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Biochimica et Biophysica Acta 1497 (2000) 148–154

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Peroxisome proliferator-activated receptor γ1 (PPARγ1) expresses in rat mesangial cells and PPARγ agonists modulate its differentiation

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Received 14 September 1999; received in revised form 7 March 2000; accepted 30 March 2000

Abstract

Thiazolidinediones, synthetic ligands of peroxisome proliferator-activated receptor γ (PPARγ), are reported to have direct beneficial effects on diabetic nephropathy without lowering blood glucose levels in human and rat. We hypothesized these effects of thiazolidinediones might be derived from PPARγ activation of kidney cells, and we examined the expression of PPARγ and the effect of PPARγ agonists, troglitazone and 15-deoxy-δ-prostaglandin J2 (15d-PGJ2), on the proliferation and differentiation in rat mesangial cells. A single band of mRNA of PPARγ with a predicted size was detected in reverse transcription-polymerase chain reaction products (RT-PCR) using established PCR probes of PPARγ. PPARγ protein in rat mesangial cells was identified as PPARγ1 by a Western blot. In a gel mobility shift assay to determine a binding activity of PPARγ, the nuclear protein from rat mesangial cells bound to a ^{32}P-labeled oligonucleotide probe, including PPAR response elements. A synthetic and a natural ligand of PPARγ, troglitazone and 15d-PGJ2, decreased thymidine incorporation in a dose dependent manner. After 7 days incubation with troglitazone and 15d-PGJ2, α-smooth muscle actin expression, a marker of mesangial cell de-differentiation, was decreased significantly compared to that of control. These results indicate that PPARγ1 is expressing in rat mesangial cells, and PPARγ1 activation with its agonists modulates the proliferation and differentiation of cultured rat mesangial cells.

Keywords: Rat mesangial cell; Peroxisome proliferator-activated receptor γ; Cell differentiation; Troglitazone

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear receptor transcriptional factors, and have three members (α, δ and γ). PPARs form heterodimers with retinoic X receptor bind to PPAR response elements (PPRE) in the promoter region of specific target genes to regulate their expressions [1,2]. PPARs play important roles in the lipid and glucose metabolisms and the differentiation of adipocytes [3,4]. Recently, the expressions of PPARγ in macrophages and vascular smooth muscle cells other than adipocytes are also reported, and seem to play important roles in the pathogenesis of atherosclerosis by regulating activation of de-differentiation of the cells [5–9].

We reported that troglitazone, one of the thiazolidinediones which are specific synthetic ligands for PPARγ, improved intracellular glucose metabolism under a high glucose condition in rat mesangial cells, in spite of the presence of increased glucose uptake

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0167-4889/00/$ – see front matter
PII: S0167-4889(00)00054-9

with an upregulation of facilitated glucose transporter 1 by the cells [10]. These findings raised a possibility of the presence of PPARγ in rat mesangial cells, and its presence may be associated with some renal diseases. Since the activation and phenotypic change in mesangial cells are considered as the features of mesangial proliferative glomerulonephritis [11] and diabetic nephropathy [12], PPARγ in mesangial cells may play important roles in pathogenesis of nephropathy. In the present study, we examined the expression of PPARγ and the effect of agonists of PPARγ on the proliferation and differentiation in rat mesangial cells.

2. Materials and methods

2.1. Isolation and culture of rat mesangial cells

Mesangial cells were isolated from kidneys of 4-week-old Sprague–Dawley rats by a differential sieving procedure as previously reported [10], and were cultured in plastic plates (NUNC Brand Products, Denmark) in a 1:1 mixture of Dulbecco's modified Eagles's medium (DMEM) and Ham's F-12 (both from Sigma, St. Louis, MO) containing 10% FBS (Gibco BRL, Grand island, NY), 10% Nu-serum (Collaborative Research, Bedford, MA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma). After having reached over 90% confluency, mesangial cells from 5–9 passages were used for the following experiments.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of rat mesangial cells was prepared using Isogen (Wako Junyaku, Osaka, Japan). To amplify a 386-base pair of cDNA fragment, the sequences of PCR primers for PPARγ: forward (5′-AACCGGAACAAATGCCAGTA-3′) and reverse (5′-TGGCAGCAGTGGAAGAATCG-3′) were synthesized and the RT-PCR was carried out as reported previously [5]. In brief, 2 μg of total RNA of rat mesangial cells was reverse-transcribed to cDNA with a reverse transcriptase (Promega, Madison, WI) at 37°C for 1 h in a standard buffer. The PCR reaction was carried out in the standard buffer with 0.5 μM of each primer, 1.5 mM $MgCl_2$ and 0.5 U Taq polymerase (Toyobo, Osaka, Japan). The temperature program for the amplification was 35 cycles of 30 s at 94°C, 30 s at 54°C and 1 min at 72°C.

2.3. Western blot of PPARγ

For the Western blot of PPARγ, rat mesangial cells were harvested and lysed in a solution containing 20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% Nonidet P-40 and 1% Triton X following a modified method of a previous report [13]. Protein extracts were obtained by centrifugation of the lysate. The protein extract (50 μg) and positive controls of PPARγ1 and PPARγ2 protein were separated on 10% non-gradient acryl amide gel using sodium dodecyl sulfate acryl amide gel electrophoresis (SDS-PAGE), and were transferred to nitrocellulose sheets. Rabbit IgG antibody against PPARγ (Santa Cruz Biotechnology, California) was reacted (1:1000 dilution), followed by peroxidase linked protein A (Amersham). Immunoreactive bands were visualized by Western blotting detection ECL reagents (Amersham) with the use of X-Omatic AR film (Eastman-Kodak, Rochester, NY). Jurkat whole cell lysate (Santa Cruz Biotechnology) was used for a positive control of PPARγ1 protein, and PPARγ2 protein was kindly gifted from Dr. Jiro Masugi [13].

2.4. Gel mobility shift assay

To obtain nuclear extracts from rat mesangial cells, the cells were lysed in 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT and 0.5% NP-40. Nuclei obtained after centrifugation at $13\,000\times g$ for 30 s were lysed in 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 1 mM DTT. After centrifugation at $13\,000\times g$ for 5 min, the supernatant was used for gel mobility shift assay. A double-stranded 23-bp oligonucleotide probe (5′-GGACCAGGACAAAGGTCACGTTC-3′), synthesized according to the sequence of rat acyl-CoA oxidase was labeled with ^{32}P-ATP (Amersham Life Science, Buckinghamshire, UK) [5]. A typical binding reaction mixture, contained oligonucleo-

tide probes and 5 μg of the nuclear extract in a final volume of 25 μl, was incubated at room temperature for 20 min, and analyzed by 5% polyacrylamide gel electrophoresis. The autoradiograph of the dried gel was determined by BAS 2000 system (Fuji film, Tokyo, Japan). To characterize specific DNA binding proteins, we incubated nuclear extracts with a specific antibody for PPARγ for 12 h at 4°C before the addition of the probe. The specific antibody used in Gel mobility shift assay was kindly gifted by Dr. Jiro Masugi [13].

2.5. Thymidine incorporation

For determination of thymidine incorporation, the mesangial cells were preincubated for 24 h in DMEM-F12 containing 1% FCS and 1% Nu-serum in 12-well plates (28 mm diameter, Nunc) with various concentrations of 15d-PGJ2 and troglitazone, and washed three times with phosphate-buffered saline (PBS). Then the cells were incubated with 2 μCi/ml of ^{3}H-thymidine for 6 h, the cells were solubilized with 500 μl of 0.5 M NaOH after washing with ice-cold PBS. The cellular radioactivities were counted after neutralization with 2 M acetic acid, using Aloka Liquid Scintillation Counter LSC 1000 (Aloka, Tokyo, Japan). Thymidine incorporation was expressed as per mg of cell protein.

2.6. Determination of α-smooth muscle actin expression

Mesangial cells in 100 mm plates were incubated for 7 days in 10 ml DMEM–F12, 1% FCS, 1% Nu-serum, and 5 mM of glucose, in the presence or absence of troglitazone (4.5 μM) and 15d-PGJ2 (2.5 μM), changing media every 2 days. The cells were harvested in a sodium dodecyl sulfate lysis buffer, then the cell lysate was sonicated and boiled. After centrifugation at $12\,000 \times g$ for 15 min at 4°C, the supernatant was used to determine α-smooth muscle actin expression by Western blots. The samples corresponding to 300 ng of the DNA contents of the same plates were separated on 10% non-gradient acryl amide gel using SDS–PAGE. Antibody against α-smooth muscle actin (Sigma) was reacted (1:1000 dilution), followed by peroxidase-linked protein A (Amersham).

2.7. Other determinations

Densitometric analyses were conducted by NIH image program. DNA contents were determined by the Hoechst 33258 dye (Sigma) method using a TKO 100 Spectrophotometer (Hoeffer Scientific Instruments, San Francisco, CA). Protein concentration was measured by the Coomassie brilliant blue method using a Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as a standard.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. Analysis of variance (ANOVA) and Student's *t*-test (two-tailed) were used for statistical determinations; a level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of PPARγ mRNA and protein

The RT-PCR products from total mRNA of rat mesangial cells analyzed by electrophoresis with ethidium bromide in 1.4% agarose gel are shown in Fig. 1. A single band with a predicted size (386 bp: indicated by an arrow) was detected in RT-PCR products, which demonstrates PPARγ mRNA expression (lane 1). The same RT-PCR reactions without re-

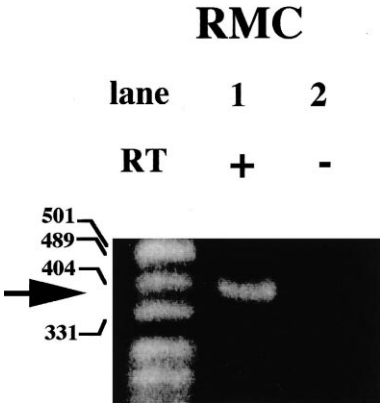

Fig. 1. Expression of mRNA of PPARγ in rat mesangial cell by the RT-PCR. A single band with predicted size (386 bp: indicated by an arrow) of PPARγ DNA was detected in lane 1. The same RT-PCR reaction, without reverse transcription (lane 2), did not make any PCR products.

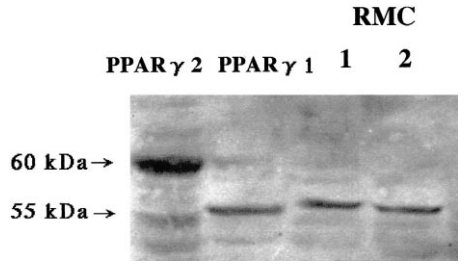

Fig. 2. Expression of PPARγ protein in rat mesangial cells by Western blot analysis. Cell lysates of rat mesangial cells were observed in a single band at a molecular weight of 55 kDa. The identity of PPARγ band was confirmed by comigration with the band seen in PPARγ1 from Jurkat cell. PPARγ2 protein was observed a single band at 60 kDa.

verse transcription (lane 2) did not make any PCR products.

Western blot analysis revealed that the rabbit PPARγ antibody reacts with both PPARγ1 (55 kDa) and PPARγ2 protein (60 kDa) (Fig. 2), PPARγ protein expressed in rat mesangial cells was identified to have the same reaction with PPARγ1.

3.2. Binding of nuclear proteins from rat mesangial cells to PPRE oligonucleotide

As shown in Fig. 3, the addition of nuclear extracts resulted in a retarded thick band (indicated by thick arrow) consistently (lane 1). This band was abolished by a 100-fold molar excess of unlabeled PPRE oligonucleotide (lane 2). Incubation of nuclear extracts with a specific antibody for PPARγ prior to the reaction produced a supershifted band (lane 3), indicating that PPARγ was the component of this DNA–protein complex.

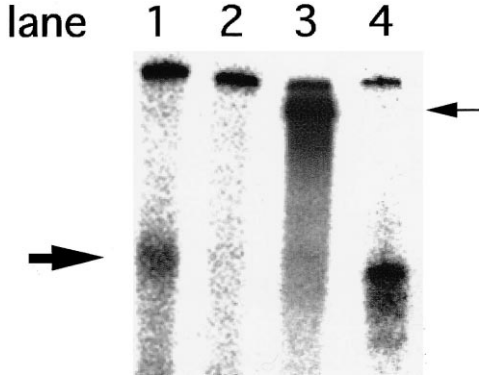

Fig. 3. Detection of binding of nuclear proteins from rat mesangial cell and PPARγ protein to PPRE oligonucleotide using gel mobility shift assay. ^{32}P-labeled PPRE oligonucleotide probe and protein complex was shown as a thick band in lane 1. This band was abolished by excess of unlabeled PPRE oligonucleotide (lane 2). Incubation of nuclear extracts with a specific antibody for PPARγ prior to the reaction produced a supershifted band (lane 3). The PPARγ protein exhibited the same band observed in lane 1 (lane 4).

3.3. Effect of PPARγ agonists on proliferation and differentiation of rat mesangial cells

The effects of troglitazone and 15d-PGJ2 on thymidine incorporation by rat mesangial cells are shown in Fig. 4. Both troglitazone (Fig. 4A) and 15d-PGJ2 (Fig. 4B) decreased ^{3}H-thymidine incorporation in a dose-dependent manner. The concentration of troglitazone was chosen to 0.23, 0.45, 2.3, 4.5,

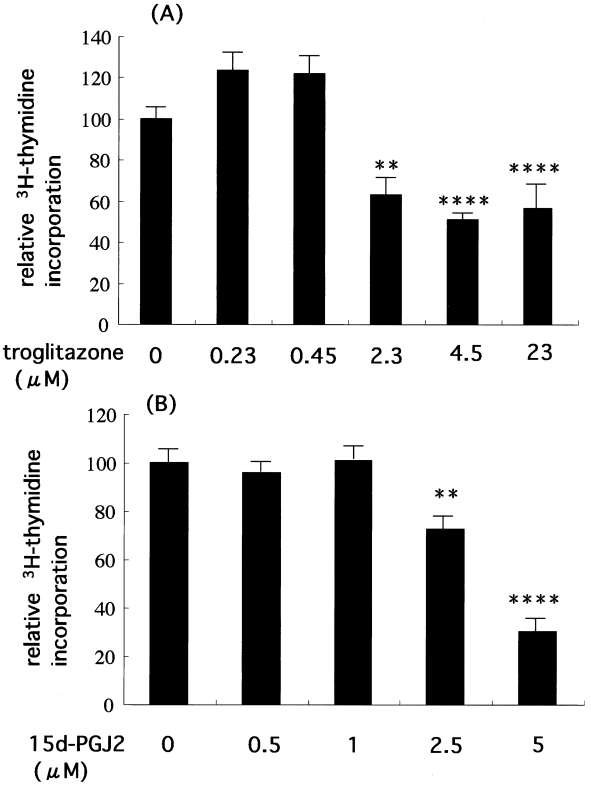

Fig. 4. Effect of troglitazone (A) and 15d-PGJ2 (B) on thymidine incorporation in rat mesangial cells. Mesangial cells were preincubated for 24 h in DMEM-F12 containing 1% FCS and 1% Nu-serum with various concentrations of 15d-PGJ2 and troglitazone. Cellular radioactivities of ^{3}H-thymidine after the incubation with 2 μCi/ml of ^{3}H-thymidine for 6 h were expressed as relative to those of control cells. Data are mean ± S.E.M., $n=4$ in each condition. $^{**}P<0.01$ vs. control. $^{****}P<0.001$ vs. control.

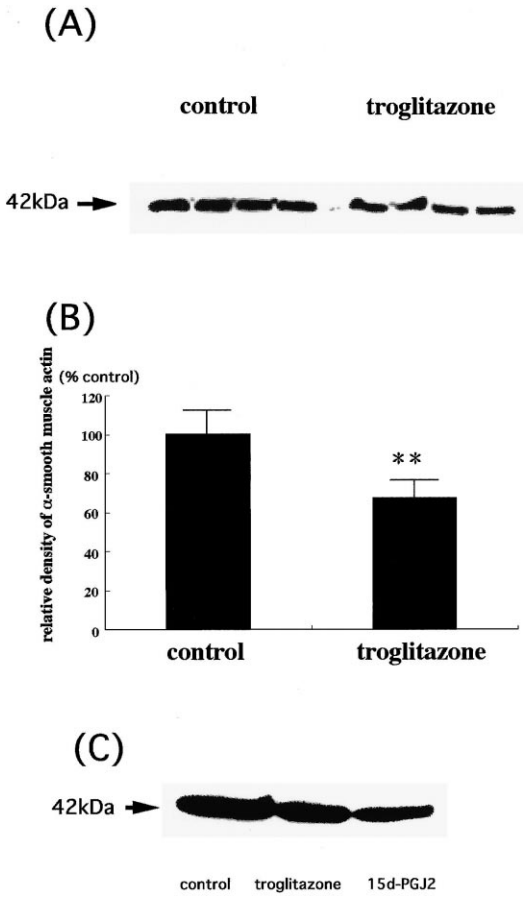

Fig. 5. The effect of troglitazone on α-smooth muscle actin expression in rat mesangial cells. Mesangial cells on 100 mm plates were incubated for 7 days in 10 ml DMEM-F12, 1% FCS, 1% Nu-serum, and 5 mM of glucose, in the presence or absence of 4.5 μM troglitazone. (A) Western blot of α-smooth muscle actin. α-Smooth muscle actin was observed as a band at 42 kDa. (B) Relative density of α-smooth muscle actin. The cell lysate corresponding to 300 ng of DNA contents were analyzed by Western blot. Data are mean ± S.E.M. $n=4$ in each condition. $^{**}P<0.01$ vs. control. (C) Comparison of effect of 2.5 μM 15d-PGJ2 and 4.5 μM troglitazone on α-smooth muscle actin expression in rat mesangial cells.

23 μM, corresponding to 0.1, 0.2, 1, 2, 10 μg/ml. Troglitazone of more than 2.3 μM and 15d-PGJ2 of more than 2.5 μM significantly decreased ^{3}H-thymidine incorporation.

The expression of α-smooth muscle actin after 7 days incubation with 4.5 μM of troglitazone was significantly decreased compared to that of control (Fig. 5A,B). Its expression did not change after 3 and 5 days incubation with troglitazone (Data not shown). 15d-PGJ2 of 2.5 μM also decreased α-smooth muscle actin after 7 days incubation (Fig. 5C).

4. Discussion

PPARγ has been reported to have three isoforms, PPARγ1, γ2 and γ3, which have specific and distinctive expression patterns in human [14]. PPARγ1 and γ3 rise to an identical protein and γ2 protein has additional amino acids in human. In the present study, we demonstrated the expression of mRNA of PPARγ by RT-PCR and PPARγ1 protein by Western blot in rat mesangial cells. These results may support the previous report that only PPARγ1 expression was observed in human kidney [15]. We also showed the protein in the nuclear extracts from rat mesangial cells bound to PPRE. These data suggest that PPARγ1 is functionally expressed in rat mesangial cells.

The α-smooth muscle actin is considered as a phenotypic marker of activated and de-differentiated mesangial cells [16]. The increase of α-smooth muscle actin is reported in mesangial proliferative glomerulonephritis [11] and diabetic nephropathy [12]. A strong expression of α-smooth muscle actin of mesangial cells by a two-dimensional culture is suppressed with the cell differentiation of the cells by a three-dimensional culture that is more near to physiological conditions [17].

Troglitazone (one of thiazolidinediones) and 15d-PGJ2 (one of metabolites of prostaglandin J2) are a synthetic ligand and a natural ligand for PPARγ, respectively. In this study, the suppressive effects of troglitazone and 15d-PGJ2 on the thymidine incorporation and production of α-smooth muscle actin by rat mesangial cells were observed, which indicate that PPARγ agonists suppress the cellular proliferation and de-differentiation of rat mesangial cells. The regulation of phenotypic changes by PPARγ is reported in smooth muscle cells and macrophages [6,9]. Since the doses of PPARγ agonists in this study seem to be high enough to upregulate transcriptional activity of PPARγ [18,19], we think the effect of PPARγ agonists on the mesangial cells in this study depends on PPARγ pathway.

PPARγ agonists are reported to have some additional effects independent of PPARγ such as an up-

regulation of NO synthesis [20,21]. The distributions of PPARs were reported in many tissues, and the presence of PPARα, δ and γ were reported in the rat glomerulus [22]. Although we did not examine PPARα and δ other than PPARγ in rat mesangial cells, these PPARs may have some effects on proliferation and differentiation of the cells, since PPARα activation is also reported to have an anti-atherogenetic effect in human smooth muscle cells [23]. Further experiments concerning PPARγ signaling and PPAR isoforms in mesangial cells are needed to clarify the effects of PPARs on the cells.

Mesangial cells play important roles in the progression of some kinds of renal disease including diabetic nephropathy, since over-production of type IV and type VI collagen by mesangial cells seem to be important to form the basement membrane thickening and mesangial expansion [24]. Recently, thiazolidinediones are reported to inhibit progression of diabetic nephropathy in rat and human without lowering glucose levels [25,26]. Phenotypic modulation of mesangial cells by an activation of PPARγ with thiazolidinediones treatment may be an additional beneficial effect on diabetic nephropathy, and may have some effect on other kidney diseases.

In conclusion, PPARγ1 is expressed in cultured rat mesangial cells, and PPARγ agonists modulates proliferation and differentiation of rat mesangial cells.

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