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Differential effects of YM440 a hypoglycemic agent on binding to a peroxisome proliferator-activated receptor γ and its transactivation

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Abstract

Peroxisome proliferator-activated receptor (PPAR) γ is a ligand-inducible transcription factor mediating glucose and lipid metabolism. Prior studies showed that YM440 ameliorated hyperglycemia in diabetic mice without affecting body fat weight or PPARγ transactivation. In this study we have examined further the effects of YM440 on PPARγ binding, transactivation and conformational change. YM440, pioglitazone and rosiglitazone displaced [^{3}H]rosiglitazone from PPARγ with K_i values of 4.0, 3.1, and 0.20 μM, indicating that YM440 was comparable to pioglitazone and 20-fold less potent than rosiglitazone. Although pioglitazone and rosiglitazone increased both PPARγ transactivation in cells expressing human full-length PPARγ2 or GAL4-PPARγ and mRNA expression of PPARγ responsive genes in 3T3-L1 cells, YM440 had weak effects on PPARγ transactivation and mRNA expression being 550- to 790-fold and 36- to 110-fold less active than rosiglitazone, respectively. YM440 and rosiglitazone induced interaction between PPARγ and the transcriptional cofactor, p300 or SRC-1, but YM440 was 151- and 1091-fold less potent than rosiglitazone, respectively. The weak transcriptional activity of YM440 was not due to poor cell permeability. Limited trypsin digestion of the full-length human PPARγ2 with YM440 or rosiglitazone showed distinct patterns of digestion, suggesting a difference in the conformational change of PPARγ. When db/db mice were treated with YM440 (100 mg/kg) for 28 days, YM440 increased hepatic glucokinase expression but not adipose tissue FABP and UCP1 expression, indicating a tissue selective expression of PPARγ-related genes. Unique properties regarding the binding-transactivation of PPARγ by YM440 may lead to the hypoglycemic activity without affecting body fat weight in diabetic mice.

Keywords: Peroxisome proliferator-activated receptor γ; Receptor binding; Transactivation; Adipocyte differentiation; Hypoglycemic agents; Thiazolidinedione

1. Introduction

A group of compounds which possess a 2,4-thiazolideinadione ring (TZDs), including pioglitazone and rosiglitazone, ameliorate hyperglycemia and hyperinsulinemia in diabetic animals with insulin resistance and patients with diabetes [1–3]. It is well established that TZDs improve insulin sensitivity by activating the peroxisome proliferator-activated receptor γ (PPARγ). TZDs bind to the ligand binding domain of PPARγ (PPARγ LBD) and activate PPARγ in a receptor assay [4]. These activities are well correlated with the potency in lowering blood glucose in diabetic mice [5].

Recently, it was reported that some TZDs and a series of L-Tyr-based PPARγ ligands do not act as typical agonists of PPARγ but ameliorate hyperglycemia in diabetic animal models with insulin resistance. These agents cause little or

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; YM440, (*Z*)-1,4-bis{4-[(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)methyl]phenoxy}but-2-ene; FABP, fatty-acid binding protein; LPL, lipoprotein lipase; UCP, uncoupling protein; SRC-1, steroid receptor coactivator-1; SPA, scintillation proximity assay; LBD, ligand binding domain; 15d-PGJ2, 15deoxy-^{12,14} prostaglandin J2; GST, glutathione *S*-transferase; TZDs, 2,4-thiazolidinediones.

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no change in adipocyte differentiation *in vitro* and have a weak effect on body weight gain and fat weight *in vivo*. Some TZDs are partial agonists of PPARγ and a series of L-Tyr-based PPARγ ligands activate PPARγ with less potency than, but a similar maximal efficacy to rosiglitazone [6–8]. NC-2100 is an analog of TZDs and a weak PPARγ activator, but significantly exhibits antidiabetic effects in KK-A^y obese mice [6]. FMOC-L-leucine (F-L-Leu) improves insulin sensitivity in diabetic mice and has weak adipogenic activity, suggesting that it is a selective PPARγ modulator that activates some (insulin sensitization), but not all (adipogenesis), PPARγ-signaling pathways [8]. Although the molecular mechanism by which PPARγ activates target genes remains largely unknown, it is important to clarify whether the state of PPARγ affects the selection of the genes transactivated, which encode proteins necessary for glucose and lipid metabolism and adipocyte differentiation.

Recently, the X-ray crystal structure of PPARγ LBD has been elucidated, revealing that ligand binding causes a conformational change within PPARγ such that the receptor is converted into an ''activated form'' that promotes the recruitment of coactivators such as steroid receptor coactivator 1 (SRC-1) and p300 [9,10]. It has been postulated that these coactivators act as bridges to transmit the nuclear receptor regulatory signals to the cellular transcriptional machinery. Studies on estrogen and progesterone receptors suggested that a range of distinct receptor conformations could be induced by the binding of different ligands [11,12]. Although less is known about PPARγ in this regard, it is possible that different PPARγ ligands induce unique conformational changes in the receptor, eliciting distinct downstream biological effects [13].

YM440, an analog of the oxadiazolidinediones (Fig. 1), has similar hypoglycemic activity to troglitazone and pioglitazone but no effect on body weight, adipocyte differentiation, or PPARγ transactivation [14]. In this study we have compared YM440 with pioglitazone and rosiglitazone for its ability to activate PPARγ in several different experimental settings, including receptor binding, transcriptional activation, coactivator recruitment, and receptor conformation. We report here that YM440 binds PPARγ with unique conformational changes.

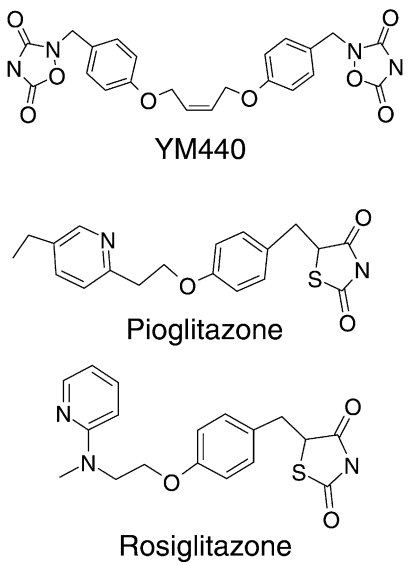

Fig. 1. Chemical structures of YM440, pioglitazone, and rosiglitazone.

2. Materials and methods

2.1. Materials

All reagents used in this study were of analytical grade and obtained commercially. (*Z*)-1,4-bis{4-[(3,5-Dioxo-1,2,4-oxadiazolidin-2-yl)methyl] phenoxy}but-2-ene (YM440), pioglitazone hydrochloride (pioglitazone) and rosiglitazone malate (rosiglitazone) were synthesized at Yamanouchi Pharmaceutical Co. Ltd. [^{3}H]rosiglitazone was purchased from American Radiolabeled Chemicals Inc.

2.2. Ligand binding assay for PPARγ

Ligand binding domain (LBD) of PPARγ was prepared and a scintillation proximity assay (SPA) for PPARγ LBD was performed according to the method reported by Nichols *et al.* [15]. Briefly, His-tagged PPARγ LBD was expressed in *Escherichia coli* strain JM-109 and the PPARγ LBD in the extracts was purified by elution through a nickel-ion agarose (Amersham Pharmacia Biotech) and Talon column (Clontech). Then, the purified PPARγ LBD was biotinylated with NHS-LC-Biotin (Pierce) and used to coat streptavidine SPA beads. Binding assays were performed in 96-well NBS plates (Corning) by incubating the PPARγ LBD-coated SPA beads, 10 nM [^{3}H]rosiglitazone and 1 nM–0.1 mM of the test compound at room temperature for 2 hr. After centrifugation at 1500 rpm, the radioactivity in each well was quantified in a Packard Topcount scintillation counter. K_i values were calculated according to the equation proposed by Cheng and Prusoff [16]: $K_i = \mathrm{IC}_{50}/(1 + [L]/K_d)$, where $[L]$ is the concentration of [^{3}H]rosiglitazone (10 nM) and the K_d for rosiglitazone is 45 nM.

2.3. PPARγ activation assay

CV-1 cells or HepG2 cells were maintained in Dulbecco's modified eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. A full-length human PPARγ2 expression vector was constructed by introducing the cDNA inserts into pcDNA3.1. A PPRE_3-TK-luciferase reporter vector containing three copies of the PPRE (GTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGAC) was also constructed [17]. The full-length

RXRα expression vector and β-galactosidase expression vector pCH110 were purchased from Invitrogen and Amersham Pharmacia Biotech, respectively. The GAL4-PPARγ expression vector contained a GAL4 DNA binding domain and human PPARγ LBD in the pSG5 expression vector (Stratagene). A RE_8-luciferase reporter vector was constructed by ligating a TATA box above eight copies of GAL4 responsive elements (RE_8) and then inserting it into the 5′-side upstream of the luciferase gene sequence in a vector for luciferase assay system [Pica Gene Vector-2, (PGV-B2), Toyo Ink Mfg]. Following transfection of the vectors using Fugene 6 (Roche Diagnostics), the cells were incubated for 24 hr with the test compound. Cell extracts were prepared and the activities of luciferase and β-galactosidase were determined. The activity of luciferase was normalized to that of β-galactosidase. Each EC_{50} value for PPARγ activation was determined by nonlinear curve fitting using a Statistical Analysis System (SAS Software, SAS Institute).

2.4. Analysis of cell differentiation in 3T3-L1 cells

3T3-L1 preadipocytes (American Type Cell Collection) were grown in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37° in a humidified atmosphere of 5% CO_2 in air [14]. To evaluate the effects of test compounds on the differentiation of 3T3-L1 preadipocytes into adipocytes, confluent 3T3-L1 preadipocytes in 6-well culture plates were incubated with various concentrations of the test compound and 25 nM dexamethasone for 96 hr.

2.5. Real-time quantitative PCR

Total RNA was extracted from the cells using ISOGEN, a mixture of acid guanidium isothiocyanate–phenol–chloroform, and treated with DNase according to the manufacturer's instructions (Nippon Gene). mRNA levels were determined using real-time quantitative RT-PCR analysis on an ABI PRISM 7700 system (Applied Biosystems) as described previously [18]. Oligonucleotide primers (forward, reverse) and TaqMan probes were designed using Primer Express™ (Applied Biosystems). The GenBank accession number and the designed primers and probes were as follows: mouse fatty-acid binding protein (FABP) (GenBank accession number K02109, forward primer 5′-AAAACACCGAGATTTCCTTCA-3′ and reverse primer 5′-CTCTTCACCTTCCTGTCGTCT-3′, TaqMan probe 5′-TGGGCGTGGAATTCGATGAAATCA-3′) [19,20], mouse lipoprotein lipase (LPL) (M60847, forward primer 5′-CGCTCCATTCATCTCTTCA-3′ and reverse primer 5′-CTTGTTGATCTCATAGCCCA-3′, TaqMan probe 5′-CTTTGAGAAAGGGCTCTGCCTGA-3′) [21], mouse uncoupling protein 2 (UCP2) (U69135, forward primer 5′-GTTCCTCTGTCTCGTCTTGC-3′ and reverse primer 5′-GGCCTTGAAACCAACCA-3′, TaqMan probe 5′-CTTCTGGGAGGTAGCAGGAAATCAG-3′) [22] and mouse β-actin (X03672, forward primer 5′-GTCATCACTATTGGCAACGAG-3′ and reverse primer 5′-CACTGTGTTGGCATAGAGGTC-3′, TaqMan probe 5′-CCATCATGAAGTGTGACGTTGACA-3′). Each EC_{50} value for PPARγ activation was determined by nonlinear curve fitting using a Statistical Analysis System.

2.6. Glutathione S-transferase (GST) pull-down assays

Full-length human PPARγ2 cDNA was expressed as a GST fusion protein (GST-PPARγ2) in *E. coli* (JM-109) according to the methods reported by Kodera *et al.* [23]. The expression of a protein of the predicted size was then monitored by SDS–PAGE. For GST pull-down assays, GST-PPARγ2 was bound to glutathione-sepharose 4B beads (Amersham Pharmacia Biotech). SRC-1 and p300 cDNA cloned into pcDNA3.1 were used to generate [^{35}S]methionine-labeled proteins using a TNT-coupled *in vitro* translation system (Promega). The [^{35}S]methionine-labeled SRC-1 or P300 proteins were incubated with beads containing GST-PPARγ2 in the presence or absence of rosiglitazone or YM440 in NET-N buffer (0.5% Nonidet P-40, 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, and 1 mM EDTA) with 1 mM phenylmethylsulfonyl fluoride. After a 1-hr incubation, the beads were washed with NET-N buffer to remove free protein. Bound proteins were extracted with loading buffer, separated by 4–20% SDS–PAGE, and visualized by autoradiography.

2.7. Cellular uptake of YM440 and rosiglitazone

The PPARγ-transfected CV-1 cells were incubated with 1 μM [^{3}H]rosiglitazone or [^{14}C]YM440 for 0.5, 2, 6, and 24 hr in DMEM buffer containing 10% FBS. After the incubation, the medium was removed and the cells were washed three times with ice-cold PBS containing 10 μM rosiglitazone or YM440. The cells were then solubilized using 0.5% sodium dodecyl sulfate and the radioactivity was measured with a scintillation counter.

2.8. Protease digestion assay

Approximately 2 μL of [^{35}S]methionine-labeled, full-length human PPARγ2 synthesized *in vitro* was preincubated with 2 μL of PBS with or without a test compound for 15 min at 25°. Then, 4 μL of distilled water (dH_2O) or dH_2O-solubilized trypsin was added. The protease digestion was allowed to proceed for 10 min at 25°, then terminated by the addition of 8 μL of denaturing gel loading buffer and boiling for 5 min. The products of digestion were separated by electrophoresis through a 1.5-mm 20–4% gradient polyacrylamide–SDS gel. The gel was then fixed in 10% acetic acid (v/v)–40% methanol (v/v) for 30 min, and dried under vacuum for 1 hr at 80°. The bands were detected with a BAS2000 bioimaging

analyzer. To measure the relative levels of the bands protected by a test compound on a gel, the intensity of the band of full-length human PPARγ2 treated without trypsin, around 50 kDa, was taken as 100%.

2.9. Animals and experimental design

Male C57BL/KsJ-db/db and db/+ mice were purchased from Charles River Japan and kept under a 12 hr light:12 hr dark cycle. Mice were fed moderately high-calorie laboratory chow (CMF, 373 kcal/100 g, Oriental Yeast Industry). YM440 and pioglitazone (100 mg/kg) were given orally to db/db mice at 8 weeks of age for 28 days. On day 29, after the mice had been sacrificed, retroperitoneal fat and liver were removed for the extraction of total RNA. The animal study was performed in accord with the legal requirements of the Animal Use Committee of Yamanouchi Pharmaceutical Co. Ltd. The levels of expression of mRNAs encoding FABP, a marker of adipose differentiation [19,20], UCP1 [19], glucokinase [24] and β-actin in mouse tissues were determined by the RNase protection method [25]. Briefly, total RNA was extracted from the tissues using ISOGEN. Extracted RNA (3 μg) was hybridized to [α-^{32}P] UTP-labeled antisense riboprobe at 50° overnight. Samples were digested with RNase A/T1 according to the manufacturer's instructions (Ambion) at 37° for 1 hr and analyzed by electrophoresis on a 3.5% polyacrylamide gel containing 8 M urea. The bands were detected with a BAS2000 bioimaging analyzer (Fuji Film). The lengths of the protected fragments were as follows; FABP (150 bp 192-342, GenBank accession number K02109), UCP1 (277 bp 244-521, U63419), glucokinase (397 bp 565-961, M58755) and β-actin (135 bp 675-809, X03672). The signal was normalized against that for β-actin.

2.10. Statistical analysis

Comparisons between experimental groups were made using Dunnett's multiple range test or *t*-test. Differences were considered significant at a *P* level of less than 0.05.

3. Results

Prior studies have shown YM440 and pioglitazone ameliorate hyperglycemia in diabetic db/db mice, and pioglitazone, but not YM440, increases body weight, fat weight and adipocyte differentiation, indicating YM440 ameliorates hyperglycemia without changing PPARγ activity [14].

3.1. Effects of YM440 on binding to and transactivation of PPARγ

The effects of YM440, pioglitazone and rosiglitazone on PPARγ binding were examined using the SPA binding assay system (Fig. 2). YM440 displaced [^{3}H]rosiglitazone

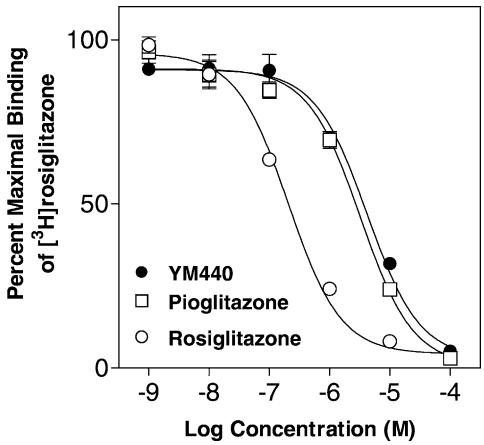

Fig. 2. Binding of YM440, pioglitazone, and rosilglitazone to PPARγ. A competition binding assay was performed for 2 hr with 10 nM [^{3}H]rosiglitazone and 100 μg of SPA beads in the presence or absence of increasingly higher concentrations of the test compound. Each point with a vertical line represents the mean ± SEM of 4–8 determinations, which were obtained in at least two independent experiments performed in duplicate. Data were normalized to the value for vehicle-control. The K_i values (95% confidence limits) are as follows: (●)YM440 4.0 (2.8–4.8) μM; (□) pioglitazone 3.1 (2.2–4.4) μM; (○) rosiglitazone 0.20 (0.15–0.26) μM.

from PPARγ-LBD with a K_i value of 4.0 μM, indicating YM440 was comparable to pioglitazone in receptor binding (K_i: 3.1 μM) and 20-fold less active than rosiglitazone (K_i: 0.20 μM). To examine the effects on PPARγ transactivation function, PPARγ reporter activity was examined in CV-1 or HepG2 cells expressing human full-length PPARγ2 (Fig. 3A and B) or in HepG2 cells expressing GAL4-PPARγ (Fig. 3C). Rosiglitazone and pioglitazone dose-dependently increased PPARγ transactivation in CV-1 and HepG2 cells. YM440, however, little affected the transactivation. The EC_{50} values of rosiglitazone, pioglitazone, and YM440 for PPARγ transactivation in the cells expressing human full-length PPARγ2 were 0.20, 0.64 and 110 μM in CV-1 cells and 0.049, 0.36 and 31 μM in HepG2 cells, respectively. The EC_{50} values of rosiglitazone, pioglitazone, and YM440 for PPARγ transactivation in HepG2 cells expressing GAL4-PPARγ were 0.042, 0.81 and 33 μM, respectively. The results indicated that YM440 was about 40- to 175-fold less potent than pioglitazone and about 550- to 790-fold less potent than rosiglitazone regarding PPARγ transactivation in CV-1 and HepG2 cells.

To examine further the effects on mRNA expression of PPARγ responsive genes in 3T3-L1 cells, mRNA expression of FABP, LPL and UCP2 was examined (Fig. 4A–C). Rosiglitazone and pioglitazone dose-dependently increased mRNA expression but YM440 produced a weak increase in the mRNA expression of these genes. The EC_{50} values of rosiglitazone, pioglitazone, and YM440 for FABP mRNA expression were 0.68, 5.8 and 80 μM, respectively. The EC_{50} values of rosiglitazone, pioglitazone, and YM440 for LPL and UCP2 mRNA expression were 0.44, 3.1 and 42 μM for LPL, and 2.8, 13 and 100 μM for UCP2, respectively. The results indicated that YM440 was about 8- to 14-fold less

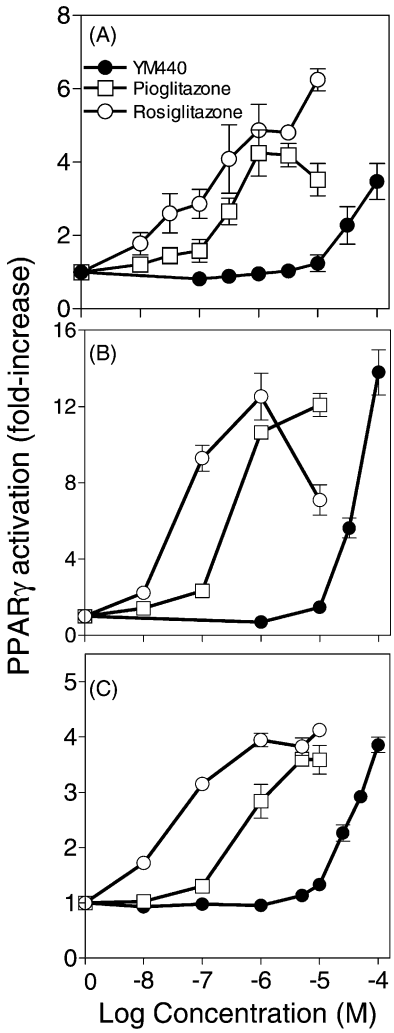

Fig. 3. Effects of YM440, pioglitazone, and rosiglitazone on PPARγ activation in CV-1 and HepG2 cells. A dose-dependent activation of human full-length PPARγ2 in CV-1 cells (A) and HepG2 cells (B), and of GAL4-PPARγ in HepG2 cells (C). YM440 (●), pioglitazone (□), and rosiglitazone (○). The experimental conditions were described in Section 2. All the data were normalized to β-galactosidase activity and plotted as fold-activation relative to untreated cells. Each point with a vertical line represents the mean ± SEM (N = 3). Each EC_{50} value is calculated under the conditions where the maximum transcriptional activity induced by rosiglitazone is 100%. The EC_{50} values (95% confidence limits) of YM440, pioglitazone and pioglitazone obtained in A, B, and C are as follows. YM440: A, 110 (79–170) μM, B, 31 (30–32) μM, C, 33 (31–36) μM; pioglitazone: A, 0.64 (0.46–0.99) μM, B, 0.36 (0.29–0.44) μM, C, 0.81 (0.54–1.2) μM; rosiglitazone: A, 0.20 (0.10–0.38) μM, B, 0.049 (0.030–0.071) μM, C, 0.042 (0.032–0.055) μM.

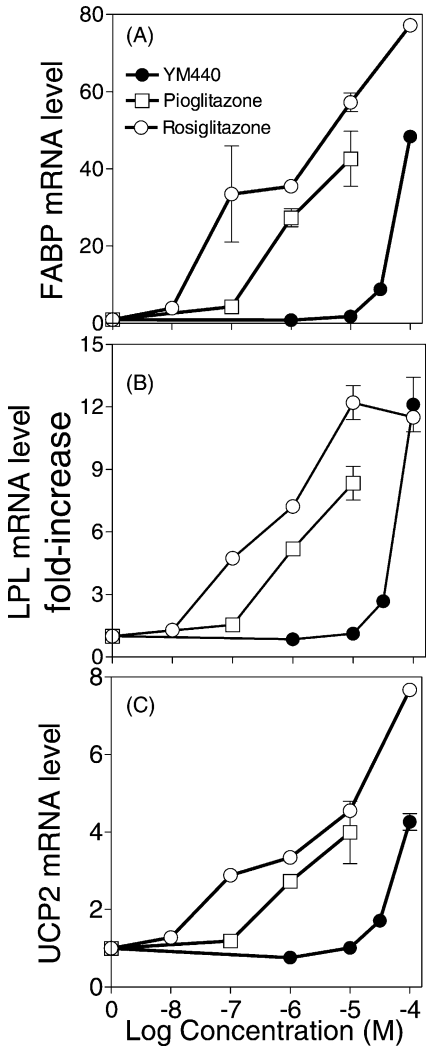

Fig. 4. Effects of YM440, pioglitazone, and rosiglitazone on PPARγ responsive genes in 3T3-L1 cells. Change in mRNA levels of FABP (A), LPL (B) and UCP2 (C) in preadipocyte differentiation. YM440 (●), pioglitazone (□) or rosiglitazone (○). Confluent 3T3-L1 cells were incubated with 0.25 mM dexamethasone and various concentrations of the test compound for 96 hr. A real-time quantitative PCR method was used to detect mRNA levels. All the data were normalized to β-actin mRNA levels and plotted as fold-activation relative to untreated cells. Each point with a vertical line represents the mean ± SEM (N = 2). Each EC_{50} value is calculated under the conditions where the maximal fold-increase in mRNA induced by rosiglitazone is 100%. The EC_{50} values (95% confidence limits) of YM440, pioglitazone and pioglitazone obtained in A, B, and C are as follows. YM440: A, 80 (79–81) μM, B, 42 (38–49) μM, C, 100 (94–110) μM; pioglitazone: A, 5.8 (3.1–14) μM, B, 3.1 (2.0–4.8) μM, C, 13 (6.2–73) μM; rosiglitazone: A, 0.68 (0.26–1.8) μM, B, 0.44 (0.26–0.74) μM, C, 2.8 (1.2–6.4) μM.

potent than pioglitazone and about 36- to 110-fold less potent than rosiglitazone regarding the mRNA expression of PPARγ responsive genes in 3T3-L1 cells.

3.2. *Effects of YM440 on recruitment of coactivators*

The recruitment of transriptional coactivators such as SRC-1 [10] and p300 [26] by PPARγ is very important to the transcriptional activity of nuclear receptors. To examine the effects of YM440 and rosiglitazone on the interactions of PPARγ with coactivators, an *in vitro* GST-fusion protein pull-down assay was performed. As shown in Fig. 5, YM440 caused only weak interaction between PPARγ and p300 or SRC-1 with an EC_{50} value of 14 and 120 μM, respectively. Rosiglitazone dose-dependently increased PPARγ interactions with p300 and SRC-1 with an EC_{50} value of 0.093 and 0.11 μM, indicating that YM440 was 151- and 1091-fold less potent than rosiglitazone.

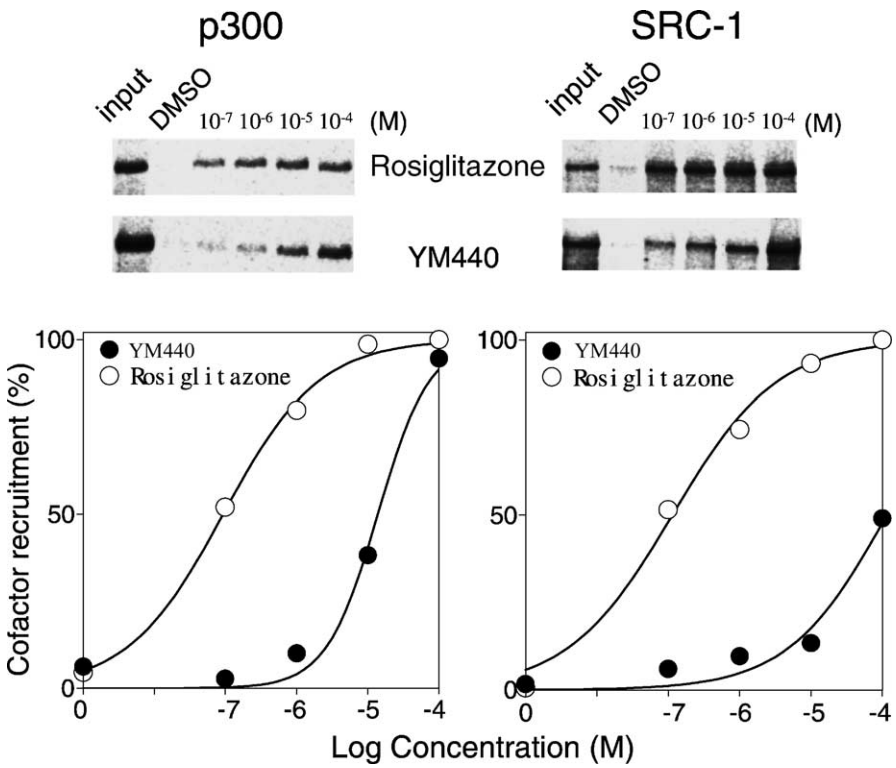

Fig. 5. Comparison of YM440- and rosiglitazone-induced recruitment of transcriptional cofactors to PPARγ. The GST-PPARγ2 fusion protein bound to glutathione-sepharose beads was incubated with increasingly higher concentrations of YM440 or rosiglitazone in the presence or absence of either [^{35}S]p300 or [^{35}S]SRC-1. Autoradiograms of SDS–polyacrylamide gels show the amount of coactivator bound to PPARγ2 in the presence of either YM440 or rosiglitazone. The concentration (*M*) of rosiglitazone or YM440 is shown at the top of each lane. Results of the quantification of the gels are shown below: YM440 (●), rosiglitazone (○). The calculated EC_{50} values (95% confidence limits) are: p300, rosiglitazone; 0.093 (0.069–0.12) μM, YM440; 14 (9.6–22) μM, SRC-1, rosiglitazone; 0.11 (0.065–0.18) μM, YM440; 120 (68–290) μM. This experiment was repeated two times with similar results.

3.3. Cellular uptake of YM440

To eliminate the possibility that YM440 failed to stimulate PPARγ transactivation because of a poor cellular uptake, we measured the cellular uptake of YM440 and rosiglitazone during 0.5 to 24 hr incubation in CV-1 cells using [^{14}C]YM440 or [^{3}H]rosiglitazone (Fig. 6). The amount of YM440 taken in by the cells increased with time and was about 4-fold that of rosiglitazone over 24 hr, suggesting that cellular uptake of YM440 was not impaired.

3.4. Difference between YM440 and rosiglitazone in sensitivity to limited trypsin digestion of PPARγ

Although YM440 is a ligand for PPARγ, it failed to promote the transactivation and recruitment of cofactors at the concentration at which it bound the receptor. These findings led us to explore further the difference in the conformational change of YM440- and rosiglitazone-bound PPARγ. To do this, we carried out the limited trypsin digestion of a full-length human PPARγ2 at increasingly higher concentrations of YM440 or rosiglitazone (Fig. 7). These agents protected 27 and 36 kDa fragments in a dose-dependent manner but rosiglitazone protected the 27 kDa fragment (54% of PPARγ without trypsin) more intensively than the 36 kDa fragment (8%). On the other hand, YM440 protected the 36 kDa fragment (38%) better than the 27 kDa fragment (29%). In addition, a strong 31 kDa fragment found in the YM440-bound receptor was faintly detected

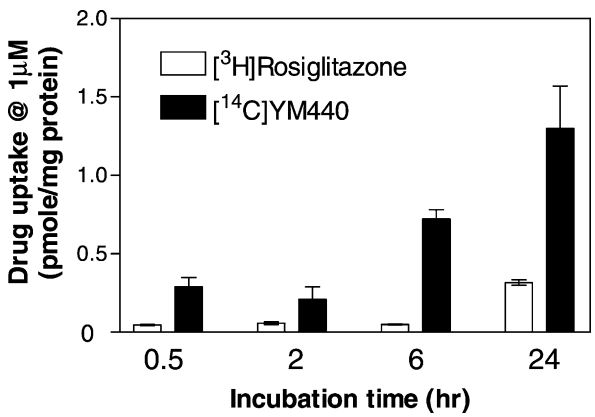

Fig. 6. Cell uptake of YM440 and rosiglitazone after various periods of incubation. The PPARγ-transfected CV-1 cells were incubated with 1 μM [^{3}H]rosiglitazone or [^{14}C]YM440 for 0.5–24 hr in medium containing 10% FBS. At various intervals, the medium was removed and the cells were washed three times with ice-cold PBS containing 100 μM unlabeled rosiglitazone or YM440. The cells were then solubilized by 0.5% sodium dodecyl sulfate and the radioactivity was determined with a scintillation counter. Each bar with a vertical line shows the mean ± SEM from triplicate assays.

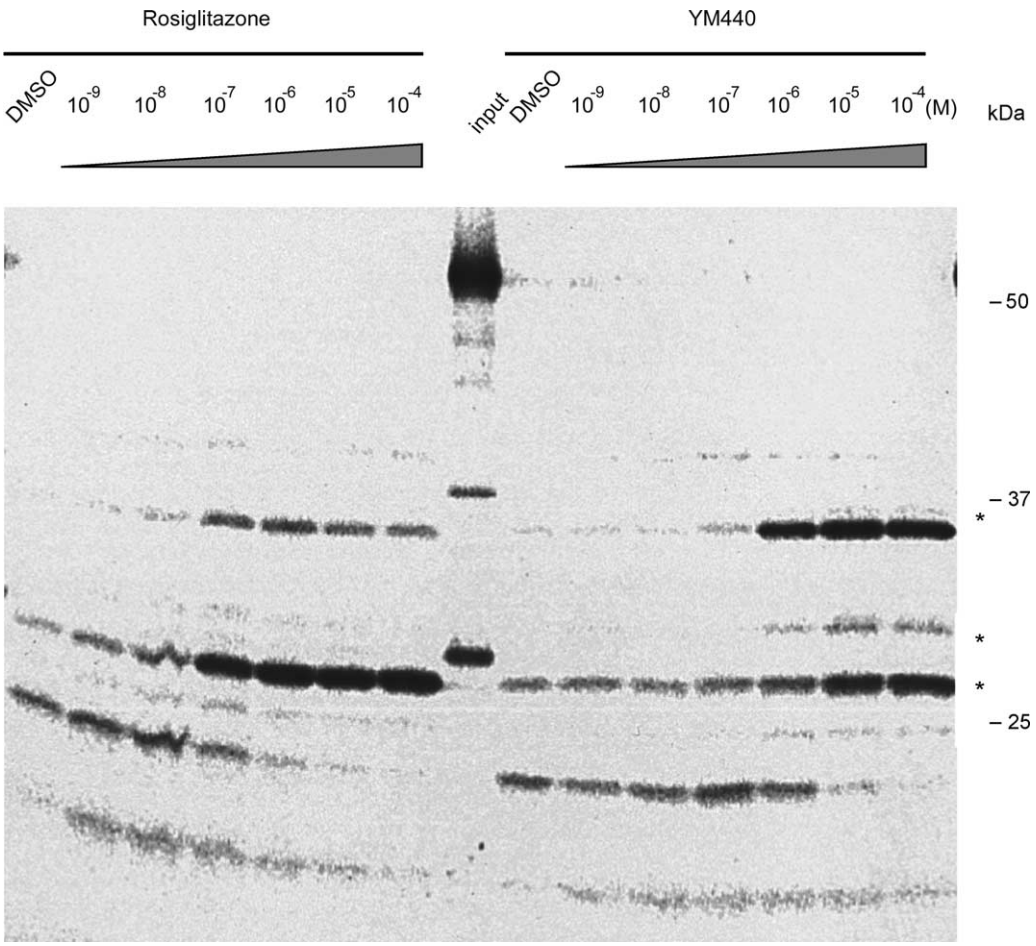

Fig. 7. Difference in protease sensitivity of YM440- and rosiglitazone-bound PPARγ. Autoradiogram of an SDS–polyacrylamide gel showing [^{35}S]methionine-labeled full-length PPARγ2 digested with trypsin in the presence or absence of increasingly higher concentrations of either YM440 or rosiglitazone. The migration of protein size markers (kDa) is indicated on the right. To measure the relative levels of the bands protected by a test compound on a gel, the intensity of the band of full-length human PPARγ2 treated without trypsin, around 50 kDa, is taken as 100%. The experiment was repeated two times with similar results. Asterisk (∗) denotes trypsin-resistant protein fragments.

in vehicle and rosiglitazone-treated receptors. These results indicate conformational differences in the receptor bound to YM440 vs. rosiglitazone.

3.5. Effects of YM440 on the mRNA expression of FABP and UCP1 in adipose tissue and of glucokinase in the liver in diabetic db/db mice

Although YM440 was less effective in stimulating adipocyte differentiation *in vitro*, we considered that its hypoglycemic activity might still involve PPARγ and relate to a favorable pharmacokinetic profile. We therefore examined the effects of YM440 on mRNA levels of FABP and UCP1 in adipose tissue and of glucokinase in liver in YM440- and pioglitazone-treated db/db mice (Fig. 8). The treatment with pioglitazone significantly induced the expression of FABP, UCP1 and glucokinase. Although YM440 did not increase the levels of FABP and UCP1 in adipose tissue, it significantly increased glucokinase expression in liver.

4. Discussion

Insulin-sensitizing TZDs are high affinity ligands for a member of the nuclear hormone receptor superfamily, PPARγ, which acts as a ligand-inducible transcription factor mediating glucose and lipid metabolism and adipocyte differentiation [27]. Prior study [14] has shown that YM440, an oxadiazolidinedione analog, as well as pioglitazone, ameliorated hyperglycemia in diabetic db/db mice and that pioglitazone, but not YM440, increased body weight and fat weight. Since YM440 failed to promote adipocyte differentiation and PPARγ transactivation in a reporter assay, it is suggested that it ameliorates hyperglycemia without affecting PPARγ activity. The purpose of this study was to clarify (1) the relationship between the binding of YM440 to PPARγ and its transactivation of the target gene, (2) the effects of YM440 and other TZDs on conformational changes of PPARγ, and (3) the involvement of PPARγ in the hypoglycemic activity induced by YM440 in diabetic db/db mice.

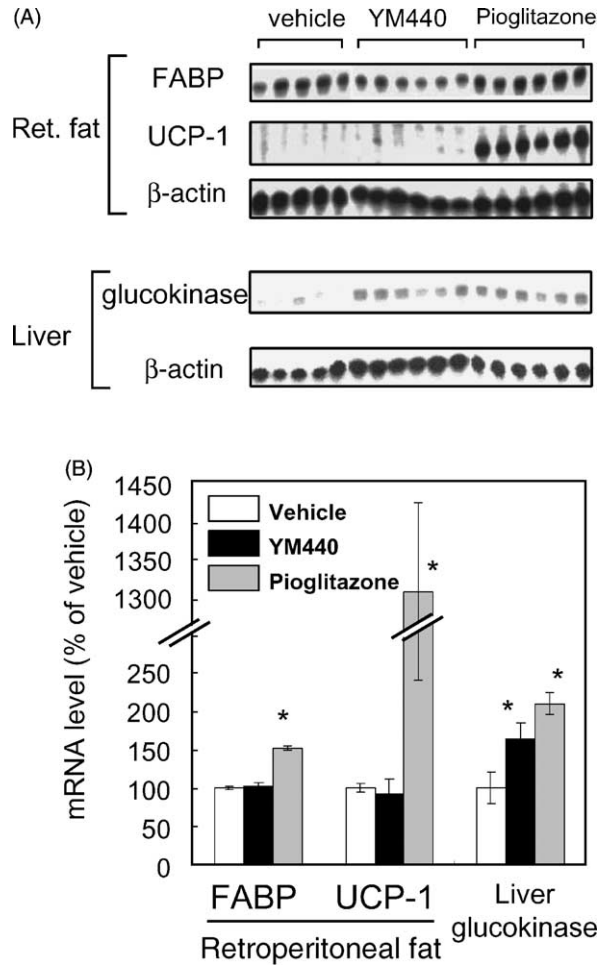

Fig. 8. Effects of YM440 and pioglitazone on mRNA expression of FABP and UCP1 in retroperitoneal fat and glucokinase in the liver of db/db mice. Male db/db mice were orally treated with either YM440 or pioglitazone (100 mg/kg) for 28 days. Total RNA isolated from retroperitoneal fat or liver (3 μg RNA) was analyzed by RNase protection assay. (A) Representative autoradiographs of FABP, UCP1, glucokinase and β-actin. (B) Relative abundance of mRNA. The signals for FABP, UCP1 and glucokinase were normalized to the β-actin mRNA expression level. Each bar with a vertical line shows the mean ± SEM from five or six animals. The statistical difference between control and treatment groups was evaluated by Dunnett's test. $^{*}P < 0.05$ vs. untreated control.

Based on the following experimental evidence, we identified YM440 as a ligand which modulated PPARγ transactivation function but its potency was much lower than its binding potency. First, YM440 bound to PPARγ LBD and induced a conformational change in PPARγ as shown by the protease protection assay. Second, YM440 increased PPARγ transactivation. Although YM440 was less potent than pioglitazone and rosiglitazone, it enhanced the maximal level of transactivation achieved by these two agents. Third, YM440 recruited coactivators, SRC-1 and p300, to PPARγ. Finally, the cell took up YM440 as well as rosiglitazone. These biological properties of YM440 are compatible with the properties of known PPARγ ligands, indicating that YM440 acted as a full agonist of PPARγ under the experimental conditions. However, we cannot totally exclude the possibility that YM440 is a partial agonist for PPARγ because in some studies, YM440 did not enhance the maximal level of transactivation and gene expression achieved by rosiglitazone even though YM440 was treated at the highest concentration used. Further study will be required in this respect. Furthermore, these properties are quite different from those of PPARγ antagonists [28–31]. It is unlikely that YM440 acted as an antagonist of PPARγ because it did not inhibit PPARγ transactivation in HepG2 cells and FABP expression in 3T3-L1 cells (Kurosaki *et al.* unpublished observations).

YM440 as well as TZDs bound to PPARγ LBD. YM440 was comparable to pioglitazone and 20-fold less potent than rosiglitazone in the binding of PPARγ. Interestingly, YM440 had 550- to 790-fold less transcriptional activity than rosigitazone in a reporter assay and was 36- to 120-fold less potent in the mRNA expression of PPARγ responsive genes in 3T3-L1 cells. In contrast, pioglitazone was 16-fold, 3.2- to 19-fold, and 4.6- to 8.5-fold less effective than rosiglitazone in receptor binding, the reporter assay, and mRNA expression of PPARγ responsive genes in 3T3-L1 cells, respectively, suggesting no difference in relative potency between PPARγ binding and transactivation. These findings indicate that the binding of pioglitazone, but not YM440, to PPARγ is closely connected with the transactivation of the gene. Compatible with the weak transactivation function of YM440, this agent showed only weak activity in recruiting coactivators to PPARγ. Since the EC_{50} value of rosiglitazone for p300 and SRC-1 was 0.093 and 0.11 μM, respectively, YM440 was 151- and 1091-fold less potent than rosiglitazone in recruiting p300 and SRC-1. These results suggest that the reduction in transactivation function is due to a decrease in the recruitment of cofactors to YM440-bound receptors.

Berger *et al.* [32] analyzed *in vivo* and *in vitro* the effects of troglitazone, pioglitazone and rosiglitazone on blood glucose levels, PPARγ binding, transactivation and conformational change in the receptor. They concluded that the hypoglycemic action of TZDs was directly mediated through PPARγ binding and the resulting active conformation of the receptor. Recently, it was reported that some hypoglycemic agents showed a dissociation between hypoglycemic activity and PPARγ activation [6–8,33]. It should be pointed out that these agents improved hyperglycemia in diabetic mice without changing body weight and fat weight. First, NC-2100, a TZD analog, reduced blood glucose and triglyceride levels in diabetic KK-A^{y} mice with only a small effect on body weight and fat weight [6,8]. Compared to pioglitazone and rosiglitazone, 10- to 30-fold higher concentrations of NC-2100 were required for maximal activation of PPARγ in a reporter assay and only high concentrations of this agent weakly induced transcription of the PPARγ target gene in mice and the adipogenesis of cultured 3T3-L1 cells. Second, F-L-Leu improved insulin sensitivity in diabetic db/db mice and had low adipogenic activity in 3T3-L1 cells [8]. F-L-Leu bound

to PPARγ with a K_i value of 15 μM, 429-fold greater than that for rosiglitazone. It was concluded that this agent was much less adipogenic than rosiglitazone, reflecting its relative affinity for the receptor. Finally, MCC-555, another TZD analog, also improved insulin sensitivity in diabetic KK-A^y mice and obese Zucker rats [7,33] and showed more antihyperglycemic activity than rosiglitazone and pioglitazone. However, this agent did not alter the body weight of obese Zucker rats. MCC-555 bound to PPARγ with 50-fold less affinity than rosiglitazone. Compatible with the receptor binding of MCC-555, it activated PPARγ with less potency than rosiglitazone and recruited the coactivator SRC-1 10- to 50-fold less effectively than rosiglitazone. MCC-555 was a partial agonist of Gal4-PPARγ.

Although YM440 shares several characteristics with the three agents mentioned above, certain features distinguish it. All three compounds as well as YM440 reduced blood glucose levels in diabetic mice with only a small effect on body weight and fat weight. They have little or no effect on adipocyte differentiation. Since the K_i value of NC-2100 for binding PPARγ has not yet been reported, it is difficult to examine the difference in potency between receptor binding and transactivation induced by this agent. However, in the case of F-L-Leu and MCC-555, we speculate that there is no difference in potency between receptor binding and transactivation. For F-L-Leu, the authors concluded that the low level of adipogenic activity of this agent reflects its weak affinity for the receptor. In addition, MCC-555 bound to PPARγ and also recruited coactivator SRC-1 in the same concentration range (10- to 50-fold less efficiently than rosiglitazone), suggesting no major difference in potency among receptor binding, transactivation and recruitment of the coactivator. In contrast, however, the binding by YM440 of PPARγ itself was not well connected with an increase in transcriptional activity and recruitment of transcriptional coactivators. There is a significant difference in potency between receptor binding and transactivation induced by YM440. Taken together, this evidence suggests that YM440 is a unique modulator of PPARγ.

As discussed above, some hypoglycemic agents which induce the transactivation of PPARγ, have less of an effect on body weight gain and adipogenesis. Since being overweight results in a decrease in insulin sensitivity, hypoglycemic agents which do not cause body weight gain are beneficial in the treatment of type 2 diabetes.

We examined the effects of YM440 and pioglitazone on the gene expression induced by PPARγ agonists, in the adipose tissue and the liver because a preliminary pharmacokinetics and tissue disposition study indicated that YM440 was distributed widely but mainly in the liver. The amount of labeled YM440 in the adipose tissue and muscle was about 2% of that in the liver (Oritani *et al.* unpublished observations). Another reason to choose the liver is that a hyperinsulinemic clamp study in obese Zucker rats indicated that YM440 suppressed hepatic glucose output rather than induced the disposal of glucose in peripheral tissues [34]. In addition, glucose intolerance in obese Zucker rats was associated with decreased hepatic glycogenesis and YM440 improved the intolerance by normalizing glycogen metabolism [35]. These observations suggest that one of the target tissues of YM440 is the liver.

Pioglitazone induced the expression of FABP and UCP1 in adipose tissue and of glucokinase in the liver in db/db mice. YM440 increased the hepatic expression of glucokinase but not FABP and UCP1 in adipose tissue. The mechanism by which YM440 acts on the liver remains unknown. However, one possibility is that YM440 increased hepatic glucokinase expression through a PPARγ-independent mechanism. Second possibility involves high concentrations of YM440 in the liver. YM440 activates a small amount of PPARγ in the liver and regulates the expression of genes related to glucose and glycogen metabolism. Although there is no report that PPARγ directly regulates gluconeogenic genes, TZDs were reported to regulate the mRNA expression [36] and the activity of gluconeogenic enzymes [37].

A third possibility was presented by Kodera *et al.* [23], that a different ligand activated a particular set of target gene promoters that exerted different biological actions based on the difference in 15d-PGJ2- and troglitazone-induced coactivator interaction with PPARγ. In the present study, rosiglitazone recruited p300 and SRC-1 to the same extent but YM440 preferentially recruited p300. Such a difference in cofactor recruitment may exert different biological effects. To examine this possibility under our experimental conditions, it is necessary to conduct *in vitro* hepatocyte experiments showing that YM440 induces PPARγ activation and increases glucokinase expression without affecting other PPARγ target genes.

In summary, YM440 bound to PPARγ responsible for adipogenesis and exhibited weak activity for both the transactivation of PPARγ and the recruitment of transcriptional cofactors relative to its binding potency. YM440-bound PPARγ caused a conformational change which was different from that of rosiglitazone. Such a unique profile regarding the binding-transactivation of PPARγ induced by YM440 may lead to hypoglycemic activity without a body weight gain in diabetic mice.

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