modulation of remnant glomerular macrophages. Treatment with TGL alone or TGL + TRX significantly decreased glomerular macrophage infiltration versus CONT.

on systemic hypertension. This indicates that additional nonhemodynamic factors contribute to sclerosis, as we have extensively investigated previously [43–46].

These renal protective effects of PPARγ agonist suggest that PPARγ agonists may provide a novel intervention strategy to prevent vascular and glomerular sclerosis. We hypothesize that the multiplicity of PPARγ actions may be due to its effects on several transcriptional factors. These effects include cell cycle regulation, decreased PAI-1 and TGF-β, and decreased macrophage infiltration.

Abnormal cell growth with associated increased matrix accumulation is one of the important factors contributing to the development of glomerulosclerosis. Cell proliferation versus hypertrophy is controlled at the nuclear level by cyclin-dependent kinases (CDKs) and cyclin kinase inhibitors (CKIs) [47]. The latter inhibit cell proliferation by causing cell cycle arrest. $p21^{CIP1/WAF1}$ and $p27^{kip1}$ belong to the CIP/Kip family of CKIs [48].

One of the possible mechanisms for the beneficial effects of PPARγ agonist on sclerosis is the observed inhibition of increased glomerular cell proliferation, which was associated with a low-level expression of p21 and p27. Previous studies have suggested a role for PPARγ receptors in the control of cell proliferation, especially in vascular smooth muscle cell (VSMC) proliferation and migration, which are critical events in the development of atherosclerosis and restenosis [49–51]. In the aortic balloon injury model in rats, TGL resulted in a significantly less neointimal/media area ratio compared with control [52], which may relate to PPARγ inhibition of early inflammatory/proliferation responses.

Recent studies have shown that mesangial cell proliferation in the Thy-1 experimental glomerulonephritis model is associated with decreased p27 levels; the resolution of mesangial cell proliferation was associated with a return to baseline levels for p27 and sustained increase for p21 [53]. Immune-mediated injury in the passive Heymann nephritis model of membranous nephropathy is associated with an increase in p21 and p27 levels, which coincide with little if any proliferation [54]. However, p21 has divergent functions. p21 can act as an assembly factor of cdk4/cyclin D complexes or as an inhibitor of CDK/cyclin [55, 56]. In mesangial cells in vitro, platelet-derived growth factor (PDGF) causes a marked increase of p21 protein [57]. The induction of p21 protein by PDGF was also reported in p53-deficient as well as in normal mouse fibroblasts, suggesting p53-independent up-regulation of p21 by PDGF. Megyesi et al has recently shown that lack of a functional p21 gene ameliorates progression to chronic renal failure [58]. They speculated that p21 regulates the balance between hyperplasia and hypertrophy after renal ablation, promoting hypertrophy and sclerosis. We found highly expressed p21 and p27 in the glomeruli of untreated 5/6 Nx rats, thus suggesting that there is active ongoing hypertrophy in these sclerotic kidneys. The decreased p21 mRNA expression and p27 protein in response to TGL indicate that these hypertrophic, prosclerotic mechanisms were dampened by TGL. Our results thus support a role for TGL, via PPARγ activation, on cell cycle events and balance of proliferation and hypertrophy.

Our study also showed that amelioration of glomerulosclerosis by PPARγ agonist was linked to down-regulated PAI-1 expression. PAI-1, a member of the superfamily of serine protease inhibitors, is a major physiological inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). These PAs activate plasminogen to plasmin and promote fibrinolysis as well as proteolysis [59, 60]. Up-regulation of PAI-1 can thus inhibit proteolysis of extracellular matrix, leading to matrix accumulation and sclerosis. PAI-1 is linked to vascular sclerosis in both animal models and humans and is induced by angiotensin [40, 41]. PAI-1 is also increased in plasma of patients with insulin resistance and is correlated with the severity of atherosclerosis in such patients [61, 62]. In vitro, it was shown that TZDs decreased basal and tumor necrosis factor-α–stimulated PAI-1 expression in human umbilical vein endothelial cells [27]. Recent data suggest that PPARγ agonist inhibited gene transcription by antagonizing the activities of the transcription factors (TFs) activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [63]. The PAI-1 promoter contains motifs for both these TFs [64, 65]. Thus, we speculate that down-regulation of PAI-1 mRNA expression may be mediated via a PPARγ effect on AP-1 and/or NF-κB–mediated transcriptional activity of PAI-1.

Transforming growth factor-β is one of the key factors contributing to extracellular matrix accumulation and glomerulosclerosis [66], and its inhibition can decrease

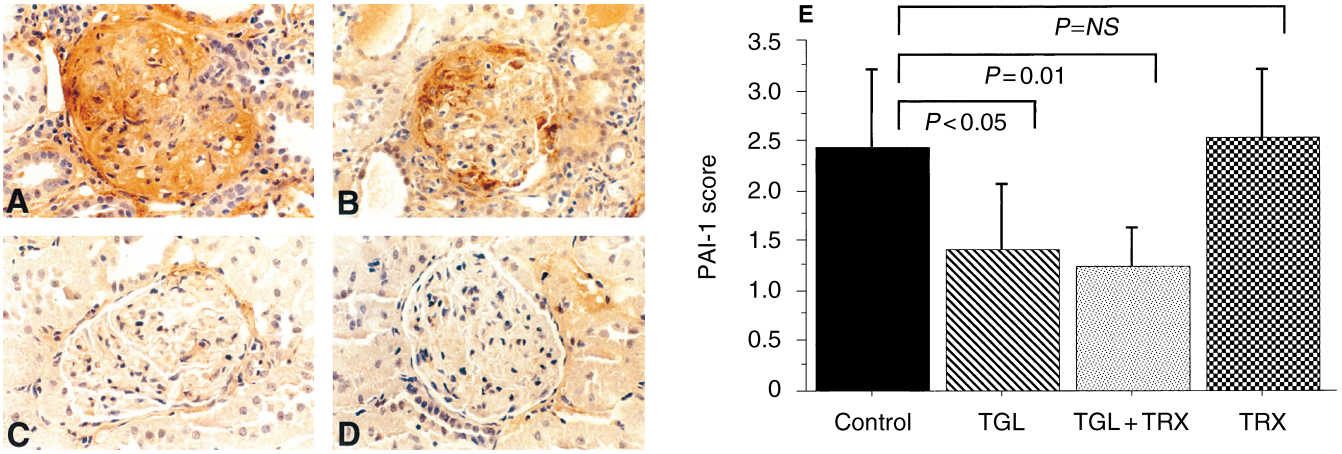

Fig. 7. PAI-1 expression detected by immunostaining. TGL alone or TGL + TRX treatment for 12 weeks significantly reduced glomerular PAI-1 expression versus control (*E*). PAI-1 was highly expressed in glomeruli in CONT (*A*) and in TRX (*B*), associated with sclerotic lesions; PAI-1 protein was only weakly expressed in some glomerular parietal epithelial cells in TGL group (*C*). There was only trace PAI-1 expression in glomeruli in the TGL + TRX (*D*) group.

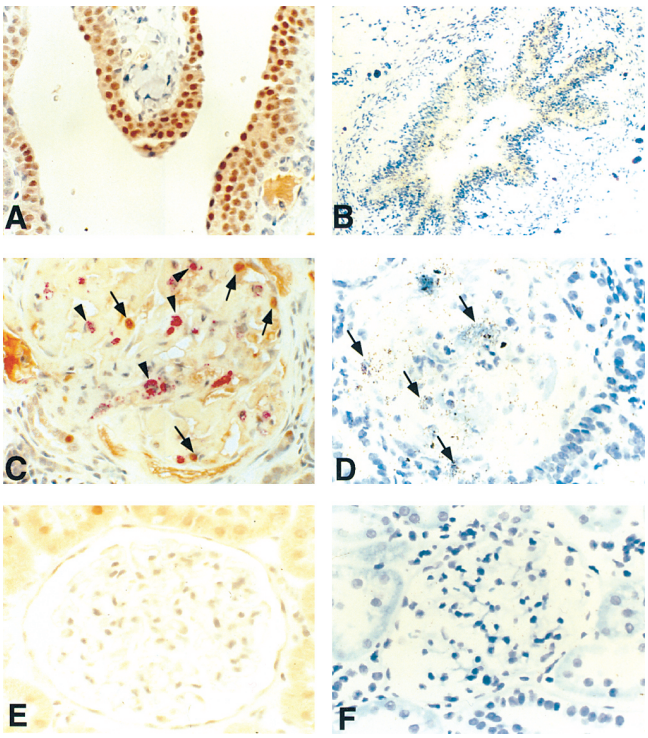

Fig. 10. Detection of PPARγ and macrophages. Transitional epithelial cells of pelvis, which express PPARγ, served as a positive control (*A*). Carotid artery plaque, another positive control, showed PPARγ-positive, CD68-positive macrophages (data not shown). Double immunostaining showed that glomerular infiltrating macrophages (*C*, red, arrowhead) did not express PPARγ, although glomerular cells did show increased PPARγ expression (C, brown, arrow). There was no detectable PPARγ in glomeruli in TGL rats (*E*). In situ hybridization showed that PPARγ mRNA expression had similar distribution as PPARγ receptor protein expression [(*D*), CONT and (*F*), TGL]. Ureteral epithelium, which expresses PPARγ, served as a positive control (*B*).

matrix accumulation [67]. In this study, we observed up-regulated TGF-β mRNA in CONT rats, and TGL diminished this TGF-β mRNA expression. These results suggest that beneficial effects of TGL on sclerosis may occur through inhibition of both PAI-1 and TGF-β, thus augmenting matrix turnover and decreasing matrix synthesis.

An additional finding in this study was the lack of PPARγ expression in macrophages infiltrating glomeruli. Monocytes and macrophages play an important role in immune and nonimmune chronic sclerosing renal diseases [68–70]. Activated macrophages are a rich source of growth factors, cytokines, vasoactive substances, proteolytic enzymes, and reactive oxygen species (ROS) [71]. Decreased ROS-related injury caused by decreased macrophages may thus have contributed to amelioration of sclerosis by TGL. Additionally, the antioxidant moiety of TGL may have decreased oxidant injury directly [72, 73].

These infiltrating macrophages did not express PPARγ, although glomerular-resident cells (glomerular visceral, parietal epithelial cells, and mesangial cells) did show increased PPARγ expression in the untreated 5/6 Nx rats. Our results indicate that there is a phenotypic diversity of macrophages in glomerular versus vascular sclerosis. Macrophages in human atherosclerotic carotid artery plaques did express PPARγ and are thought to promote foam cell transformation and plaque formation [74]. Because glomerular resident cells contribute actively to glomerulosclerosis [41], our results also suggest that glomerular intrinsic cells are a target of PPARγ agonist. PPARγ expressed in these glomerular cells may act to counterbalance the existing overwhelming proinflammatory and prosclerotic effects. A reduction of glomerular macrophages by TGL treatment in our study could reflect

TGL's effects in inflammation regulation. Possible mechanisms include TGL inhibition of expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, resulting in significantly reduced monocyte/macrophage homing to atherosclerotic plaques [75]. PPARγ ligands also inhibited the inflammatory response and reduced colonic epithelial injury in a mouse model of colitis with PPARγ expression primarily in the colonic epithelium [76]. Interestingly, in our study, glomerular PPARγ was undetectable by in situ hybridization in TGL-treated animals. We speculate that this finding could represent ligand-mediated receptor down-regulation, which has been demonstrated for other cytoplasmic and nuclear receptors [77, 78].

In summary, this study shows that PPARγ activation in vivo in a nondiabetic, glomerulosclerotic model leads to beneficial effects, disproving the hypothesis suggested by in vitro studies that PPARγ activation promoting macrophage foam cell transformation results in increased vascular sclerosis. PPARγ's beneficial effects appear to be related to the regulation of glomerular cell proliferation and hypertrophy, decreased macrophage infiltration, and decreased PAI-1 and TGF-β and are not mediated by improving insulin sensitivity. Our results further indicate that there is a phenotypic diversity of macrophages in glomerular versus vascular sclerosis, with PPARγ expression in macrophages occurring only in the latter. We speculate that amelioration of development of glomerulosclerosis could be mediated via a PPARγ effect on AP-1 and/or NF-κB–mediated transcriptional activity of PAI-1. Our results also suggest that PPARγ agonists will provide a novel approach with therapeutic potential in nondiabetic glomerulosclerosis.

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Reprint requests to Agnes B. Fogo, M.D., MCN C3310, Department of Pathology, Vanderbilt University Medical Center, 21st and Garland Avenue, Nashville, Tennessee 37232-2561, USA.
E-mail: agnes.fogo@mcmail.vanderbilt.edu

APPENDIX

Abbreviations used in this study are: AP-1, activated protein-1; CDK, cyclin-dependent kinase; CKI, cyclin kinase inhibitor; CONT, control; DAB, diaminobenzidine; 15-deoxy PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; FFAs, free fatty acids; HUVEC, human umblical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; LDL, low density lipoprotein; NF-κB, nuclear factor-κB; NSAIDs, nonsteroidal anti-inflammatory drugs; Nx, nephrectomy; PAI-1, plasminogen activator inhibitor-1; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PPARγ, peroxisome proliferator-activated receptor-γ; PPREs, peroxisome proliferator response elements; RXR, retinoic X receptor; SI, sclerosis index; SPB, systolic blood pressure; TF, transcription factor; TGF-β, transforming growth factor-β; TGL, troglitazone group; TNF-α, tumor necrosis factor-α; t-PA tissue-type plasminogen activator; TRX, triple antihypertensive drug therapy group; TZDs, thiazolidinediones; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cell; u-PA, urokinase-type plasminogen activator.

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