PPAR- γ agonist attenuates renal interstitial fibrosis and inflammation through reduction of TGF- β

Toru Kawai¹, Takao Masaki², Shigehiro Doi¹, Tetsuji Arakawa¹, Yukio Yokoyama¹, Toshiki Doi¹, Nobuoki Kohno¹ and Noriaki Yorioka²

Thiazolidinediones (TZDs), synthetic peroxisome proliferator-activated receptor (PPAR)- γ ligands, have a central role in insulin sensitization and adipogenesis. It has been reported that TZDs exert protective effects in both diabetic and nondiabetic models of renal disease, although the exact mechanism is not well understood. In particular, only a few studies have reported the renoprotective effects of TZDs in nondiabetic models of tubulointerstitial fibrosis and inflammation. Therefore, we investigated the anti-fibrotic and anti-inflammatory effects of the TZD troglitazone in the mouse model of unilateral ureteral obstruction (UUO). C57BL/6J mice underwent UUO and were studied after 3 and 7 days. Animals were divided into three groups and received control vehicle, troglitazone (150 mg/kg per day) or troglitazone (300 mg/kg per day) by gavage. Kidneys were harvested for morphological, mRNA and protein analysis. Reverse-transcriptase-PCR was used to assess the expression of transforming growth factor- β 1 (TGF- β 1) and the TGF- β 1 type I receptor (TGF β R-I). Protein expression was assessed by western blotting (TGF β R-I) and immunostaining (TGF β R-I, α -smooth muscle actin (α -SMA), type I collagen (collagen I), F4/80, and proliferating cell nuclear antigen (PCNA)). The expression of α -SMA, collagen I, and F4/80 was decreased in mice treated with troglitazone compared with the control group. The numbers of PCNA-positive interstitial cells were decreased in mice treated with troglitazone. TGF- β 1 mRNA and TGF β R-I mRNA and protein expression were decreased in the group treated with troglitazone compared with the control group. The beneficial effects of troglitazone treatment were also dose dependent. PPAR- γ agonist significantly reduced TGF- β and attenuated renal interstitial fibrosis and inflammation in the model of UUO.

Laboratory Investigation (2009) **89**, 47–58; doi:10.1038/labinvest.2008.104; published online 10 November 2008

KEYWORDS: PPAR- γ ; thiazolidinedione; fibrosis; inflammation; UUO

Progressive interstitial fibrosis represents a common final pathway of nearly all forms of chronic kidney disease.^{1,2} Following unilateral ureteral obstruction (UUO), renal tubular cell injury results from mechanical stretch, ischemia, hypoxia, or exposure to toxins such as oxygen free radicals. As a consequence, renal tubular epithelial cells may either undergo cell death by apoptosis or necrosis or undergo a phenotypic transformation and acquire mesenchymal characteristics.^{3,4} Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a ligand-dependent transcription factor involved in the production of profibrotic and proinflammatory mediators. However, the role of PPAR- γ in the inhibition of fibrosis *in vivo* has not been described.

PPAR- γ belongs to the nuclear hormone receptor superfamily.⁵ PPAR- γ is selectively expressed in medullary

collecting ducts, glomeruli, and proximal tubular cells.^{6–8} PPAR- γ has a central role in regulating insulin sensitivity, adipocyte differentiation, cell growth, and inflammation.⁹ Thiazolidinediones (TZDs) are synthetic PPAR- γ agonists and are widely used as insulin-sensitizing agents in patients with type 2 diabetes. The beneficial effects of TZD treatment include blood pressure lowering, triglyceride reduction and high-density lipoprotein-cholesterol elevation as well as improving glycemic control.^{10,11}

There are several clinical studies demonstrating a beneficial effect in patients with type 2 diabetes treated with TZDs, with patients exhibiting a reduction in albuminuria.^{12,13} In addition, a beneficial effect on urine albumin or protein excretion has been reported in animal models of insulin resistance, diabetes, and hypertension following treatment with TZDs^{14,15} with these

¹Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan and ²Department of Advanced Nephrology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Correspondence: Dr T Masaki, MD, Department of Advanced Nephrology, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City 734-8551, Japan.

E-mail: masaki@hiroshima-u.ac.jp

Received 22 April 2008; revised 18 September 2008; accepted 19 September 2008

benefits appearing to be independent of glycemic control. The renoprotective benefit of TZDs is further suggested by studies in nondiabetic models of renal injury. For example, activation of PPAR- γ reduced glomerulosclerosis and apoptosis in various models including the 5/6 nephrectomy model, passive Heymann nephritis model, crescentic glomerulonephritis model, acute mesangial proliferative glomerulonephritis model, and renal ischemia-reperfusion injury model.^{16–21} *In vitro* studies have focused on the effect on mesangial cells, where PPAR- γ activation exerts antiproliferative and antifibrotic effects^{22,23} via the modulation of transforming growth factor- β 1 (TGF- β 1)-mediated pathways.²⁴ TZDs also reduced the secretion of macrophage chemotactic protein-1 (MCP-1), a pro-inflammatory chemokine, in human HK-2 cells exposed to high concentrations of glucose.⁷ However, there are few studies of the renoprotective effect of PPAR- γ agonists in tubulointerstitial fibrosis and inflammation that develops in nondiabetic models, as tubulointerstitial disease is widely accepted as a final common pathway of chronic kidney disease that leads ultimately to end-stage renal failure.

The aim of this study was to evaluate the effect of troglitazone, a specific PPAR- γ ligand, on tubulointerstitial fibrosis and inflammation in a model of UUO.

MATERIALS AND METHODS

Experimental Model and Administration of Drug Treatment

Total of 56 female C57BL/6J mice weighing 20–25 g were obtained from Charles River Laboratories Japan (Yokohama, Japan). The animals were housed in the animal facility of Hiroshima University with free access to food and water. The Institutional Animal Care and Use Committee at Hiroshima University (Hiroshima, Japan) approved all the animal protocols and the experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Troglitazone was kindly provided by Sankyo Daiichi Co. Ltd. (Tokyo, Japan). Troglitazone was prepared as a suspension in distilled water and administered by oral gavage (0.1 ml per mouse) at a dose of either 150 or 300 mg/kg per day twice per day. The control group was administered an equal volume of distilled water by gavage. In all groups, medication was started 7 days before surgery and continued until sacrifice. The last treatment was administered 3 h before sacrifice. Surgery was performed under general anesthesia induced by ketamine and xylazine as described previously.²⁵ The abdomen was opened using a midline incision and the left ureter was located and ligated twice with 4-0 nylon suture. Groups of eight mice were sacrificed on days 3 and 7 after UUO and renal tissue harvested.²⁶

RNA Extraction and Quantitative Real-Time Reverse Transcriptase-PCR (qRT-PCR)

Total RNA was extracted from murine renal tissue using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Equal

amounts (2 μ g) of total RNA from each sample were converted to cDNA using moloney murine leukemia virus RT RnaseH with oligo dT20 primer in a 20 μ l reaction volume. Quantitative real-time RT-PCR analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Gene-specific oligonucleotide primers and probes for TGF- β 1 (assay ID: Mm03024053_m1) and its type I receptor (TGF β R-I) (assay ID: Mm03024015_m1) as well as the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID: Mm99999915_g1) were obtained as TaqMan® Gene Expression Assays (Applied Biosystems). Amplification was performed with TaqMan® universal PCR master mix and universal cycling conditions (Applied Biosystems). Data analysis was performed using 7500 Fast System Sequence Detection Software version 1.4 (Applied Biosystems). The results of TGF- β 1 and TGF β R-I were normalized to the expression level of GAPDH.

Antibodies

The following primary antibodies were used in the study: mouse monoclonal FITC-conjugated anti- α -smooth muscle actin (α -SMA) antibody (Sigma-Aldrich, St Louis, MO, USA), mouse monoclonal anti- α -tubulin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rat monoclonal anti-F4/80 antigen antibody (Serotec, Kidlington, UK), rabbit polyclonal anti-collagen I (Abcam, Cambridge, UK), antiproliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology Inc.), and anti-TGF β R-I, antibodies (Santa Cruz Biotechnology Inc.). Immunostaining with α -SMA, collagen I, F4/80, and TGF β R-I was used to identify myofibroblasts, collagen I, infiltrating macrophages, and TGF β R-I expression, respectively.

The following secondary polyclonal antibodies were used in the study: goat anti-mouse immunoglobulin (IgG) conjugated with HRP (Dako Cytomation, Glostrup, Denmark), biotinylated goat anti-rabbit immunoglobulin (IgG, H + L) (Zymed, South San Francisco, CA, USA), and biotinylated rabbit anti-rat immunoglobulin (IgG, H + L) (Zymed).

Western Blot Analysis

For detection of TGF β R-I, one-third of a kidney was homogenized on ice in 1 ml of lysis buffer (Cell Signaling Technology) and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15,000 g for 20 min to remove tissue debris and the supernatant was divided into aliquots and stored at -80°C . The protein content of cell lysates was determined by a BCA protein assay (Pierce, Rockford, IL, USA). Western blotting was performed as described previously.^{27,28}

The blots were incubated overnight at 4°C with anti-TGF β R-I antibody (1:1000 dilution) followed by washing and incubation with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution). Monoclonal anti- α tubulin antibody was used as the internal control for the analysis of TGF β R-I expression. The intensity of each band was estimated using

National Institutes of Health Image software (NIH, Bethesda, MD, USA; version 1.6).

Immunohistochemistry

Immunohistochemical staining was performed on tissues fixed in 10% formalin and embedded in paraffin, as described previously.²¹ The direct method was used for the detection of α -SMA and the ABC method was used for the detection of collagen I, F4/80, PCNA, and TGF β R-I. For α -SMA staining, sections were blocked in 5% bovine serum albumin (BSA) and 10% normal sheep serum in phosphate-buffered saline (PBS) for 60 min and then incubated overnight at 4°C with the primary antibody diluted in 10% normal sheep and 5% normal mouse serum in PBS. After washing, endogenous peroxidase was blocked by incubation in 0.6% H₂O₂ in methanol for 20 min. Sections were incubated sequentially with peroxidase-labeled anti-fluorescein (Vector Laboratories, Burlingame, CA, USA) for 45 min. Sections were developed with diaminobenzidine (Sigma) to give a brown color or with Vector SG (Vector Laboratories) to give a blue/gray color. Tissue sections requiring antigen retrieval treatment were placed in 0.01 M citrate buffer, pH 6.0, and heated for 15 min in a microwave oven. This treatment was used for detection of collagen I, F4/80, PCNA, and TGF β R-I. For collagen I, PCNA, and TGF β R-I (rabbit polyclonal antibodies) or F4/80 (rat monoclonal antibody) staining, sections were blocked in 5% BSA and 10% normal sheep or rabbit serum in PBS for 60 min, and then incubated overnight at 4°C with the primary antibody in 10% normal sheep or rabbit serum and 5% normal mouse serum in PBS. After washing, endogenous peroxidase was blocked by incubation in 0.6% H₂O₂ in methanol for 20 min. Sections were blocked in Avidin–Biotin blocking kit (Vector Laboratories) and then incubated sequentially with biotinylated goat anti-rabbit or rabbit anti-rat IgG for 45 min followed by incubation with the Vectastain Elite kit (Vector Laboratories). Sections were developed with diaminobenzidine to give a brown color.

Two color immunostaining was used to detect colocalization of α -SMA and PCNA. After staining for α -SMA followed by developing with Vector SG to give a blue/gray color, sections were placed in 0.01 M citrate buffer, pH 6.0, and heated for 15 min in a microwave oven. Sections were then blocked as above and incubated overnight at 4°C with the polyclonal anti-PCNA antibody. Sections were then washed and blocked in Avidin–Biotin blocking kit and then incubated sequentially with biotinylated goat anti-rabbit IgG for 45 min, followed by incubation with the Vectastain Elite kit and then developed with diaminobenzidine to give a brown color.²⁹

Quantification of Immunohistochemical Staining

The area of α -SMA, collagen I, and F4/80-positive staining was assessed in predetermined high power fields ($\times 200$) of the cortex (eight fields) and the corticomedullary junction (eight fields) that were captured by a digital camera and the

area stained determined using Lumina Vision 2.20 (Mitani Corporation, Osaka, Japan). Immunostaining for PCNA was quantified by examination of predetermined high power fields ($\times 400$) of the cortex (10 fields) and the corticomedullary junction (10 fields). Scoring was performed using an eye-piece graticule and the number of PCNA-stained interstitial cells was expressed as cells per 0.5 mm².

Measurement of Plasma Troglitazone and Blood Sugar Levels

Blood samples were taken by cardiac puncture before harvesting tissue samples. The samples were centrifuged at 3000 g for 10 min at 4°C and supernatants were aliquoted and stored at –80°C. The troglitazone levels before UUO and at day 7 of the UUO model were measured by high-performance liquid chromatography at Daiichi Sankyo RD Associate Co. Ltd. (Tokyo, Japan). The lower limit of quantification is 0.2 μ g/ml. In addition, the blood sugar levels of mice were measured using a Hitachi 7180 Automatic Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan) at SRL Inc. (Tokyo, Japan).

Statistical Analysis

Results are expressed as means $\pm$ s.e. for each group of eight mice. Statistical analysis was performed with ANOVA followed by Tukey's *post hoc* test unless otherwise stated. Data differences were deemed significant at $P < 0.05$.

RESULTS

Troglitazone Suppressed the Interstitial Expression of α -SMA in UUO

The expression of α -SMA is the molecular hallmark of myofibroblasts, with the localization and quantification of myofibroblasts denoting the intensity of the fibrogenic response after UUO. α -SMA was expressed in the smooth muscle cells of renal arterioles but was rarely evident in the renal interstitium of normal kidneys (Figure 1A-a). Although ureteral obstruction resulted in a gradual though striking increase in the interstitial expression of α -SMA (Figure 1A-b and e), troglitazone treatment resulted in a reduction in interstitial α -SMA expression (Figure 1A-c, d, f and g). Quantification of immunostaining in renal tissue is shown in Figure 1B and indicates the marked increase in the α -SMA-positive area in the experimental control group after UUO. Treatment with troglitazone significantly reduced interstitial expression of α -SMA in the treatment groups at both 3 and 7 days after UUO and these effects were dose dependent.

Troglitazone Suppressed the Interstitial Expression of Collagen I in UUO

The process of tubulointerstitial fibrosis involves the loss of renal tubules and the accumulation of extracellular matrix (ECM) proteins. Collagen I is one of these ECM proteins and in this study we showed collagen I was expressed in the renal arterioles, but was rarely evident in the renal interstitium of

Figure 1 Immunohistochemical staining for α -SMA. (A) Representative photomicrographs of immunohistochemical staining for α -SMA (arrows) in the cortex of the control group before UUO (a), 3 days after UUO in the control group (b), 3 days after UUO in the group receiving troglitazone 150 mg/kg per day (c), 3 days after UUO in the group receiving troglitazone 300 mg/kg per day (d), 7 days after UUO in the control group (e), 7 days after UUO of the group receiving troglitazone 150 mg/kg per day (f), and 7 days after UUO in the group receiving troglitazone 300 mg/kg per day (g). (B) Graphical depiction of the α -SMA-positive area of the cortex and the corticomedullary junction before and after UUO in the control group (white bars), the group receiving troglitazone 150 mg/kg per day (gray bars) and the group receiving troglitazone 300 mg/kg per day (black bars). Values are expressed as the mean $\pm$ s.e. of seven groups of animals (8 mice per group). Magnification: $\times 200$. * $P < 0.05$ and ** $P < 0.01$ by ANOVA with Tukey's *post hoc* test.

normal kidneys (Figure 2A-a). Although ureteral obstruction resulted in a gradual but striking increase in the interstitial expression of collagen I (Figure 2A-b and e), troglitazone

treatment resulted in a reduction in interstitial collagen I expression (Figure 2A-c, d, f and g). Quantification of immunostaining in renal tissue is shown in Figure 2B, which

Figure 2 Immunohistochemical staining for collagen I. **(A)** Representative photomicrographs of immunohistochemical staining for collagen I (arrows) in the cortex of the control group before UUO (**a**), 3 days after UUO in the control group (**b**), 3 days after UUO in the group receiving troglitazone 150 mg/kg per day (**c**), 3 days after UUO in the group receiving troglitazone 300 mg/kg per day (**d**), 7 days after UUO in the control group (**e**), 7 days after UUO in the group receiving troglitazone 150 mg/kg per day (**f**), and 7 days after UUO in the group receiving troglitazone 300 mg/kg per day (**g**). **(B)** Graphical depiction of the collagen I-positive area of the cortex and the corticomedullary junction before and after UUO in the control group (white bars), the group receiving troglitazone 150 mg/kg per day (hatched bars) and the group receiving troglitazone 300 mg/kg per day (black bars). Values are expressed as the mean $\pm$ s.e. of seven groups of animals (8 mice per group). Magnification: $\times 200$. ****P < 0.01** by ANOVA with Tukey's *post hoc* test.

Figure 3 Immunohistochemical staining for F4/80. **(A)** Representative photomicrographs of immunohistochemical staining for F4/80 (arrows) in the cortex of the control group before UUO (**a**), 3 days after UUO in the control group (**b**), 3 days after UUO in the group receiving troglitazone 150 mg/kg/day (**c**), 3 days after UUO in the group receiving troglitazone 300 mg/kg per day (**d**), 7 days after UUO in the control group (**e**), 7 days after UUO of the group receiving troglitazone 150 mg/kg per day (**f**), and 7 days after UUO in the group receiving troglitazone 300 mg/kg per day (**g**). **(B)** Graphical depiction of the F4/80-positive area of the cortex and the corticomedullary junction before and after UUO in the control group (white bars), the group receiving troglitazone 150 mg/kg per day (gray bars) and the group receiving troglitazone 300 mg/kg per day (black bars). Values are expressed as the mean $\pm$ s.e. of seven groups of animals (8 mice per group). Magnification: $\times 200$. ** $P < 0.01$ by ANOVA with Tukey's *post hoc* test.

demonstrates the marked increase in the collagen I-positive area in the experimental control group after UUO. Treatment with troglitazone significantly reduced interstitial expression of collagen I in the treatment groups at both 3 and 7 days after UUO and these effects were dose dependent.

The Effect of Troglitazone on Renal Inflammation after UUO

One of the early events in the development of tubulointerstitial fibrosis is the recruitment of inflammatory cells. Immunohistochemical staining for macrophages using

Figure 4 (A) Representative photomicrographs of the immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in the cortex of the control group before UUO (a), 3 days after UUO in the control group (b), 3 days after UUO in the group receiving troglitazone 150 mg/kg per day (c), 3 days after UUO in the group receiving troglitazone 300 mg/kg per day (d), 7 days after UUO in the control group (e), 7 days after UUO in the group receiving troglitazone 150 mg/kg per day (f), and 7 days after UUO in the group receiving troglitazone 300 mg/kg per day (g). Arrows show PCNA-positive interstitial cells. (B) Quantification of interstitial cell proliferation in obstructed mouse kidney. Graphical presentation showing the number of PCNA-positive interstitial cells in the control group (white bars), the group receiving troglitazone 150 mg/kg per day (gray bars) and the group receiving troglitazone 300 mg/kg per day (black bars). Values are expressed as the mean $\pm$ s.e. of seven groups of animals (8 mice per group). $**P < 0.01$ by ANOVA with Tukey's *post hoc* test. (C) Localization of PCNA in obstructed mouse kidney using two color immunohistochemistry. Day 7 following UUO indicates PCNA staining (brown) of interstitial α -SMA (blue/gray)-positive cells. Magnification: $\times 200$.

Figure 5 (a) Induction of TGF- β 1 mRNA in mouse kidney. Graph depicting the relative mRNA levels of TGF- β 1 normalized to GAPDH before and after UUO. Values are expressed as the mean $\pm$ s.e. of the seven groups of animals (8 mice per group). * P < 0.05 and ** P < 0.01 by ANOVA with Tukey's *post hoc* test. (b) Induction of TGF β type I receptor (TGF β R-I) mRNA in mouse kidney. Graph depicting the relative mRNA levels of TGF β R-I normalized to GAPDH before and after UUO. Values are expressed as the mean $\pm$ s.e. of the seven groups of animals (8 mice per group). * P < 0.05 and ** P < 0.01 by ANOVA with Tukey's *post hoc* test.

Figure 6 TGF- β type I receptor (TGF β R-I) expression in lysates of mouse kidneys. The upper panels were probed with antibody recognizing the TGF β R-I. The lower panels were probed with antibody recognizing α tubulin, with α tubulin expression used as an internal control. Densitometric analysis presented as the ratio of TGF β R-I to α tubulin expression. Values are presented as the mean $\pm$ s.e. of seven groups of animals (8 mice per group). * P < 0.05 and ** P < 0.01 by ANOVA with Tukey's *post hoc* test.

anti-F4/80 antibody confirmed these findings. F4/80-positive macrophages were present in both normal (Figure 3A-a) and control kidneys after UUO (Figure 3A-b and e). F4/80-positive macrophages increased gradually in control kidneys after UUO with troglitazone treatment resulting in reduced

interstitial F4/80 expression (Figure 3A-c, d, f and g). Quantification of immunostaining is shown in Figure 3B and indicates the increase in F4/80-positive staining in the experimental control group after UUO. Treatment with troglitazone significantly reduced interstitial F4/80 expression in the treatment groups at 3 and 7 days after UUO with the inhibitory effect being dose dependent.

Troglitazone Inhibits the Proliferation of Interstitial Cells after UUO

Control kidneys demonstrated only scant proliferation of both tubular and interstitial cells (Figure 4A-a). However, UUO resulted in the induction of marked proliferation of both tubular and interstitial cells (Figure 4A-b and e). PCNA-positive interstitial cells increased gradually in control kidneys after UUO, with troglitazone treatment resulting in reduced interstitial PCNA expression (Figure 4A-c, d, f and g). However, troglitazone treatment did not reduce the number of PCNA-positive tubular cells. Quantification of PCNA immunostaining in renal tissue is shown in Figure 4B and indicates a marked increase in the number of PCNA-positive interstitial cells in the experimental control group after UUO. Treatment with troglitazone significantly inhibited the proliferation of interstitial cells in the treatment groups at 3 and 7 days after UUO with the antiproliferative effects of troglitazone treatment being dose dependent. We also performed double staining of α -SMA and PCNA in control mice at 7 days after UUO in order to determine the proliferation of myofibroblasts (Figure 4C) and this demonstrated the presence of proliferating myofibroblasts after UUO.

Figure 7 Representative micrographs showed the expression and localization of TGF β 1. Immunohistochemical staining for TGF β 1 (arrows) in the cortex before UUO in the control group (a), 7 days after UUO in the control group (b), 7 days after UUO in the group receiving troglitazone 150 mg/kg per day (c), and 7 days after UUO in the group receiving troglitazone 300 mg/kg per day (d). Magnification: $\times 200$.

Table 1 Plasma levels of troglitazone and blood sugar

UUO (day)	Troglitazone (mg/kg per day)	Drug level (μ g/ml)	Blood sugar (mg/dl)
Before	0	LLOQ	173.38 $\pm$ 10.32
7	0	LLOQ	180.50 $\pm$ 6.97
7	150	0.388 $\pm$ 0.07	190.88 $\pm$ 11.13
7	300	0.562 $\pm$ 0.05	169.75 $\pm$ 5.49

The lower limit of quantification (LLOQ) is 0.2 μ g/ml.

Troglitazone Inhibits the Expression of TGF- β 1 and the TGF- β 1 Type I Receptor

We evaluated the effect of troglitazone on TGF- β 1 mRNA, TGF β R-I mRNA, and TGF β R-I protein expression using real-time RT-PCR, western blotting, and immunostaining. The expression of TGF- β 1 mRNA in the experimental control group gradually increased after UUO. In contrast, troglita-

zone treatment significantly inhibited TGF- β 1 mRNA expression in the treatment groups at 3 and 7 days after UUO. The effects of troglitazone treatment on the expression of TGF- β 1 mRNA were dose dependent (Figure 5a). Expression of TGF β R-I mRNA in the experimental control group increased gradually after UUO. Troglitazone treatment significantly inhibited the upregulation of TGF β R-I mRNA

expression in the treatment groups at both 3 and 7 days after UUO in a dose dependent manner (Figure 5b). Western blotting analysis identified TGF β R-I and α tubulin in lysates of kidney tissue. Quantification of TGF β R-I protein expression by densitometric analysis demonstrated increased expression in the control group after UUO, with troglitazone treatment resulting in the significant inhibition of TGF β R-I protein expression in the treatment groups at 3 and 7 days after UUO. The effects of troglitazone treatment on the protein expression of TGF β R-I were also dose dependent (Figure 6). Similar results were obtained when TGF β R-I expression by immunohistochemistry was analyzed (Figure 7). Of note, TGF β R-I expression was localized in the tubular epithelial cells.

Plasma Levels of Troglitazone and Blood Sugar

The mean plasma levels of troglitazone in the control group before UUO and at 7 days after UUO were at the lower limit of quantification, whereas the levels in the treatment groups receiving troglitazone at a dose of 150 or 300 mg/kg per day were 0.388 ± 0.07 and 0.562 ± 0.05 μ g/ml, respectively. The mean blood sugar level in the control group before UUO and at 7 days after UUO was 173.38 ± 10.32 and 180.50 ± 6.97 mg/dl, respectively. The mean blood sugar level in the treatment groups receiving troglitazone at a dose of 150 or 300 mg/kg per day was 190.88 ± 11.13 and 169.75 ± 5.49 mg/dl, respectively. There was no significant difference in the plasma blood sugar levels between the control group and the treatment groups (Table 1).

DISCUSSION

We demonstrated that expression of α -SMA, collagen I, and F4/80 was decreased in the group treated with troglitazone compared with the control group. In addition, the number of PCNA-positive interstitial cells was decreased in mice treated with troglitazone. We also showed that troglitazone inhibited the expression of TGF- β 1 and TGF β R-I in mouse kidneys following UUO. These results indicate that troglitazone can effectively reduce interstitial fibrosis and inflammation in the model of UUO. The plasma troglitazone concentration is reported as a peak level measured after oral and intravenous administration in mice^{30,31} and the dosage used in this study is not excessive in view of the short-term administration of 14 days. However, the troglitazone dosages we used in this study were greater than those used in humans. We chose these dosages as a pilot study had shown they resulted in normal blood glucose levels in the mice, whereas other studies had administered troglitazone at dosages greater than 500 mg/kg per day.^{32,33}

The expression of α -SMA is considered specific for myofibroblasts that may be derived from epithelial mesenchymal transdifferentiation (EMT). Elongated myofibroblasts expressing α -SMA were found in the fibrotic interstitium of obstructed kidneys and these cells may produce interstitial ECM components such as collagen I and fibronectin.³⁴ The increased expression of TGF- β undoubtedly is important in

the induction of EMT and stimulates progressive interstitial fibrosis during the pathogenesis of interstitial nephritis and fibrosis.³⁵ We demonstrated clearly that the expression of α -SMA, collagen I, and PCNA was decreased by treatment with PPAR- γ agonists. Moreover, by using double immunostaining for α -SMA with PCNA, we noted an increased number of double-positive myofibroblasts present within the interstitium. These data thus suggest that PPAR- γ agonists inhibit renal fibrosis in UUO.

We also examined the level of macrophage infiltration in UUO. Interstitial inflammation develops early in the course of obstructive nephropathy as a consequence of upregulation of the rennin-angiotensin system and production of proinflammatory cytokines and chemokines. This results in the interstitial infiltration by activated macrophages that have a crucial role in the renal inflammatory response during UUO. Previous *in vivo* studies demonstrated that PPAR- γ agonists reduced interstitial macrophage infiltration in progressive renal disease in the rat and a model of murine sepsis.^{17,18,36} On the other hand, Panzer reported that MCP-1 expression and monocyte-macrophage infiltration were enhanced during the induction phase in a model of experimental glomerulonephritis.³⁷ These different findings may be attributable to the different times studied after induction of glomerulonephritis. The experimental glomerulonephritis model is a short-term model of the 24 h period following induction, whereas the model used in our study investigated the period of at least 72 h after onset. The time of administration was also different from the experimental glomerulonephritis model. In fact, our study showed that pretreatment of the mouse model for 7 days before surgery was more effective than 3 days of pretreatment. As expected, pretreatment of the mice 3 days before surgery was more effective than mice who received no treatment before surgery. We demonstrated clearly that the interstitial expression of F4/80 and PCNA was decreased by treatment with PPAR- γ agonists. These data suggest that PPAR- γ agonists act to limit the extent of renal inflammation in UUO.

Finally, we examined the cause of renal interstitial fibrosis and inflammation in UUO. Many studies have demonstrated that obstructive nephropathy leads to activation of the intrarenal rennin-angiotensin system. The consequent increase in the level of angiotensin II has crucial direct and indirect roles in the initiation and progression of obstructive nephropathy by stimulating the production of molecules such as TGF- β 1, tumor necrosis factor- α , osteopontin, vascular cell adhesion molecule-1, and nuclear factor- κ B that contribute to renal fibrosis and renal cell apoptosis.³⁸ Of these cytokines, TGF- β 1 and its receptor TGF β R-I have a particularly important role in the progression of renal injury.³⁹ Indeed, Miyajima *et al*⁴⁰ demonstrated previously that a function blocking monoclonal antibody to TGF- β reduced the extent of renal fibrosis and apoptosis in the obstructed kidneys of rats. Moon *et al*³⁵ demonstrated previously that the TGF β R-I kinase activin receptor-like

kinase (ALK)5 inhibitor had protective effects on renal interstitial fibrosis following UUO in rats. In summary, these studies showed that TGF- β is a key player in renal interstitial disease. Also, in other organs, the administration of PPAR- γ agonists suppresses the fibrogenic effects of hepatic stellate cells and lung fibroblasts via effects on TGF- β .^{41,42} Moreover, PPAR- γ agonists can directly decrease glomerular TGF- β expression in both diabetic and nondiabetic model rats independent of its effect on glycemic control.^{19,43} *In vitro* studies by Hong *et al* demonstrated that cytoplasmic phosphorylation of Smad2/3 that act to stimulate TGF- β -dependent nuclear events was unaffected by PPAR- γ agonists, whereas PPAR- γ agonists were found to inhibit Smad2/3 phosphorylation either through direct interaction with TGF β R-I or with nonphosphorylated Smads.⁴⁴ In this study, we demonstrated that a PPAR- γ agonist significantly reduced the expression of both TGF- β 1 and TGF β R-I at 7 days after UUO. Taken together, these data suggest that PPAR- γ agonists inhibit renal interstitial disease in UUO by reducing TGF- β expression with subsequent beneficial effects on fibrosis and inflammation.

Previous studies indicated that the increased renal production of TGF- β 1 and TGF β R-I is mainly in the proximal tubular cells of rats with UUO.⁴⁵ Our studies showed that TGF β R-I was localized predominantly in the tubular epithelial cells and suggested that tubular epithelial cells are the primary targets of PPAR- γ agonists in UUO.

In this study, we used troglitazone as an example of a synthetic PPAR- γ ligand. In addition to our study, several other clinical and *in vivo* studies have demonstrated troglitazone has a renal protective effect.^{12,15,21} Studies have also shown TZDs have the potential to activate a third pathway, mitogen-activated protein kinase, and specifically phosphorylated extracellular signal-regulated kinase, in addition to the PPAR- γ and mitochondrial pathways *in vitro*.^{46–48} It is therefore possible that the renal protective effect of troglitazone we observed in our study may be attributable to factors other than the PPAR- γ pathway.

In conclusion, pretreatment with a PPAR- γ agonist attenuated renal interstitial fibrosis and inflammation in the model of UUO through reduction of TGF- β expression. The decreased interstitial fibrosis results from less severe tubular phenotypical changes, reduced accumulation of interstitial myofibroblasts, and collagen I and downregulation of renal TGF- β 1 expression. These anti-inflammatory effects were accompanied by a decrease in the level of interstitial macrophage infiltration. Therefore, the administration of exogenous TZDs may act to limit the progression of chronic fibrotic kidney disease.

ACKNOWLEDGEMENT

This work was partly supported by the Ryokufukai Research Grant.

1. Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol* 2000;15:290–301.

2. Liu Y. Renal fibrosis: new insights into the pathogenesis and therapeutics. *Kidney Int* 2006;69:213–217.
3. Chevalier RL. Pathogenesis of renal injury in obstructive uropathy. *Curr Opin Pediatr* 2006;18:153–160.
4. Chevalier RL. Specific molecular targeting of renal injury in obstructive nephropathy. *Kidney Int* 2006;70:1200–1201.
5. Moras D, Gronemeyer H. The nuclear receptor ligand-binding domain: structure and function. *Cell Biol* 1998;10:384.
6. Guan Y, Breyer MD. Peroxisome proliferator-activated receptors (PPARs): novel therapeutic targets in renal disease. *Kidney Int* 2001;60:14–30.
7. Panchapakesan U, Pollock CA, Chen XM. The effect of high glucose and PPAR-gamma agonists on PPAR-gamma expression and function in HK-2 cells. *Am J Physiol Renal Physiol* 2004;287:F528–F534.
8. Zafiriou S, Stanners SR, Polhill TS, *et al*. Pioglitazone increases renal tubular cell albumin uptake but limits proinflammatory and fibrotic responses. *Kidney Int* 2004;65:1647–1653.
9. Guan Y, Zhang Y, Breyer MD. The role of PPARs in the transcriptional control of cellular processes. *Drug News Perspect* 2002;15:147–154.
10. Czoski-Murray C, Warren E, Chilcott J, *et al*. Clinical effectiveness and cost-effectiveness of pioglitazone and rosiglitazone in the treatment of type 2 diabetes: a systematic review and economic evaluation. *Health Technol Assess* 2004;8:1–91.
11. Chiquette E, Ramirez G, Defronzo R. A meta-analysis comparing the effect of thiazolidinediones on cardiovascular risk factors. *Arch Intern Med* 2004;19:2097–2104.
12. Imano E, Motomura M, Kanda T, *et al*. Effect of troglitazone on microalbuminuria in patients with incipient diabetic nephropathy. *Diabetes Care* 1998;21:2135–2139.
13. Bakris GL, Ruilope LM, McMorn SO, *et al*. Rosiglitazone reduces microalbuminuria and blood pressure independently of glycemia in type 2 diabetes patients with microalbuminuria. *J Hypertens* 2006;24:2047–2055.
14. Buckingham RE, Al-Barazanji KA, Toseland CD, *et al*. Peroxisome proliferator-activated receptor-gamma agonist, rosiglitazone, protects against nephropathy and pancreatic islet abnormalities in Zucker fatty rats. *Diabetes* 1998;47:1326–1334.
15. Fujiwara K, Hayashi K, Ozawa Y, *et al*. Renal protective effect of troglitazone in Wistar fatty rats. *Metabolism* 2000;49:1361–1364.
16. Ma LJ, Marcantoni C, Linton MF, *et al*. Peroxisome proliferator-activated receptor-gamma agonist troglitazone protects against nondiabetic glomerulosclerosis in rats. *Kidney Int* 2001;59:1899–1910.
17. Benigni A, Zoja C, Tomasoni S, *et al*. Transcriptional regulation of nephrin gene by peroxisome proliferator-activated receptor-gamma agonist: molecular mechanism of the antiproteinuric effect of pioglitazone. *J Am Soc Nephrol* 2006;17:1624–1632.
18. Haraguchi K, Shimura H, Onaya T. Suppression of experimental crescentic glomerulonephritis by peroxisome proliferator-activated receptor (PPAR)gamma activators. *Clin Exp Nephrol* 2003;7:27–32.
19. Westerweel PE, den Ouden K, Nguyen TQ, *et al*. Amelioration of anti-Thy1-glomerulonephritis by PPAR-gamma agonism without increase of endothelial progenitor cell homing. *Am J Physiol Renal Physiol* 2008;294:379–384.
20. Sivarajah A, Chatterjee PK, Patel NS, *et al*. Agonists of peroxisome-proliferator activated receptor-gamma reduce renal ischemia/reperfusion injury. *Am J Nephrol* 2003;23:267–276.
21. Doi S, Masaki T, Arakawa T, *et al*. Protective effects of peroxisome proliferator-activated receptor gamma ligand on apoptosis and hepatocyte growth factor induction in renal ischemia-reperfusion injury. *Transplantation* 2007;84:207–213.
22. Ghosh SS, Gehr TW, Ghosh S, *et al*. PPAR- γ ligand attenuates PDGF-induced mesangial cell proliferation: role of MAP kinase. *Kidney Int* 2003;64:52–62.
23. Routh RE, Johnson JH, McCarthy KJ. Troglitazone suppresses the secretion of type I collagen by mesangial cells *in vitro*. *Kidney Int* 2002;61:1365–1376.
24. Weigert C, Brodbeck K, Bierhaus A, *et al*. c-Fos-driven transcriptional activation of transforming growth factor β -1: inhibition of high glucose-induced promoter activity by thiazolidinediones. *Biochem Biophys Res Commun* 2003;304:301–307.
25. Kuratsune M, Masaki T, Hirai T, *et al*. Signal transducer and activator of transcription 3 involvement in the development of renal interstitial fibrosis after unilateral ureteral obstruction. *Nephrology* 2007;12: 565–571.

26. Masaki T, Foti R, Hill PA, *et al*. Activation of the ERK pathway precedes tubular proliferation in the obstructed rat kidney. *Kidney Int* 2003;63:1256–1264.
27. Kiribayashi K, Masaki T, Naito T, *et al*. Angiotensin II induces fibronectin expression in human peritoneal mesothelial cells via ERK1/2 and p38 MAPK. *Kidney Int* 2005;67:1126–1135.
28. Naito T, Masaki T, Nikolic-Paterson DJ, *et al*. Angiotensin II induced thrombospondin-1 production in human mesangial cells via p38 MAPK and JNK: a mechanism for activation of latent TGF- β . *Am J Physiol Renal Physiol* 2004;286:F278–F287.
29. Hirai T, Masaki T, Kuratsune M, *et al*. PDGF receptor tyrosine kinase inhibitor suppresses mesangial cell proliferation involving STAT3 activation. *Clin Exp Immunol* 2006;144:353–361.
30. Izumi T, Enomoto S, Hoshiyama K, *et al*. Pharmacokinetics of troglitazone, an anti-diabetic agent: prediction of *in vivo* stereoselective sulfation and glucuronidation from *in vitro* data. *J Pharmacol Exp Ther* 1997;280:1392–1400.
31. Kawai K, Kawasaki-Tokui Y, Odaka T, *et al*. Disposition and metabolism of the new oral anti-diabetic drug troglitazone in rats, mice and dogs. *Arzneim-Forsch/Drug Res* 1997;47:356–368.
32. Kubota T, Koshizuka K, Williamson EA, *et al*. Ligand for peroxisome proliferator-activated receptor (troglitazone) has potent antitumor effect against human prostate cancer both *in vitro* and *in vivo*. *Cancer Res* 1998;58:3344–3352.
33. Elstner E, Müller C, Koshizuka K, *et al*. Ligands for peroxisome proliferator-activated receptor and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc Natl Acad Sci USA* 1998;95:8806–8811.
34. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 2004;15:1–12.
35. Moon JA, Kim HT, Cho IS, *et al*. IN-1130, a novel transforming growth factor-beta type I receptor kinase (ALK5) inhibitor, suppresses renal fibrosis in obstructive nephropathy. *Kidney Int* 2006;70:1234–1243.
36. Lee S, Kim W, Kang KP, *et al*. Agonist of peroxisome proliferator-activated receptor-gamma, rosiglitazone, reduces renal injury and dysfunction in a murine sepsis model. *Nephrol Dial Transplant* 2005;20:1057–1065.
37. Panzer U, Schneider A, Guan Y, *et al*. Effects of different PPARgamma-agonists on MCP-1 expression and monocyte recruitment in experimental glomerulonephritis. *Kidney Int* 2002;62:455–464.
38. Klahr S, Morrissey J. Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 2002;283:F861–F875.
39. Chevalier RL. Obstructive nephropathy: towards biomarker discovery and gene therapy. *Nat Clin Pract Nephrol* 2006;2:157–168.
40. Miyajima A, Chen J, Lawrence C, *et al*. Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. *Kidney Int* 2000;58:2301–2313.
41. Galli A, Crabb DW, Ceni E, *et al*. Anti-diabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation *in vivo* and *in vitro*. *Gastroenterology* 2002;122:1924–1940.
42. Burgess HA, Daugherty LE, Thatcher TH, *et al*. PPAR-gamma agonists inhibit TGF-beta induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2005;288:1146–1153.
43. Isshiki K, Haneda M, Koya D, *et al*. Thiazolidinedione compounds ameliorate glomerular dysfunction independent of their insulin-sensitizing action in diabetic rats. *Diabetes* 2000;49:1022–1032.
44. Hong KM, Belperio JA, Keane MP, *et al*. Differentiation of human circulating fibrocytes as mediated by transforming growth factor-beta and peroxisome proliferator-activated receptor gamma. *J Biol Chem* 2007;282:22910–22920.
45. Fukuda K, Yoshitomi K, Yanagida T, *et al*. Quantification of TGF-beta1 mRNA along rat nephron in obstructive nephropathy. *Am J Physiol Renal Physiol* 2001;281:F513–F521.
46. Oliver III R, Friday E, Turturro F, *et al*. Troglitazone's rapid and sustained activation of ERK1/2 induces cellular acidosis in LLC-PK1-F+ cells: physiological responses. *Am J Physiol Renal Physiol* 2005;288:F1257–F1266.
47. Takeda K, Ichiki T, Tokunou T, *et al*. 15-Deoxy-delta 12,14-prostaglandin J2 and thiazolidinediones activate the MEK/ERK pathway through phosphatidylinositol 3-kinase in vascular smooth muscle cells. *J Biol Chem* 2001;276:48950–48955.
48. Turturro F, Oliver III R, Friday E, *et al*. Troglitazone and pioglitazone interactions via PPAR-gamma-independent and -dependent pathways in regulating physiological responses in renal tubule-derived cell lines. *Am J Physiol Cell Physiol* 2007;292:C1137–C1146.