Troglitazone Inhibits Isolated Cell Proliferation, and Induces Apoptosis in Isolated Rat Mesangial Cells

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Key Words

Troglitazone · Mesangial cell · Peroxisome proliferator-activated receptor-gamma · Cell proliferation · Mitogen-activated protein kinase · Apoptosis

Abstract

Background/Aims: Troglitazone is one of thiazolidinedione derivatives as a high affinity ligand for peroxisome proliferator-activated receptor-gamma (PPAR- γ). The in vivo studies demonstrated that troglitazone ameliorated microalbuminuria. There have been few reports about direct effect of thiazolidinedione derivatives on mesangial cell function. We determined the effect of troglitazone on isolated rat mesangial cell proliferation. **Methods:** We determined PPAR- γ mRNA expression in isolated rat mesangial cells. Chronic effects of 10^{-6} to 10^{-4} mol/l troglitazone on mesangial cell proliferation and mitogen-activated protein (MAP) kinase activity were also determined. The effects of troglitazone on apoptosis were investigated in rat mesangial cells. **Results:** Rat PPAR- γ mRNA was detected in isolated rat mesangial cells. Living cell number, assessed by colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazol-

ium bromide] (MTT) assay, was significantly decreased with 10^{-4} mol/l troglitazone. The addition of 10^{-6} to 10^{-4} mol/l troglitazone dose-dependently inhibited 5-bromo-2'-deoxyuridine (BrdU) uptake into isolated rat mesangial cells. The addition of 10^{-4} and 10^{-5} mol/l troglitazone significantly reduced MAP kinase activity. Troglitazone at the concentrations of 10^{-6} to 10^{-4} mol/l dose-dependently increased DNA fragmentation rates, indicating that troglitazone may cause apoptosis in rat mesangial cells. Bax and Bcl-xL proteins were not changed, although Bcl-2 proteins increased with troglitazone. **Conclusions:** The present data demonstrated that troglitazone inhibits cell proliferation, and induces apoptosis in rat mesangial cells, raising a possibility that it directly affects renal function.

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Introduction

Plenty of investigators have reported that chronic hyperglycemia contributes to the development of diabetic micro-angiopathy. Histological lesion of diabetic nephropathy is characterized by mesangial cell proliferation and extracellular matrix expansion [1]. These structural

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changes produce renal dysfunction in the patient with diabetes mellitus [2]. Mesangial cell proliferation is one of important features of chronic glomerular disease and involves the development of diabetic glomerulopathy in poorly controlled diabetic patients. It was demonstrated that mild hyperglycemia stimulates cultured murine mesangial cells [3]. Chronic hyperglycemia is the major causal factor in the development of diabetic nephropathy, and can mediate their adverse effects through multiple pathways. Recent studies have reported that polyol pathway exists in mesangial cells and may play a role in the development of mesangial cell dysfunction caused by hyperglycemia [4, 5]. The activation of protein kinase C may play a critical role in the development of diabetic nephropathy characterized by mesangial cell proliferation and matrix expansion [6, 7]. Protein kinase C is activated by hyperglycemia-induced increases in diacylglycerol level, partly due to de novo synthesis [8, 9]. Advanced glycation end products may also play a role in mesangial extracellular matrix expansion and cell proliferation [10]. However, the exact mechanism of mesangial cell proliferation is not completely understood in diabetes mellitus and effective treatment on diabetic nephropathy has not been established except an improvement of glycemic control.

Troglitazone, one of the thiazolidinedione derivatives, is a new antidiabetic agent that improves hyperglycemia by the attenuation of peripheral insulin resistance in animal models of type II diabetes mellitus and non-insulin-dependent diabetic patients [11–13]. It is well known that troglitazone is a high-affinity ligand for peroxisome proliferator-activated receptor-gamma (PPAR- γ). PPAR- γ is supposed to be a target for the therapeutic actions of troglitazone [14]. Recently, troglitazone treatment of streptozotocin-induced diabetic rats significantly decreased diabetes-associated albuminuria without affecting blood glucose level, blood pressure and creatinine clearance [15, 16]. Clinically, troglitazone ameliorated microalbuminuria in patients with incipient diabetic nephropathy [17]. These beneficial effects of troglitazone may be attributable to the improvement of hyperglycemia accompanied by improving hyperinsulinemia. There remains a possibility that troglitazone may directly affect renal function to improve diabetic nephropathy, since PPAR exists in mesangial cell [17]. However, there are only few reports about direct effect of PPAR ligands on mesangial cell function [18, 19]. The present study was undertaken to examine the direct effect of troglitazone on proliferation and apoptosis in isolated rat mesangial cell proliferation.

Materials and Methods

Isolation and Primary Culture of Rat Mesangial Cell

Male Wistar rats weighing about 200 g were obtained from Imai Animal Laboratories (Saitama, Japan). The rats were decapitated at 7 weeks of age and bilateral kidneys were immediately dissected out. Kidney was sliced and renal cortex was collected in buffer. The obtained renal cortex was minced and filtrated through stainless mesh (pore size 106 μ m) and nylon mesh (pore size 133 μ m) in Hanks' balanced salt solution containing 100 U/ml penicillin G and 100 μ g/ml streptomycin (HBSS). Then, the suspension was filtered through nylon mesh (pore size 77 μ m) and the cells remaining on the mesh were re-suspended in HBSS. The suspension was centrifuged twice at 400 g for 2 min. The cell pellet was dispersed by pipetting in HBSS containing 375 U/ml collagenase type 1 and incubated at 37°C for 15 min. The solution was centrifuged at 400 g for 2 min and the supernatant was discarded. The cell pellet was re-suspended in culture medium and the suspension was centrifuged twice at 400 g for 2 min. The culture medium was a mixture of 80% RPMI 1640 medium and 20% fetal bovine serum (FBS) containing 100 ng/ml insulin, 55 ng/ml transferrin, 50 pg/ml selenium acid, and antibiotics. The cells were incubated with this culture medium in a 5% CO₂-95% O₂ incubator at 37°C and the medium was exchanged every 2 days. Mesangial cells were identified by morphological characterization. Cells at passage 1 to 4 were used for the following experiments.

Rat PPAR- γ mRNA Expression Determination by RT-PCR Method

To obtain total RNA from isolated rat mesangial cells, 10⁶ mesangial cells were sonicated in 0.8 ml of Isogene (Nippon Gene, Tokyo, Japan) and centrifuged at 12,000 g for 10 min. Total RNA was extracted from the supernatant. Rat PPAR- γ mRNA was measured by RT-PCR, using the GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, USA). The synthetic rat PPAR- γ primer sequences used in the present study were as follows: sense primer 5'-GCGAATGCCACAGGCCGA-3'; antisense primer 5'-TTCCACGGATCGAAACTGGCACC-3', according to the rat PPAR- γ cDNA sequence obtained from GenBank. On the basis of the results from our preliminary experiment, following the reverse transcription step at 60°C for 30 min, PCR was performed for 40 cycles using 1 min denaturation step at 94°C, 1 min annealing and extension step at 60°C. An additional 7 min extension step at 60°C was added after 40 cycles. The PCR product was loaded on to a 6% acrylamide gel and the intensity of fluorescence of the band stained by ethidium bromide was recorded.

Assay of Living Cell Number Using Colorimetric [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] (MTT)

Living cell number of isolated rat mesangial cells was assayed by the method of Mosmann [20]. The incubation medium was completely aspirated after the incubation with 10⁻⁶ to 10⁻⁴ mol/l troglitazone for 5 days. The MTT-formazon product was dissolved in a phosphate buffer solution. Following the addition of RPMI 1640 medium containing 10% MTT, the cells were incubated at 37°C for 4 h, the medium was aspirated, and the cells were lysed by the addition of 100 μ l DMSO. Then, 10 μ l was collected from each sample and diluted in 90 μ l of fresh DMSO. After the sample was mixed with a mechanical plate mixer, the optical density of each sample was measured by the Kinetic Microplate-Reader (Molecular Devices Co., Calif., USA), using test and reference wavelengths of 550 and 650 nm.

5-Bromo-2'-Deoxyuridine (BrdU) Uptake Assay

BrdU uptake into isolated rat mesangial cells and its incorporation into DNA [21] were determined by using the Cell Proliferation ELISA system (Amersham International plc, England). The incubation medium was completely aspirated after the incubation with 10^{-6} to 10^{-4} mol/l troglitazone for 5 days. Following the addition of 10 μ mol/l-BrdU containing the culture medium without FBS, the cells were incubated at 37°C for 2 h. The medium was removed, the cells were incubated for 30 min at room temperature in fixative solution and then in the blocking reagent included in the assay kit. Peroxidase-labeled anti-BrdU monoclonal antibody (from mouse cells) solution was added and incubated for an additional 90 min at room temperature. After the cells were washed three times with PBS for 5 min, substrate solution was added and the cells were incubated for 30 min at room temperature. The reaction was stopped by the addition of 1 mol/l sulphuric acid and the optical density of each sample was measured by Kinetic Microplate-Reader at 450 nm.

Assay of Mitogen-Activated Protein (MAP) Kinase Activity

MAP kinase activity was measured with the p42/p44 MAP Kinase Enzyme Assay System (Amersham International plc, England) in primary cultured rat mesangial cells. Primary cultured rat mesangial cells were seeded into 6-well plates and used for the experiment when the cells had reached about 90% confluence. The cells were incubated at 37°C for 5 days in the presence of 10^{-5} or 10^{-4} mol/l troglitazone. The cells were then lysed with 1,000 μ l/well lysing buffer in 10 mmol/l TRIS, 150 mmol/l NaCl, 2 mmol/l EGTA, 2 mmol/l dithiothreitol, 1 mmol/l orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, pH 7.4 measured at 4°C, and homogenized. Cellular debris was precipitated at 13,500 rpm for 20 min at 4°C. Following the addition of 10 μ l of the substrate buffer and 5 μ l of magnesium 32 P-ATP buffer (1.0 μ Ci/tube) to 15 μ g supernatant, each sample was incubated for 30 min at 30°C. The reaction was terminated by the addition of 10 μ l of stop reagent and 30 μ l of the sample was put on the center of the binding paper disc. The discs were twice washed with 1% acetic acid for 2 min, and followed by washing twice with distilled water for 2 min. Then, each disc was cut out and placed in 10 ml liquid scintillation cocktail (Ultima GoldTM, Packard Instrument Co., Meriden, Conn., USA) and counted in β -scintillation counter for phosphorus-32. Protein concentration was determined by Lowry [22].

DNA Fragmentation Assay

As previously reported [23], the Cellular DNA Fragmentation ELISA Kit (Boehringer Mannheim GmbH, Germany) was used for the determination of DNA fragmentation induced by the addition of troglitazone. Primary cultured rat mesangial cells were incubated with 10 μ mol/l BrdU overnight at 37°C, centrifuged at 250 g for 10 min, adjusted to 1×10^5 cells/ml in the culture medium without FBS and plated out in 96 multi-well plates. The cells were incubated with 10^{-6} to 10^{-4} mol/l troglitazone at 37°C for 5 days and the supernatant was completely removed from each well. The cells were lysed by the addition of the incubation buffer enclosed in the kit for 30 min at room temperature. The multiplate was centrifuged at 250 g for 10 min and the supernatant was transferred directly to the well of a multiplate pre-coated with anti-DNA antibody. Then, samples were incubated for 90 min at room temperature. After being washed, the samples were denatured and fixed by microwave (500 W) for 5 min and frozen at -20°C for 10 min. Peroxidase-conjugated anti-BrdU solution was added and incubated for an additional 90 min at room

Fig. 1. Expression of rat PPAR- γ mRNA in isolated rat mesangial cells. The RNAs extracted from male Wistar rat subcutaneous adipose tissue (lane 1) and primary cultured rat mesangial cells (lane 2) were analyzed by RT-PCR.

temperature. Then substrate solution was added and the mixture was incubated at room temperature in the dark on a plate shaker at 250 rpm for 10 min. The reaction was stopped by the addition of 0.5 mol/l sulfuric acid to each well and the plate was shaken for 1 min at 250 rpm. The absorbance was measured at 450 nm (reference wavelength: 690 nm) against substrate solution as a blank.

Immunoblot Analysis

Mesangial cells were lysed with 62.5 mmol/l TRIS, 2% SDS, 10% glycerol, 50 mmol/l dithiothreitol and 0.1% bromophenol blue after the incubation with 10^{-6} to 10^{-4} mol/l troglitazone for 5 days. Following protein determination by the method of Lowry [22], 20 μ g of each sample protein was subjected to 12.5% SDS-PAGE electrophoresis. Each protein was transferred electrically to a nitrocellulose membrane. Filters were blocked with 5% non-fat milk at room temperature and incubated for 2 h with Bax (1:200 final dilution), Bcl-2 (1:100 final dilution) or Bcl-xL (1:100 final dilution) (Santa Cruz Biotechnology Inc., USA). Then, antigen-antibody reaction was detected after a 1-hour incubation with goat anti-rabbit IgG (1:5,000 final dilution) and visualized with ECL detection system (Amersham International plc, England).

Statistical Analysis

All data represent means $\pm$ SEM. The statistical analysis of the means was performed by analysis of variance (ANOVA), followed by Duncan's multiple range test for the individual comparisons of the means.

Results

Expression of PPAR- γ and Effect of Troglitazone on Cell Proliferation in Rat Mesangial Cells

Rat PPAR- γ mRNA was expressed in isolated rat mesangial cells by RT-PCR method (fig. 1). Then, we determined the effect of troglitazone on isolated rat mesangial cell proliferation. Figures 2 and 3 demonstrate tro-

Fig. 2. Changes in living cell number induced by 10^{-6} to 10^{-4} mol/l troglitazone. Living cell number was assessed by MTT assay in isolated rat mesangial cells. $n = 6$ in each group. OD = Optical density.

Fig. 3. Changes in BrdU uptake into isolated rat mesangial cells elicited by 10^{-6} to 10^{-4} mol/l troglitazone. $n = 10$ in each group. OD = Optical density.

glitazone-induced changes in cell proliferation, as measured by colorimetric MTT assay and BrdU uptake into isolated rat mesangial cells. Living cell number, assessed by colorimetric MTT assay, was significantly decreased in mesangial cells incubated with 10^{-4} mol/l troglitazone for 5 days (fig. 2). As shown in figure 3, the addition of troglitazone dose-dependently inhibited isolated rat mesangial cell proliferation, measured by BrdU uptake. The inhibitory effect of troglitazone on BrdU uptake in isolated rat mesangial cells was statistically significant at the concentrations of 10^{-5} and 10^{-4} mol/l. Then, we determined the effect of troglitazone on MAP kinase activities in isolated rat mesangial cells (fig. 4). The addition of 10^{-5} mol/l and 10^{-4} mol/l troglitazone significantly decreased MAP kinase activity by 51.6 and 47.5%, respectively.

Effect of Troglitazone on Apoptotic Death in Rat Mesangial Cells

To examine the involvement of apoptosis in the reduction of living cell number by troglitazone (fig. 2), the effect of troglitazone on DNA fragmentation was also investigated in isolated rat mesangial cells (fig. 5). The addition of troglitazone dose-dependently increased DNA fragmentation rate and the effect of troglitazone was statistically significant at the concentrations of 10^{-5}

Fig. 4. Reduction of MAP kinase activity elicited by 10^{-5} and 10^{-4} mol/l troglitazone in isolated rat mesangial cells. $n = 4$ in each group.

Fig. 5. Effect of 10^{-6} to 10^{-4} mol/l troglitazone-induced DNA fragmentation in isolated rat mesangial cells. $n = 10$ in each group. OD = Optical density.

and 10^{-4} mol/l. Therefore, it was indicated that troglitazone may cause apoptosis in isolated rat mesangial cells. Next, we determined the effect of troglitazone on regulator protein expressions of apoptotic death program in isolated rat mesangial cells (fig. 6). Bax protein, a regulator to accelerate apoptotic death [24], was not changed by the addition of 10^{-6} to 10^{-4} mol/l troglitazone. With regards to repressing apoptotic death program, Bcl-xL protein [25] was not changed, but Bcl-2 protein [26] was obviously increased by the addition of troglitazone.

Discussion

PPAR- γ is expressed in low abundance in rat renal cortex (glomerulus, proximal tubule) [27]. Electrophoretic mobility of a labeled PPAR- γ response element was retarded in the presence of mesangial cell extract, suggesting that PPAR- γ is functional in cultured mesangial cells. The present studies confirmed that PPAR- γ mRNA is expressed in primary cultured rat mesangial cells. Troglitazone is one of thiazolidinedione derivatives as a high affinity ligand for PPAR- γ [14]. Recently, in vivo study demonstrated that troglitazone ameliorated microalbuminuria without affecting blood glucose level in strepto-

Fig. 6. Effect of 10^{-6} to 10^{-4} mol/l troglitazone on Bax and Bcl-xL protein level in isolated rat mesangial cells. Each protein was extracted from mesangial cells treated with none (lane 1) and 10^{-6} mol/l (lane 2), 10^{-5} mol/l (lane 3) and 10^{-4} mol/l (lane 4) of troglitazone. These samples were analyzed by the immunoblot method using specific antiserum against Bax (upper panel), Bcl-xL (middle panel) and Bcl-2 (lower panel) protein.

zotocin-induced diabetic rats [15, 16] and patients with incipient diabetic nephropathy [17]. Rosiglitazone, one of the insulin-action-enhancing thiazolidinediones, also protects against diabetic nephropathy in Zucker fatty rats [28]. Inflammatory marker, C-reactive protein (CRP), was elevated in microalbuminuric subjects, indicating that microalbuminuria might be associated with chronic inflammation [29]. Rosiglitazone reduces serum CRP levels in patients with type 2 diabetes [30]. These previous findings indicate the beneficial effects of thiazolidinedione derivatives on diabetic nephropathy.

The present data demonstrated that the addition of troglitazone significantly inhibited isolated rat mesangial cell proliferation independent of circumstantial glucose and insulin levels. This result is compatible with previous observation that troglitazone decreased thymidine incorporation in a dose-dependent manner [18]. It is known that a maximal physiological concentration of troglitazone is about 10^{-5} mol/l in the serum after oral administration in clinical usage [31]. The finding that troglitazone at 10^{-5} mol/l concentration suppresses BrdU uptake indicates that clinical use of troglitazone should be beneficial to block the development of diabetic nephropathy by inhibiting mesangial cell proliferation. In addition, the present data suggested that the observed reduction of mesangial cell proliferation by troglitazone may relate to both reduced MAP kinase activity and increased apoptosis of mesangial cells. While MAP kinase activities were inhibited at both concentrations of 10^{-4} mol/l and 10^{-5} mol/l, mesangial cell proliferation rate was dose-

dependently decreased. This discrepancy can be explained by dose-dependent increase of apoptosis by this agent.

Alteration in mesangial cell function induced by high glucose levels is implicated in the development of diabetic nephropathy. Glucose stimulates rat mesangial cell glucose transporter (GLUT) 1 expression [32]. Troglitazone enhances glucose uptake through GLUT 1 with an acceleration of glycolysis, and improves the abnormal intracellular glucose metabolism under high-glucose conditions in rat mesangial cell [33]. Raising the ambient glucose concentration from 100 mg/dl to 450 mg/dl stimulates cultured murine mesangial cell proliferation after 24 to 48 h [3]. It is supposed that MAP kinase may play an important role in mesangial cell proliferation [34, 35]. The translocation of protein kinase C and the activation of MAP kinase under high glucose conditions (27.8 mmol/l) might be underlying mechanisms of the functional disturbance of mesangial cells in diabetes mellitus [36]. The present data added a new finding that troglitazone inhibited MAP kinase activities in cultured mesangial cells.

The recent reports suggested that troglitazone induces apoptosis in various cell lines, for example, human breast cancer cells [37], gastric cancer cells [38], or promyelocytic leukemia cells [39]. We have also demonstrated the possibility that the addition of troglitazone may cause apoptosis in HIT-T 15 cells [40]. Additionally, the *in vivo* study demonstrated that troglitazone increased the number of small adipocytes without changing white adipose tissue mass and decreased the number of large adipocytes with increasing apoptotic nuclei in obese Zucker rats [41]. From our DNA fragmentation data, it is supposed that troglitazone may cause apoptosis in isolated rat mesangial

cells. The supposed increase of apoptotic mesangial cells may partially contribute to a reduction of living mesangial cell number measured by MTT assay in addition to reduced proliferation rate measured by BrdU uptake. From the present data, there is a possibility that troglitazone at the concentration of 10^{-5} mol/l may cause apoptosis of mesangial cell *in vivo*. No significant changes in Bax and Bcl-xL expressions were observed by 10^{-6} to 10^{-4} mol/l troglitazone treatment, but Bcl-2 expression increased. Hepatocyte-growth factor (HGF) induces apoptosis, but Bax and Bcl-xL are not changed, with a slight increase of Bcl-2. HGF is supposed to exert its apoptotic effect by fostering the formation of Bax-Bax homodimers, without an actual change in protein levels, or that the constitutive expression of Bax may sensitize these cells to apoptosis [42]. Troglitazone may exert its effects by a similar mechanism as HGF.

The present data indicated that troglitazone inhibits mesangial cell proliferation. This finding appears to be important in the prevention of diabetic nephropathy by thiazolidinedione derivatives. Troglitazone modulates mesangial cell proliferation, perhaps via both MAP kinase cascade and unknown apoptotic death programs. However, further studies should be necessary to clarify the differences in morphological effects between thiazolidinedione derivatives and other inhibitors of angiotensin convertase or protein kinase C.

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