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Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor γ for monocyte recruitment and endothelial regeneration

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Abstract

Thiazolidinediones, a new class of antidiabetic drugs that increase insulin sensitivity, have been shown to be ligands for peroxisome proliferator-activated receptor γ (PPARγ). Recent studies demonstrating that PPARγ occurs in macrophages have focused attention on its role in macrophage functions. In this study, we investigated the effect of thiazolidinediones on monocyte proliferation and migration in vitro and the mechanisms involved. In addition, we examined the therapeutic potentials of thiazolidinediones for injured atherosclerotic lesions. Troglitazone and pioglitazone, the two thiazolidinediones, as well as 15-deoxy-Δ12,14-prostaglandin J2 inhibited in a dose-dependent manner the serum-induced proliferation of THP-1 (human monocytic leukemia cells) and of U937 (human monoblastic leukemia cells), which permanently express PPARγ. These ligands for PPARγ also significantly inhibited migration of THP-1 induced by monocyte chemoattractant protein-1 (MCP-1). Troglitazone and 15-deoxy-Δ12,14-prostaglandin J2 significantly suppressed the mRNA expression of the MCP family-specific receptor CCR2 (chemokine CCR2 receptor) in THP-1 at the transcriptional level. Furthermore, troglitazone significantly inhibited MCP-1 binding to THP-1. Oral administration of troglitazone to Watanabe heritable hyperlipidemic (WHHL) rabbits after balloon injury suppressed acute recruitment of monocytes/macrophages and accelerated re-endothelialization. These results suggest that thiazolidinediones have therapeutic potential for the treatment of diabetic vascular complications.

Keywords: Thiazolidinedione; PPARγ; MCP-1; CCR2; Macrophage; Insulin resistance

1. Introduction

Recruitment of circulating monocytes and their proliferation and differentiation into macrophages are not only the central events for initiation and progression of atherosclerosis, but have also been recently recognized as crucial pathogenic events in both diabetic micro- and macroangiopathy. Monocyte chemoattractant protein (MCP)-1 is a member of the C-C branch (or β) of the chemokine family and a potent monocyte and lymphocyte chemoattractant, which is expressed abundantly in atherosclerosic lesions (Nelken et al., 1991). MCP-1 initiates signal transduction through binding to the chemokine CCR2 receptor (CCR2) (Charo et al., 1994). In a study of CCR2 knockout mice, markedly fewer macrophages were present in the aorta of CCR2−/−, apoE−/− double knockout mice than in that of apoE−/− mice (Boring et al., 1998). Moreover, an independent study demonstrated that MCP-1−/− mice, when crossed with LDL receptor−/− mice, had smaller lesions and a significant reduction of macrophages in the lesions (Gu et al., 1998). These findings indicate the direct role of MCP-1 and CCR2 in monocyte recruitment and atherosclerosis.

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Thiazolidinediones are a new class of antidiabetic agents that increase sensitivity to insulin (Nolan et al., 1994). Insulin resistance has been attracting attention as the common casual factor not only for diabetes mellitus but also for hypertension, hyperlipidemia and obesity, all of which are risk factors for atherosclerosis (DeFronzo and Ferrannini, 1991). Recently, thiazolidinediones have been shown to be the ligands for peroxisome proliferator-activated receptor γ (PPARγ), which is a member of the nuclear receptor superfamily of ligand-activated transcription factors and has been identified as the functional receptor in antidiabetic action of thiazolidinediones (Lehmann et al., 1995).

PPARγ and the retinoid X receptor contain a heterodimer to bind regulatory elements in the promoter region of a number of adipocyte-specific genes and stimulate transcription (Tontonoz et al., 1994). In a previous study, we cloned rat PPARγ and detected down-regulation of PPARγ mRNA by several cytokines (Tanaka et al., 1999). Recent studies have demonstrated that PPARγ is expressed in cells of monocyte/macrophage lineage (Ricote et al., 1998; Tontonoz et al., 1998), and that oxidized low density lipoprotein (oxLDL), which plays a central role in atherogenesis, can regulate PPARγ-dependent gene transcription (Nagy et al., 1998). We recently reported that oxLDL potentiates, through the activation of PPARγ, the expression of vascular endothelial growth factor (VEGF) in human endothelial cells and in monocytes/macrophages (Inoue et al., 2001). Another study demonstrated that the administration of troglitazone, one of the thiazolidinediones, to Watanabe heritable hyperlipidemia (WHHL) rabbits and high fat-fed low density lipoprotein receptor or apo E knockout mice inhibits progression of atherosclerosis (Shiomi et al., 1999; Chen et al., 2001; Collins et al., 2001). All of these studies indicate the significance of PPARγ in monocyte and macrophage functions and atherogenesis.

The objective of the study presented here was to determine the effect of thiazolidinediones on the migration and proliferation of monocytes/macrophages and to investigate the molecular mechanism of the effect of thiazolidinediones on MCP-1-induced monocyte migration, with the focus on the expression of CCR2. Furthermore, we used WHHL atherosclerotic rabbits for an in vivo investigation of the therapeutic potentials of thiazolidinediones for acute monocyte recruitment and infiltration as well as for endothelial regeneration after acute vascular injury.

2. Materials and methods

2.1. Cell culture

THP-1 (human monocytic leukemia cells) and U937 (human monoblastic leukemia cells) were obtained from ATCC and cultured as previously reported (Inoue et al., 2001), with or without the following agents: troglitazone (Sankyo, Tokyo, Japan), pioglitazone (Takeda Chemical Industries, Osaka, Japan), 15-deoxy-Δ12,14-prostaglandin J2 (Sigma, St. Louis, MO), which is one of the natural ligands of PPARγ, or 9-*cis*-retinoic acid (Sigma), which is the ligand of the retinoid X receptor.

2.2. Northern blot analysis

Total cellular RNA was isolated from cultured cells using TRIzol reagents (Gibco BRL, Gaithersburg, MD). Northern blot analysis was performed as described elsewhere (Tanaka et al., 1999). The human PPARγ probe consisted of an 858-base pair fragment of the cDNA corresponding to nucleotides 329–1186 of the human PPARγ1 cDNA. The human CCR2 probe consisted of a 939-base pair fragment of the CCR2 cDNA corresponding to nucleotides 1–939. A human β-actin probe (Wako, Japan) was used to monitor the amount of total RNA in each sample.

2.3. Establishment of U937 cells permanently expressing PPARγ

U937 cells permanently expressing PPARγ were established by using the PPARγ expression vector (pCMX-mPPARγ), which contains a cytomegalovirus enhancer and mouse full-length PPARγ cDNA, as we previously reported and explained in detail (Inoue et al., 2001).

2.4. Chemotaxis assay

The cell migration was evaluated with the modified Boyden chamber technique using a 96-well chemotaxis chamber (Neuroprobe, Cabin John, MD) with 50 μl of cell suspension (2×10^7 cells/ml cells in Roswell Park Memorial Institute medium (RPMI)), as previously reported by us (Sawada et al., 2000).

2.5. Equilibrium binding analysis

The cells were suspended at a density of 2×10^7 cells/ml in 200 μl of binding buffer containing 0.1% bovine serum albumin. The cells were incubated with 0.02 nM ^{125}I-MCP-1 and various amounts of unlabelled ligand for 90 min at 25 °C. All assays were done in triplicate, and binding data were examined with the Ligand Assistance Program (Ligand Pharmaceuticals, Charlotte, NC) or Scatchard analysis.

2.6. Balloon angioplasty and troglitazone administration

Homozygous male WHHL rabbits (10 months old, 3.6±0.1 kg) were used for this study. The rabbits were supplied by Sankyo Pharmaceutical. All animals used in the present study were treated with humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH

publication No. 85-23, revised 1985). Each WHHL rabbit was fully anesthetized with sodium pentobarbital (25 mg/kg body weight). A 4F Fogarty balloon catheter was inserted from the left femoral artery, and after the balloon was inflated with air (0.3 ml in the thoracic region and 0.2 ml in the abdominal region), the intima of the thoracic and abdominal aorta was denuded by three passages of the catheter, as we previously reported (Doi et al., 2001). One group (n=8) received troglitazone at a concentration of 100 mg/kg body weight/day from 2 weeks before the angioplasty until 6 weeks after the balloon treatment and the other group (n=8) was given a control solvent. Two rabbits in the control group died during the catheterization and were dropped from the study.

2.7. Pathological examination, immunohistochemical analysis and evaluation of re-endothelialization

The rabbits were fully anesthetized and then killed 6 weeks after the angioplasty. Thirty minutes before they were killed, the animals received an intravenous injection of 6 mL of 0.5% Evans blue dye delivered via the ear vein to identify the remaining non-endothelialized area, as previously described by us (Doi et al., 2001). The area of the intimal surface that was stained blue after the application of Evans blue dye was considered to represent the portion of the arterial segment that remained endothelium deficient. Computerized planimetry (NIH image ver 1.61) was used for analysis. Next, two segments each from the thoracic aorta and the abdominal aorta were obtained from each rabbit (four sites per rabbit). The segments were fixed in methanol–Carnoy's fixative and processed routinely, embedded in paraffin and sectioned into 5-μm-thick slices. The serial sections from each segment were stained with hematoxylin–eosin or with the anti-smooth muscle actin monoclonal antibody (mAb) (1A4; Deckman) or with anti-rabbit macrophage mAb RAM11 (Dako), as we previously reported (Inoue et al., 1998). The acute recruitment or infiltration of macrophages after the angioplasty was evaluated by counting the number of RAM-11^{+} macrophages on the surface of the aorta (see Fig. 6).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) for VEGF

The aorta was frozen in liquid N2 immediately after sacrifice and stored at −80 °C until further study. The frozen aorta was homogenized in cold TRIzol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. cDNA synthesis was performed with 1 μg of total RNA, oligo(dT)20 and ThermoScript (Invitrogen). Incubation lasted for 40 min at 55 °C. The sense primer for rabbit VEGF was 5′GTGGACATCTT CCAGG AGTA-3′and the antisense primer 5′TCTTTGGTCTGCATTCAC A-3′as described previously (Skorjanc et al., 1998). For rabbit G3PDH, the sense primer was 5′ACCACGGTGCACGCCATCAC-3′ and the antisense primer was 5′TCCACCA CCCTGTTGCTGTA-3′. PCR was performed with 2 μL of cDNA template, 2.5U Platinum Taq DNA Polymerase (Invitrogen) and 0.4 μM of the sense and antisense primers. The annealing temperature was 57 °C for VEGF and 60 °C for G3PDH and the number of cycles was 30 for both. Product detection [226 nucleotides for VEGF and 450 for G3PDH] was performed after electrophoresis on 2% agarose gel using ethidium bromide staining.

2.9. Statistical analysis

All values were expressed as mean±S.E.M. Factorial analysis of variance (ANOVA) followed by the Fischer's protected least significant difference test was used to identify significant differences in multiple comparisons.

3. Results

3.1. Induction of PPARγ expression by PPARγ and retinoid X receptor ligands in monocytes/macrophages

PPARγ mRNA was not detected in U937 whether treated or not with PPARγ and retinoid X receptor ligands. While unstimulated THP-1 expressed PPARγ mRNA at a low level, treatment of THP-1 with PPARγ ligands (10^{-5} mol/l troglitazone, 10^{-5} mol/l pioglitazone, or 10^{-5} mol/l 15-deoxy-Δ12,14-prostaglandin J2) or retinoid X receptor ligand (10^{-7} mol/l 9-*cis*-retinoic acid) resulted in a significant increase of PPARγ expression in THP-1. Stimulation with a combination of troglitazone and 9-*cis*-retinoic acid resulted in further up-regulation of PPARγ mRNA expression (Fig. 1).

3.2. Inhibition of proliferation of THP-1 by PPARγ and retinoid X receptor ligands

Cells (1.0×10^{5} cells/ml) were cultured for 5 days in the presence of various doses of one of the thiazolidinediones, 15-deoxy-Δ12,14-prostaglandin J2, 9-*cis*-retinoic acid or combination thereof. When cells were treated with vehicle alone, the number of cells significantly increased 7.2 times after 5 days in culture. Troglitazone, pioglitazone, 15-deoxy-Δ12,14-prostaglandin J2 or 9-*cis*-retinoic acid caused a concentration-dependent suppression of cell growth. With 10^{-5} mol/l troglitazone, pioglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 10^{-7} mol/l 9-*cis*-retinoic acid alone, cell proliferation was inhibited by 60%, 51%, 56% and 39%, respectively, after 5 days. The simultaneous treatment of cells with both 10^{-5} mol/l troglitazone and 10^{-7} mol/l 9-*cis*-retinoic acid produced a 77% inhibition of cell growth. No effect was observed with troglitazone (10^{-5} mol/l) when used alone or in combination with 9-*cis*-retinoic acid (10^{-7} mol/l) in U937 cells in which PPARγ was not detected.

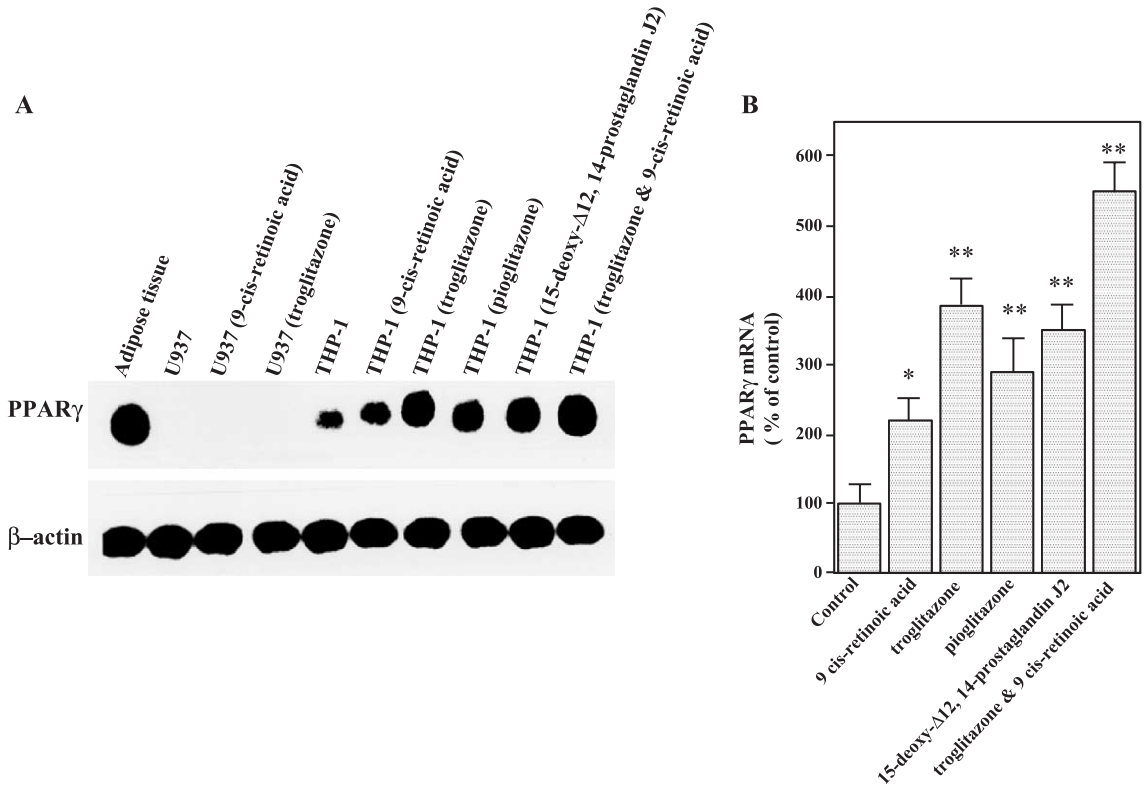

Fig. 1. Induction of PPARγ mRNA expression by PPARγ and retinoid X receptor ligands in monocytes/macrophages. U937 and THP-1 were incubated with 10^{-5} mol/l of troglitazone, pioglitazone, and 15-deoxy-Δ12,14-prostaglandin J2 and with 10^{-7} mol/l of 9-*cis*-retinoic acid for 24 h and their effects on PPARγ mRNA expression were evaluated by Northern blot analysis. (A) Northern blot analysis of PPARγ mRNA in adipose tissue, U937, and THP-1. Twenty micrograms of total RNA per lane were used for the analysis. (B) Quantitative measurements of PPARγ mRNA levels in THP-1. **P*<0.05 and ***P*<0.01 vs. corresponding controls (*n*=4).

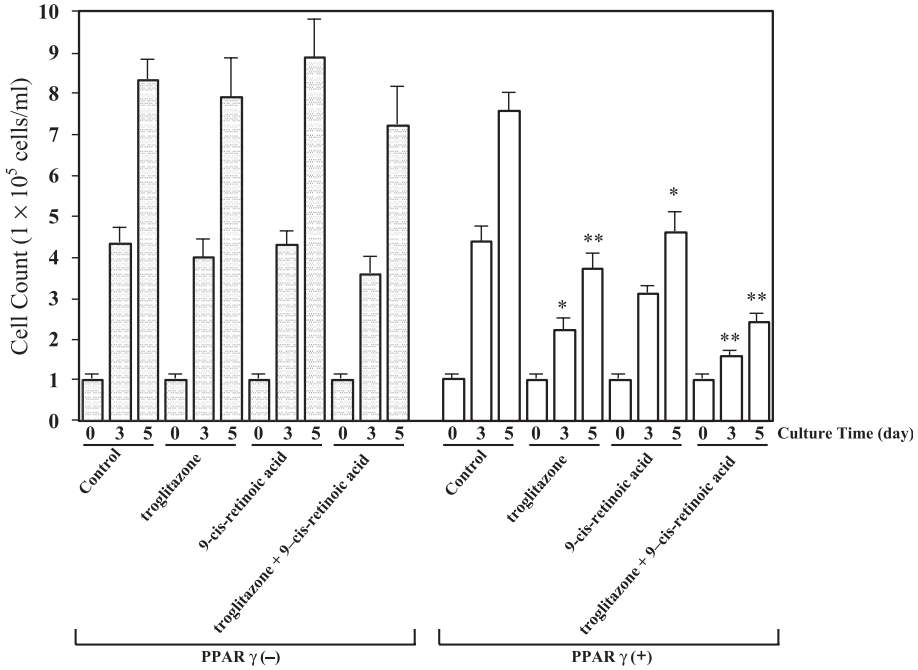

Fig. 2. Inhibition of proliferation of U937 cell expressing PPARγ by troglitazone and 9-*cis*-retinoic acid. Wild-type U937 cells and U937 cells stably transfected with PPARγ expression vector were plated at 1.0×10^5 cells/ml and cultured for 5 days in the presence of 10^{-5} mol/l of troglitazone and 10^{-7} mol/l of 9-*cis*-retinoic acid alone or in combination. Cell numbers were counted after 3 and 5 days. **P*<0.05 and ***P*<0.01 vs. corresponding controls (*n*=4).

3.3. Restoration of responsiveness to troglitazone and 9-cis RA in U937 expressing PPARγ

To further characterize the role of PPARγ in monocytes/macrophages proliferation, we utilized the permanent cell line of U937 expressing PPARγ. As shown in Fig. 2, troglitazone or 9-*cis*-retinoic acid treatment of these cell lines resulted in a marked inhibition of cell growth, similar to that of THP-1. With 10^{-5} mol/l of troglitazone alone, cell proliferation was inhibited by 56% after 5 days, and with 10^{-7} mol/l 9-*cis*-retinoic acid alone by 45%. Moreover, treatment of the cells with a combination of 10^{-5} mol/l troglitazone and 10^{-7} mol/l 9-*cis*-retinoic acid resulted in a 71% inhibition of cell growth (Fig. 2).

3.4. Inhibition of MCP-1-induced migration of THP-1 by PPARγ and retinoic X receptor ligands

The chemotactic response of THP-1 to MCP-1, troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-*cis*-retinoic acid was assessed during a 2-h incubation. The treatment with MCP-1 resulted in a dose-dependent induction in the migration of THP-1 (5 To 50 ng/ml), but THP-1 cells did not show a significant migratory response to troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 or 9-*cis*-retinoic acid. Pretreatment with troglitazone for 24 h significantly inhibited the migration of THP-1 induced by MCP-1 (25 ng/ml), and with 10^{-7} mol/l troglitazone, the migration of THP-1 was inhibited by 44%. Maximal inhibition (66%, $P<0.01$) of THP-1 migration was observed in response to treatment with 10^{-4} mol/l troglitazone (Fig. 3A). Pretreatment with 15-deoxy-Δ12,14-prostaglandin J2 or 9-*cis*-retinoic acid also inhibited the migration of THP-1 under the same conditions (Fig. 3B,C), while troglitazone and 9-*cis*-retinoic acid (10^{-7} mol/l) had an even more profound inhibitory effect on migration (Fig. 3D).

3.5. Regulation of CCR2 mRNA expression by troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-cis RA In THP-1

We also examined the effect of troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-*cis*-retinoic acid on CCR2

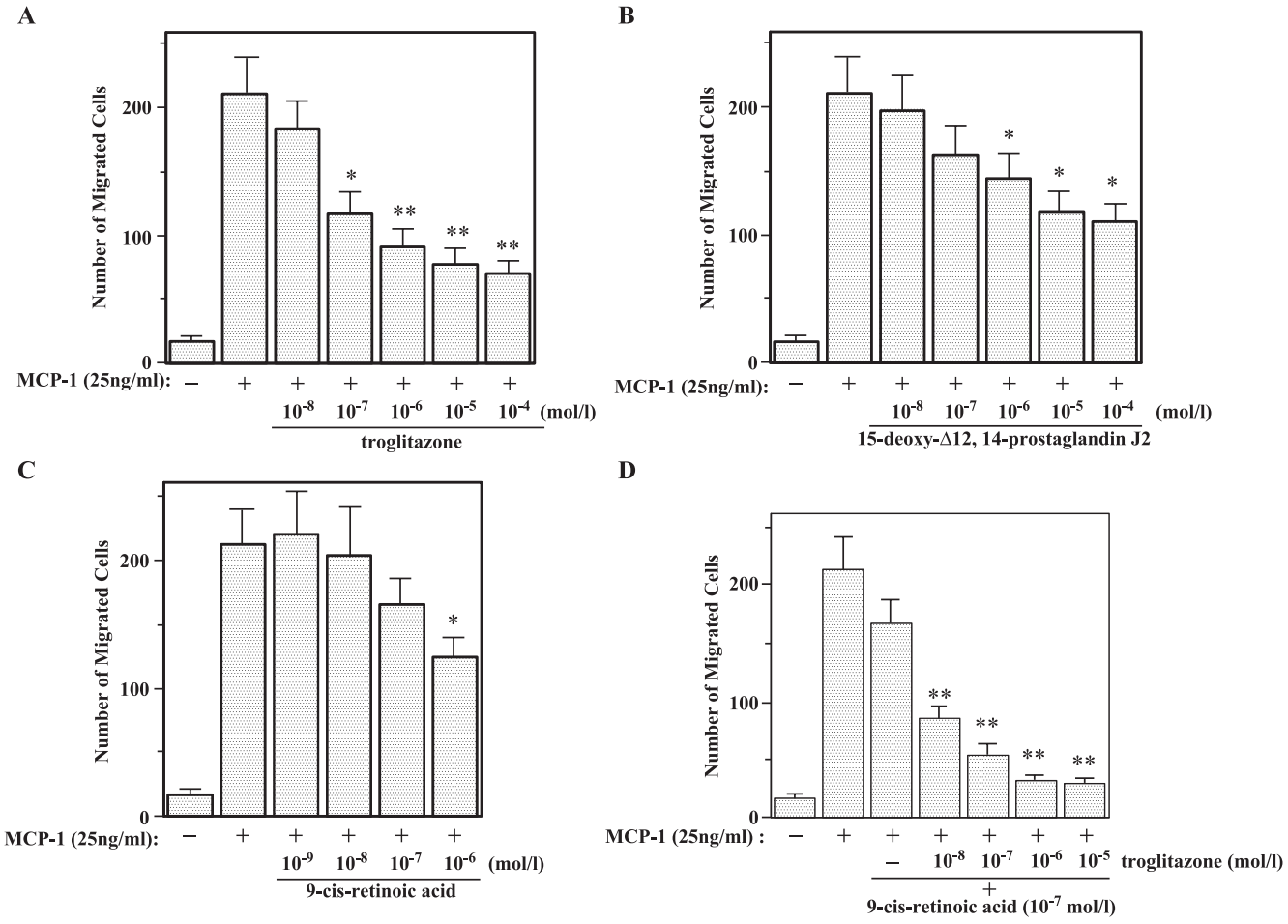

Fig. 3. Inhibition of MCP-1-induced migration of THP-1 by PPARγ and retinoid X receptor ligands. THP-1 were pretreated with different concentrations of troglitazone (A), 15-deoxy-Δ12,14-prostaglandin J2 (B), 9-*cis*-retinoic acid (C) alone or in combination with troglitazone and 9-*cis*-retinoic acid (D) for 24 h at 37 °C. MCP-1 (25 ng/ml) was added to the lower wells as the chemoattractant and 2 h migration assays were performed ($n=6$). *$P<0.05$ and **$P<0.01$ vs. 25 ng/ml of MCP-1 alone.

expression. treatment with 10^{-5} mol/l troglitazone resulted in a time-dependent reduction in the expression of CCR2 mRNA. The inhibitory effect of 10^{-5} mol/l of troglitazone was first observed after a 6-h incubation and persisted for at least a 48-h exposure (Fig. 4A). Treatment with troglitazone (10^{-7} mol/l to 10^{-4} mol/l) suppressed CCR2 mRNA expression in a dose-dependent fashion (Fig. 4B,C). The maximum decrease of 93% occurred in response to 10^{-4} mol/l of troglitazone. Treatment with 15-deoxy-Δ12,14-prostaglandin J2 (10^{-6}–10^{-4} mol/l) and with 9-*cis*-retinoic acid (10^{-7}–10^{-6} mol/l) also suppressed the CCR2 mRNA level in a dose-dependent fashion (Fig. 4B,C).

To determine whether the down-regulation of CCR2 is a transcriptional or post-transcriptional event, we analyzed the effect of troglitazone on the expression of CCR2 mRNA in the presence of actinomycin D (3 μg/ml, Sigma), a transcriptional inhibitor. When treated with actinomycin D, a linear decrease in the level of CCR2 mRNA without the treatment of troglitazone was observed (T1/2; 3 h). With the treatment of troglitazone, the level of CCR2 mRNA also

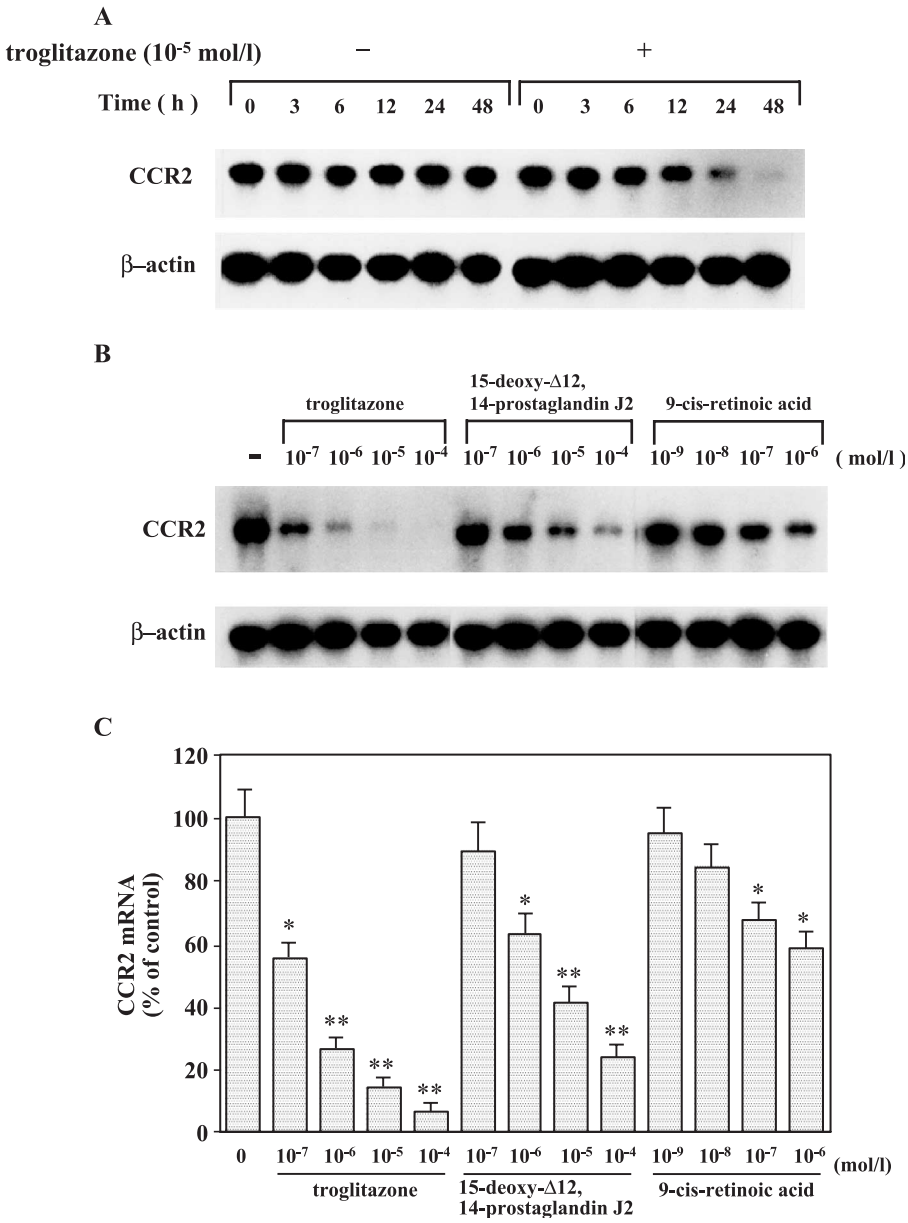

Fig. 4. Regulation of CCR2 mRNA expression by troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 or 9-*cis*-retinoic acid in THP-1. (A) Time-dependent effect of troglitazone on CCR2 mRNA expression. THP-1 cells were incubated with or without 10^{-5} mol/l of troglitazone and harvested after various incubation times for RNA isolation. Twenty micrograms of total RNA per lane was used for the analysis. Similar results were obtained in three independent experiments. (B) Concentration-dependent effect of troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-*cis*-retinoic acid on CCR2 mRNA expression. THP-1 were incubated with or without various concentrations of troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-*cis*-retinoic acid harvested 24 h after incubation. Twenty micrograms of total RNA per lane was used for the analysis. (C) Quantitative measurements of CCR2 mRNA levels after administration of troglitazone, 15-deoxy-Δ12,14-prostaglandin J2, or 9-*cis*-retinoic acid. **P*<0.05 and ***P*<0.01 vs. corresponding controls (*n*=4).

decreased, but at a similar rate to that of the control not treated with troglitazone (Fig. 5A,B).

3.6. Inhibition of MCP-1 binding to THP-1 by troglitazone and 9-cis RA

Under the same experimental condition as described above, we also examined the effects of troglitazone on the binding of MCP-1 to THP-1. THP-1 was pretreated for 24 h with or without 10^{-5} mol/l of troglitazone. As illustrated in Fig. 6A, Scatchard analysis showed that K_d values were similar for the control and troglitazone-treated groups (0.61±0.13 nmol/l for the control and 0.65±0.16 nmol/l for the troglitazone-treated group). In contrast, the troglitazone-treated THP-1 expressed 4.3±0.8 fmol of receptors/10^6 cells, while the control THP-1 expressed 11.7±1.6 fmol/10^6 cells. Thus, troglitazone (10^{-5} mol/l) reduced the number of MCP-1 receptor on the cell surface by 64%. Fig. 6B shows the time course of the effect of troglitazone and 9-*cis*-retinoic acid on the binding of MCP-1 to THP-1. At 10^{-5} mol/l of troglitazone alone, the binding was inhibited by 51% after 12 h and by 66% after 24 h, and the simultaneous treatment of cells with 10^{-5} mol/l troglitazone and 10^{-7} mol/l 9-*cis*-retinoic acid resulted in 85% inhibition.

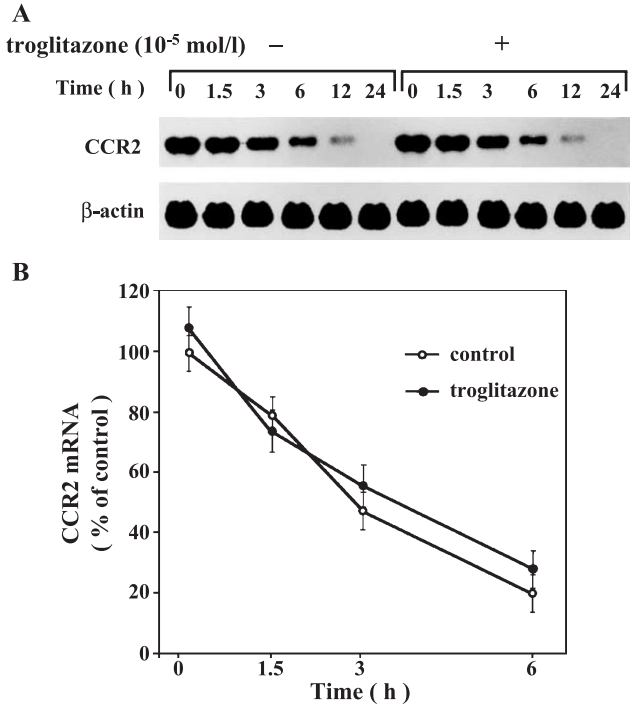

Fig. 5. Effect of troglitazone on the expression of CCR2 mRNA in the presence of actinomycin D. (A) THP-1 were incubated with 3 μg/ml of actinomycin D with or without 10^{-5} mol/l of troglitazone for 24 h and harvested after various incubation times for RNA isolation. Twenty micrograms of total RNA per lane was used for the analysis. (B) Quantitative measurements of CCR2 mRNA levels after treatment with 3 μg/ml of actinomycin D with or without 10^{-5} mol/l of troglitazone (n=3).

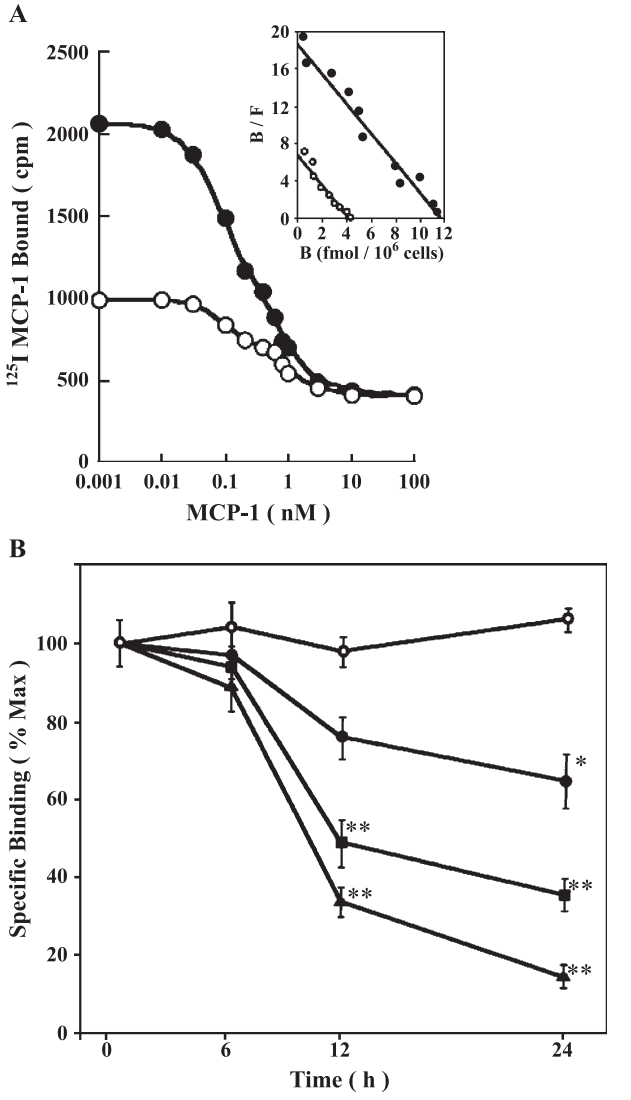

Fig. 6. Inhibition of MCP-1 binding to THP-1 by troglitazone and 9-*cis*-retinoic acid. (A) ^{125}I-labelled MCP-1 binding curve and Scatchard plot analysis. THP-1 were pretreated with (white circles) or without (black circles) 10^{-5} mol/l of troglitazone for 24 h at 37 °C. Insets show Scatchard analysis of the specific binding data. The results are representative of three independent experiments. (B) Time course of troglitazone and 9-*cis*-retinoic acid-induced reduction of MCP-1 binding to THP-1. The cells were incubated for up to 24 h at 37 °C without treatment (white circle) or with 10^{-5} M of troglitazone (black square) or 10^{-7} mol/l of 9-*cis*-retinoic acid (black circle) alone or in combination (black triangle). The binding of ^{125}I-MCP-1 was determined in the presence or absence of 100 nmol/l of unlabeled MCP-1. *P<0.05 and **P<0.01 vs. corresponding controls.

3.7. Suppression by troglitazone of monocyte and macrophage recruitment onto the balloon-injured aorta of WHHL rabbits

At the end of the study, 10-month-old WHHL rabbits belonging to the control group exhibited severe atherosclerotic lesions in thoracic aortae (61±11% of the total surface area) and patchy lesions in abdominal aortae (37±9%). Treatment with troglitazone for a total of 8 weeks did not

noticeably change the gross appearance of the atherosclerotic lesions (66±8% in thoracic aortae and 32±7% in abdominal aortae). The immunohistochemical analysis showed that the troglitazone treatment had no significant effect on the total content of $1A4^{+}$ vascular smooth muscle cells (1A4-positive area/atheromatous area: 11.4±3.9% in

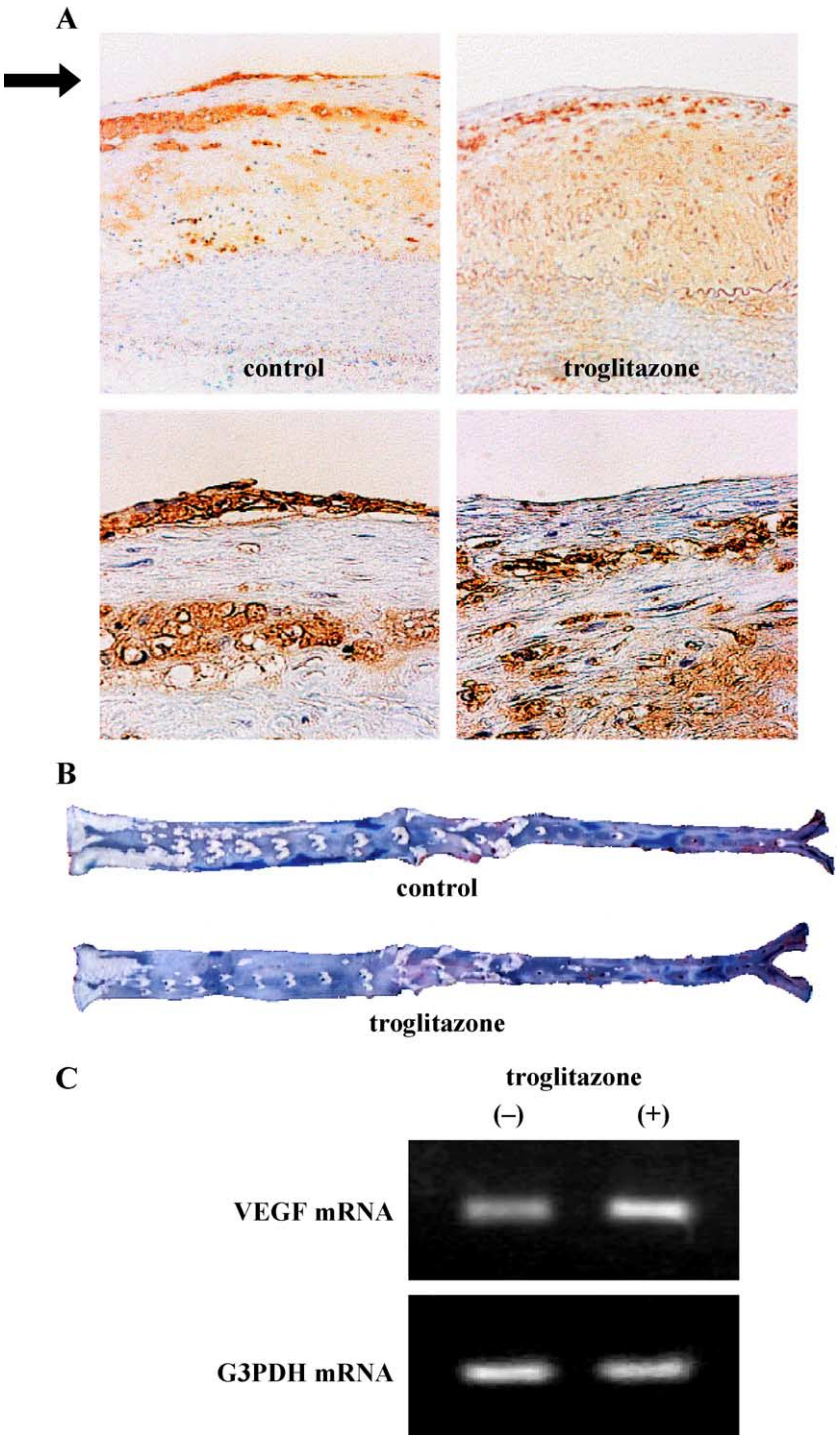

Fig. 7. Effect of troglitazone administration to balloon-injured WHHL rabbits. (A) Suppression of recruitment of monocytes/macrophages onto the surface of the aorta of WHHL rabbits 6 weeks after balloon injury. Troglitazone was administered every day for 8 weeks, from 2 weeks before injury until sampling. RAM11-positive immunostaining shows monocytes/macrophages in brown. Top: Lower magnification of the whole blood vessel. Bottom: Higher magnification of the surface of the balloon-injured aortas (the portion indicated by arrow in top). (B) Macroscopic appearance of balloon-injured aortas of WHHL rabbits after the injury. Area of re-endothelialization is not stained by Evans blue dye and appears white. Control: vehicle-treated group; troglitazone: troglitazone-treated group. (C) Analysis of VEGF gene expression. Pooled frozen aortic samples (vehicle-treated group: n=6; troglitazone-treated group: n=8) were subjected to RT-PCR for VEGF.

the abdominal and 9.3±1.1% in the thoracic aortae of the control group; 6.3±0.7% in the abdominal and 7.8±1.7% in the thoracic aortae of the troglitazone-treated group), although there was a tendency for the vascular smooth muscle cell content to decrease as a result of the troglitazone treatment, which is compatible with the results of previous studies with different experimental protocols (Shiomi et al., 1999; Law et al., 1996). Nor did the treatment significantly affect the total content of $RAM11^+$ monocytes/macrophages (RAM11-positive area/atheromatous area: 11.6±3.7% in the thoracic and 14.3±4.8% in the abdominal aortae of the control group; 14.9±3.1% in the thoracic and 12.8±2.5% in the abdominal aortae of the troglitazone-treated group).

However, the number of acutely recruited monocytes/macrophages onto the surface of the balloon injured aorta was significantly lower in the abdominal aorta of the troglitazone-treated group (39±5% of the control, $P<0.05$), although in the thoracic aorta of this group the reduction in numbers did not reach statistical significance (82±7% of the control) (Fig. 7A).

3.8. Re-endothelialization in balloon-injured aorta of WHHL rabbits accelerated by troglitazone

Evans blue staining demonstrated that the denuded area was significantly smaller in the aorta of the troglitazone-treated group than of the control group (Fig. 7B; Evans blue-stained area/non-atheromatous area: 0.4±0.2% for the troglitazone-treated and 4.8±1.2% for the control group; $P<0.05$). RT-PCR for VEGF showed that VEGF gene expression had increased in the aorta of the troglitazone-treated group (Fig. 7C).

4. Discussion

The study presented here demonstrated that PPARγ and retinoid X receptor ligands caused a concentration-dependent suppression of cell growth in PPARγ-expressing THP-1 cells. In contrast, troglitazone and 9-*cis*-retinoic acid had no effect on the proliferation of wild-type U937 which lack PPARγ expression. We further demonstrated that U937 cell lines with stable PPARγ expression restored the responsiveness to troglitazone and 9-*cis*-retinoic acid for growth suppression. These findings indicate that activation of PPARγ in monocytes/macrophages causes growth suppression of these cell lines.

We could also demonstrate that PPARγ and retinoid X receptor specific ligands strongly inhibited the MCP-1-induced migration of THP-1. This result is compatible with those reported in previous publications (Zhu et al., 1999; Kintscher et al., 2000). To investigate the potential mechanism of inhibition of MCP-1-induced migration by PPARγ activation, we also examined changes in the functional expression of CCR2 in THP-1. We found that troglitazone, 15-deoxy-Δ12,14-prostaglandin J2 and 9-*cis*-retinoic acid had a strong down-regulatory effect on CCR2 mRNA expression at the transcriptional level. We further confirmed that MCP-1 binding activity was also reduced and the number of MCP-1 receptors (CCR2) declined as a result of exposure to troglitazone and 9-*cis*-retinoic acid. Since it has been reported that in an in vitro chemotaxis assay, monocytes derived from CCR2 knockout mice failed to migrate in response to MCP-1 (Boring et al., 1997), the suppressive action of PPARγ and retinoid X receptor ligands on MCP-1-induced migration can be interpreted as representing the down-regulation of CCR2 expression.

Other studies have demonstrated that CCR2 or MCP-1 knockout mice are less susceptible to atherosclerosis and showed low monocyte recruitment in vascular lesions (Boring et al., 1998; Gu et al., 1998). The potent suppressive action of PPARγ and retinoid X receptor ligands on MCP-1-induced migration of THP-1 observed in our study suggests therefore the protective role of PPARγ in the development of atherosclerosis.

We also investigated in vivo the therapeutic effectiveness of troglitazone on acute recruitment of monocytes/macrophages onto the atheromatous lesion after balloon injury in WHHL rabbits. Since we utilized 10-month-old WHHL rabbits with fully developed atherosclerosis to examine the effect of troglitazone on the acute recruitment of monocytes onto atheromatous lesions, the effect of troglitazone on pre-existing atheromas was not as prominent as that reported by another study, which examined the effect of troglitazone on the development of atherosclerosis in younger WHHL rabbits (Shiomi et al., 1999). However, compatible with the in vitro effect of thiazolidinediones on monocytes/macrophages observed in our study, we also found in vivo evidence of suppression of acute adhesion and/or subsequent transendothelial migration in response to troglitazone treatment. Han et al. (2000) recently showed that oxLDL reduces circulating monocyte CCR2 expression through activation of PPARγ and postulated that oxLDL may promote the arrest of newly recruited monocytes in the arterial wall. However, our findings contradict their hypothesis, that is, the administration of the PPARγ agonist suppressed attachment and/or proliferation of monocytes/macrophages on atherosclerotic lesions at the site of balloon injury.

A previous study of ours found that thiazolidinediones stimulate endothelial proliferation and induce regeneration in vitro within clinically relevant doses (Fukunaga et al., 2001). The significant acceleration of re-endothelialization in the aorta after balloon injury observed in the study presented here was thus highly compatible with our previous in vitro findings. Accelerated re-endothelialization may be ascribed to the enhanced expression of pro-angiogenic factors previously demonstrated by us (Inoue et al., 2001; Itoh et al., 1999). In fact, troglitazone administration used in the current study showed increased gene expression of VEGF in the aorta of the WHHL rabbits.

In conclusion, we showed that PPARγ and retinoid X receptor specific ligands inhibited proliferation and migration of monocytes/macrophages as well as suppressed the functional expression of the MCP-1 receptor, CCR2 in THP-1. The administration of thiazolidinediones to WHHL rabbits inhibited monocyte/macrophage recruitment and induced endothelial regeneration after balloon injury. These results indicate the involvement of PPARγ in modulating monocyte proliferation, recruitment and transmigration through the endothelial cell layers under various pathological conditions and suggest the therapeutic potential of thiazolidinediones for diabetic vascular complications, which has been implied by some human studies (Minamikawa et al., 1998).

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