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Antagonism of peroxisome proliferator-activated receptor γ prevents high-fat diet-induced obesity in vivo

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Abbreviations:
PPARγ, peroxisome proliferator-activated receptor γ
GW9662, 2-chloro-5-nitrobenzanilide
SRC-1, steroid receptor coactivator-1
SPA, scintillation proximity assay
LBD, ligand binding domain
GST, glutathione S-transferase
TZDs, 2,4-thiazolidinediones

ABSTRACT

Peroxisome proliferator-activated receptor γ (PPARγ) has been reported to play an important role to regulate adiposity and insulin sensitivity. It is not clear whether antagonism of PPARγ using a synthetic ligand has significant effects on adipose tissue weight and glucose metabolism in vivo. The aim of this study is to examine the effects of a synthetic PPARγ antagonist (GW9662) on adiposity and glycemic control in high-fat (HF) diet-fed mice. First the properties of GW9662 as a PPARγ antagonist were estimated in vitro. GW9662 displaced [^{3}H]rosiglitazone from PPARγ with K_i values of 13 nM, indicating that the affinity of GW9662 for PPARγ was higher than that of rosiglitazone (110 nM). GW9662 had no effect on PPARγ transactivation in cells expressing human PPARγ. Treatment of 3T3-L1 preadipocytes with GW9662 did not increase aP2 expression or [^{14}C]acetic acid uptake. GW9662 did not recruit transcriptional cofactors to PPARγ. Limited trypsin digestion of the human PPARγ/GW9662 complex showed patterns of digestion distinct from those of rosiglitazone. This suggests that the binding characteristics between GW9662 and PPARγ are different from those of rosiglitazone. Treatment of HF diet-fed mice with GW9662 revealed that this compound prevented HF diet-induced obesity without affecting food intake. GW9662 suppressed any increase in the amount of visceral adipose tissue, but it did not change HF diet-induced glucose intolerance. These data indicate that antagonism of PPARγ using a synthetic ligand suppresses the increased adiposity observed in HF diet-induced obesity, and that a PPARγ antagonist could possibly be developed as an anti-obesity drug.

1. Introduction

Adipose tissue plays a central role in regulating the body's energy balance. Adipose tissue helps control energy homeostasis (including food intake), metabolic efficiency, and energy expenditure via the hormones it secretes. The quantity of body fat present in mammals varies greatly. This variability can also be easily observed between individuals of the same

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species, which highlights the complexity of the interplay of factors that control fat deposition. This huge range of fat mass variation is a phenomenon unlike any other seen in the body. It is determined by both an individual's genetic background and lifestyle factors such as diet and physical activity. Having a significant amount of excess body fat (obesity) is a major health problem that increases the risk of developing diabetes, hypertension, and coronary artery disease [1–3].

The cellular and molecular mechanisms behind adipocyte differentiation have been studied extensively [4]. A number of key transcription factors that participate in the complex transcriptional cascade during adipocyte differentiation have been identified, including some peroxisome proliferator-activated receptor (PPAR) family proteins (α, δ and γ) [5].

PPARγ is abundantly expressed in adipose tissue, where it is a key regulator of adipocyte differentiation. Thus, PPARγ regulates energy homeostasis in this way [6–9].

Thiazolidinediones (TZDs), antidiabetics currently used as insulin sensitizers, are well-known synthetic PPARγ ligands in terms of specificity and affinity. TZDs can bind directly to activate PPARγ and stimulate adipocyte differentiation [10–13]. This activity is well correlated with the ability to lower blood glucose in diabetic mice [14]; however, the physiological role of PPARγ in mature adipocytes and the regulation of insulin sensitivity in vivo remain largely unclear. In vivo deletion of PPARγ function is an effective means of investigating its physiological role. Generation of PPARγ-deficient mice by gene targeting is a useful method for investigating the role of PPARγ in vivo. Since PPARγ knockout is lethal for mouse embryos due to a defect in placental development [15], several groups have generated tissue-specific PPARγ deletion mice using the Cre/lox P system [16–21].

One of the other methods used to analyze the function of PPARγ in vivo is the dosing of a synthetic PPARγ antagonist. Several groups have reported in vitro and in vivo studies on PPARγ antagonists, but because these studies used a partial antagonist, these reports did not sufficiently elucidate the role of PPARγ [22–26]. GW9662, however, is a full synthetic PPARγ antagonist. Leesnitzer et al. [27] reported previously that GW9662 suppressed adipocyte differentiation in vitro, and several other in vitro studies showed that GW9662 is a full PPARγ antagonist [28–30]. Unfortunately, there have been no reports on the in vivo use of GW9662.

In this report we analyzed high-fat (HF) diet mice treated with GW9662 in order to investigate the role of PPARγ in vivo. GW9662 treatment suppressed HF diet-induced obesity, but did not change glucose intolerance. This study provides evidence that PPARγ antagonism prevents the increased adiposity induced by a HF diet.

2. Materials and methods

2.1. Materials

All reagents used in this study were of analytical grade and obtained commercially. 2-Chloro-5-nitrobenzanilide (GW9662) and rosiglitazone maleate (rosiglitazone) were synthesized at Astellas Pharma Inc. (Tokyo, Japan). [^{3}H]rosiglitazone was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

2.2. Ligand binding assay for PPARγ

The 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. [31]. 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, Piscataway, NJ, USA) and Talon column (Clontech, Mountain View, CA, USA). Then, the purified PPARγ LBD was biotinylated with NHS-LC-Biotin (Pierce, Rockford, IL, USA) and used to coat streptavidine SPA beads. Binding assays were performed in 96-well NBS plates (Corning, Corning, NY, USA) by incubating the PPARγ LBD-coated SPA beads with 10 nM [^{3}H]rosiglitazone and 1 nM–100 μM of the test compound at room temperature for 2 h. After centrifugation at 1500 rpm, the radioactivity in each well was quantified in a Packard TopCount scintillation counter (Packard Instrument Company, Meriden, CT, USA). K_i values were calculated according to the equation proposed by Cheng and Prusoff [32]: $K_i = IC_{50}/(1 + [L]/K_d)$, 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

HepG2 cells were maintained in Dulbecco's modified eagle 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 PPRE3-TK-luciferase reporter vector containing three copies of the PPRE (GTCGACAGGGGACCAGGACAAAGGTCACGTTCGGGAGTC-GAC) was also constructed [33]. The full-length RXRα expression vector and β-galactosidase expression vector pCH110 were purchased from Invitrogen (Carlsbad, CA, USA) and Amersham Pharmacia Biotech, respectively. Cells were plated at the rate of 2.5×10^4/well in a 96-well tissue culture plate. A DNA mixture containing each expression vector (PPRE-TK-Luc, 6 μg/well; full-length human PPARγ2, 3 μg/well; full-length RXRα, 1 μg/well; and β-galactosidase, 1 μg/well) were transfected by using Fugene 6 (Roche Diagnostics, Basel, Switzerland). The cells were incubated for 6 h after transfection. The test compound was added to each well. After 24 h, cell extracts were prepared, and the activities of luciferase and β-galactosidase were determined using a luciferase assay kit (Pica Gene, Toyo Ink Mfg., Tokyo, Japan) and a β-galactosidase enzyme assay system (Promega, Madison, WI, USA), respectively. 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 Institute, Cary, NC, USA).

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

Assays for the analysis of cell differentiation were performed according to previously described methods [34]. 3T3-L1

preadipocytes (American Type Cell Collection, Manassas, VA, USA) were grown in basal medium (DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 in air [35]. Cells were plated at $1.5 \times 10^4/cm^2$ in a 96-well tissue culture plate coated with type I collagen (Iwaki, Tokyo, Japan). After the cells had reached confluence, they were cultured for four more days in differentiation medium [basal medium supplemented with 5% FBS, 100 ng/mL insulin, 0.1 mM isobutylmethylxanthine (IBMX), and 1 mM dexamethasone] supplemented with various concentrations of compounds. The compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration 1000 times higher than the final concentration and then added to the differentiation medium at a concentration of 0.1% (v/v). DMSO was also present in the control culture at a concentration of 0.1% (v/v). The medium was then replaced with maintenance medium (basal medium supplemented with 5% of FBS and 100 ng/mL of insulin), and the cells were cultured for two more days.

Degree of adipogenesis stimulation was determined by [1-^{14}C]acetic acid uptake. The maintenance medium was replaced with fresh maintenance medium supplemented with 7.4 kBq/mL [1-^{14}C]acetic acid. After 1 h of incubation, the maintenance medium was discarded and the cells were washed twice with phosphate-buffered saline (PBS). The cells were then air-dried, and scintillation cocktail (200 μL) (Microscint-20, Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA) was added to the wells. Scintillation counts were then measured with a Packard TopCount microplate scintillation counter. Each ED_{50} value for PPARγ activation was determined by nonlinear curve fitting using a Statistical Analysis System.

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, Tokyo, Japan). mRNA levels were determined using real-time quantitative RT-PCR analysis on an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) as previously described [36]. 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/aP2) (GenBank accession number K02109, forward primer 5′-AAAACACCGAGATTTCCTTCA-3′ and reverse primer 5′-CTCTTCACCTTCCTGTCGTCT-3′, TaqMan probe 5′-TGGGCGTGGAATTCGATGAAATCA-3′) [37,38].

The aP2 mRNA expression levels were normalized to internal GAPDH expression levels using TaqMan rodent GAPDH control reagent (Applied Biosystems).

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 previously described methods [39]. 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/CBP 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/CBP proteins were incubated with beads containing GST–PPARγ2 in the presence or absence of either rosiglitazone or GW9662 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 1 h of incubation, the beads were washed with NET-N buffer to remove the free protein. Bound proteins were extracted with loading buffer, separated by 4–20% SDS-PAGE, and visualized using autoradiography.

2.7. Protease digestion assay

Protease digestion assay were carried out according to the methods previously described [40]. 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 °C. Then, 4 μL of distilled water (dH_2O) or dH_2O-solubilized trypsin was added. Protease digestion was allowed to proceed for 10 min at 25 °C, and was 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 4–20% 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 h at 80 °C. The bands were detected with a BAS2000 bioimaging analyzer (Fuji Film, Tokyo, Japan). To measure the relative levels of the bands on the gel that were protected by a test compound, the intensity of the band of full-length human PPARγ2 treated without trypsin, around 50 kDa, was taken as 100%.

2.8. Animals and experimental design

Male C57BL6/J mice were purchased from Charles River Japan and kept under a 12 h light:12 h dark cycle. Mice were randomly divided into three groups and fed one of the following diets from 8 to 16 weeks of age: (I) high-carbohydrate (HC) diet, (II) high-fat (HF) diet, and (III) HF diet + GW9662. The components of these diets were determined based on Ikemoto's method [41]. Briefly, the HF diet contains 32% safflower oil, 33.1% casein, 17.6% sucrose, 1.4% vitamin mixture, 9.8% mineral mixture, 5.6% cellulose powder, and 0.5% DL-methionine. The HC diet contains 4% safflower oil, 23.7% casein, 10% sucrose, 50% α-starch, 1% vitamin mixture, 7% mineral mixture, 4% cellulose powder, and 0.4% DL-methionine. Casein, sucrose, starch, vitamin mixture, mineral mixture, and cellulose powder were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan); Safflower oil from Benibana Food (Tokyo, Japan); and DL-methionine from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). The caloric densities of these diets are 343 kcal/100 g for the HC diet and 490 kcal/100 g for the HF diet. GW9662 was given as a 0.1% (w/w) admixture. Oral glucose tolerance tests (OGTT) were conducted at week eight of feeding. Blood samples for glucose,

insulin, triglyceride, non-estrified fatty acid (NEFA), and cholesterol assays were collected from the mice. Food intake in each experimental group was measured for 12 h (from 8 p.m. to 8 a.m.) in separate 3 days at 8 weeks. The animals were then sacrificed 3 days after the OGTT, and body weight, liver weight, and wet visceral adipose tissue (right and left epididymal/retroperioneal fat pads) weight were measured. Blood glucose, plasma triglyceride, NEFA, and cholesterol levels were measured using the glucose-CII test, triglyceride-E test, NEFA-C test, and cholesterol-C test, respectively (Wako Pure Chemical). Plasma insulin concentrations were measured using radioimmunoassay kits (Amersham Pharmacia Biotech). The animal study was performed in accordance with the legal requirements of the Animal Use Committee of Astellas Pharma Inc.

2.9. Oral glucose tolerance test

Mice fed each diet for 8 weeks were fasted overnight, and then D-glucose (1.5 g/kg of body weight) was administered via a stomach tube. Blood samples were obtained by snipping the tail vain both before and 0.5, 1, 2, and 4 h after glucose administration.

2.10. Statistical analysis

Comparisons between the experimental groups were made using the one-way ANOVA followed by Fisher's least significant difference (LSD) test. Comparisons of each parameter in the time course studies were analyzed using the two-way ANOVA followed by Fisher's LSD test. Differences were considered significant at P levels of less than 0.05.

3. Results

3.1. GW9662 is a ligand for PPARγ, shows anti-adipogenic activity, but does not activate PPARγ-mediated transcription

The effects rosiglitazone and GW9662 on PPARγ binding were examined using the SPA binding assay system (Fig. 1). GW9662 displaced [^{3}H]rosiglitazone from PPARγ LBD with a K_i value of 13 nM (95% confidence limits: 9.3–18 nM), indicating that GW9662 was 8.5-fold more active than rosiglitazone (K_i = 110 nM, 95% confidence limits: 69–160 nM).

Rosiglitazone dose dependently increased PPARγ transactivation in HepG2 cells. The EC_{50} value of rosiglitzone in the cells expressing human full-length PPARγ2 was 51 nM (95% confidence limits: 35–80 nM). GW9662, however, had no effect on the transactivation (Fig. 2).

PPARγ agonists are known to promote differentiation of 3T3-L1 preadipocytes into fat droplet-containing mature adipocytes. The effects of these PPARγ ligands on adipocyte differentiation was studied in this cell line and assessed through monitoring increases in mRNA for aP2 and [^{14}C]acetic acid uptake. Treatment of 3T3-L1 with rosiglitazone increased aP2 mRNA expression and [^{14}C]acetic acid uptake in a dose-dependent manner (EC_{50} = 4.9 nM, 95% confidence limits: 0.021–22 nM). In contrast, GW9662 did not increase aP2 expression or [^{14}C]acetic acid uptake at 100 μM (Fig. 3A and B).

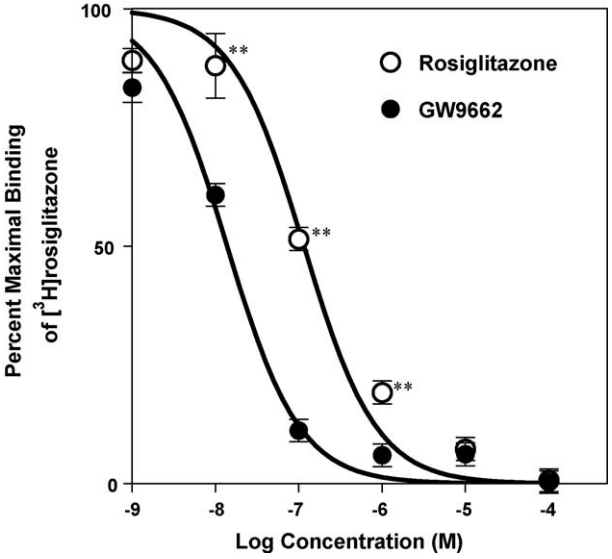

Fig. 1 – Binding of GW9662 and rosiglitazone to PPARγ. A 2-h competitive binding assay was performed using 10 nM [^{3}H]rosiglitazone and 100 μg of SPA beads in the presence or absence of increasingly higher concentrations of the test compound. Each point with a vertical line represents the mean ± S.E.M. of three to six determinations, which were obtained from at least two independent experiments performed in duplicate. Data were normalized to the vehicle-control value. $^{**}P < 0.01$ vs. GW9662.

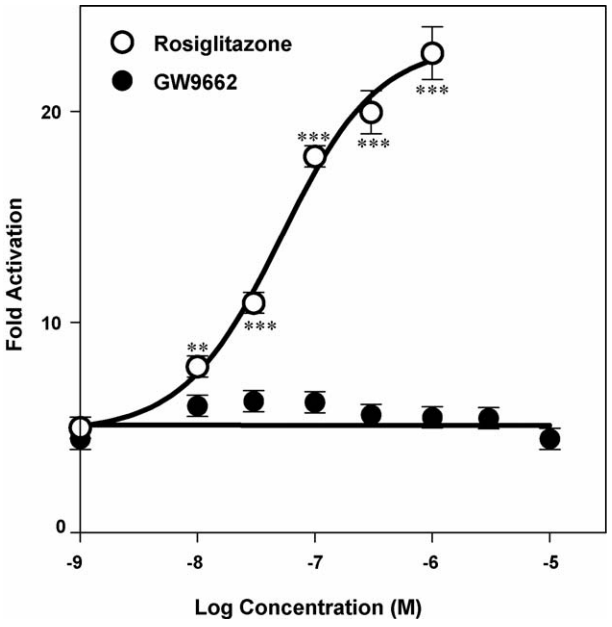

Fig. 2 – Effects of GW9662 and rosiglitazone on PPARγ activation in HepG2 cells. Dose-dependent activation of human full-length PPARγ2 in HepG2 cells (experimental conditions described in Section 2). All data were normalized to β-galactosidase activity and plotted as fold-activation relative to untreated cells. Each point with a vertical line represents the mean ± S.E.M. (N = 3). Each EC_{50} value is calculated under the assumption that the maximum transcription activity induced by rosiglitazone is 100%. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. GW9662.

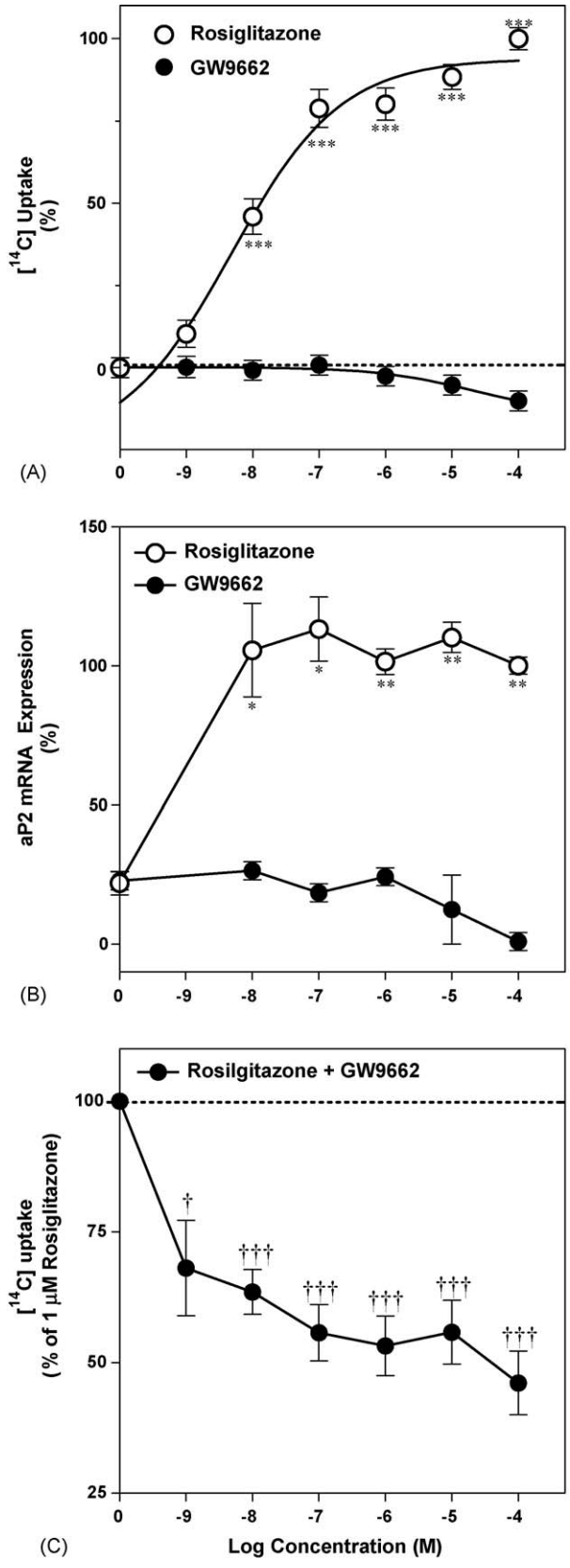

Fig. 3 – Effects of GW9662 and rosiglitazone on adipogenesis. (A) Effect of GW9662 and rosiglitazone on differentiation of 3T3-L1 cells. Cells were cultured for 4 days in differentiation medium supplemented with various concentrations of compounds. The medium was replaced with maintenance medium, and culturing continued for 2 more days. After 6 days of culturing, the level of adipogenesis stimulation was determined by [1-C^{14}]acetic acid uptake. (B) Effect of these compounds on the expression of aP2 mRNA. RNA was isolated from 3T3-L1 cells treated under the same conditions as the differentiation assay. A real-time quantitative PCR method was used to detect mRNA levels. All the data were normalized to GAPDH mRNA levels. (C) Inhibition of rosiglitazone-induced adipogenesis by GW9662. 3T3-L1 cells were cultured with differentiation medium supplemented with 1 μM rosiglitazone and various concentrations of GW9662. Culture conditions and determination of adipogenesis activity are described above. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ vs. 1 μM rosiglitazone.

When cells were cultured in the presence of GW9662, the rosiglitazone (1 μM)-induced increases in [^{14}C]acetic acid uptake decreased dose dependently (Fig. 3C). Thus, in adipogenesis assays, GW9662 acts as a PPARγ antagonist.

3.2. GW9662 has no effect on the recruitment of coactivators

The recruitment of transcriptional coactivators such as SRC-1 [42,43] and p300/CBP [44] by PPARγ is very important to the transcripitonal activity of nuclear receptors. To determine the effects of rosiglitazone and GW9662 on coactivator interaction, an in vitro GST fusion protein pull-down assay was performed on SRC-1 and p300/CBP. As shown in Fig. 4, rosiglitazone dose dependently increased the interaction of PPARγ with p300/CBP and SRC-1 with an EC_{50} value of 111 nM (95% confidence limits: 60–190 nM) and 93 nM (95% confidence limits: 54–140 nM), respectively. On the other hand, GW9662 had no effect on the recruitment of these coactivators. Similar results were observed by using TIF-II as a cofactor (data not shown).

3.3. Rosiglitazone and GW9662 impose a sensitivity differential on the limited trypsin digestion of PPAR gamma

Although GW9662 is a ligand for PPARγ, it had no effect on the recruitment of cofactors or 3T3-L1 adipocyte differentiation at the concentration that bound the receptor. The fact that these results differ from those for rosiglitazone could be due to conformational changes in the receptor induced by the binding of these two ligands. To determine if conformational differences for PPARγ binding do indeed occur with these compounds, a limited trypsin digestion of a full-length human PPARγ2 was carried out at increasingly higher concentrations of rosiglitazone and GW9662 (Fig. 5A). Rosiglitazone strongly protected 27 and 36 kDa fragments in a dose-dependent manner. Low doses (10^{-9} to 10^{-7} M) of rosiglitazone protected 20 kDa fragments (Fig. 5B). GW9662, though, protected 26 kDa fragments only weakly (Fig. 5C). These results indicate conformational differences in the receptor that binds rosiglitazone and GW9662.

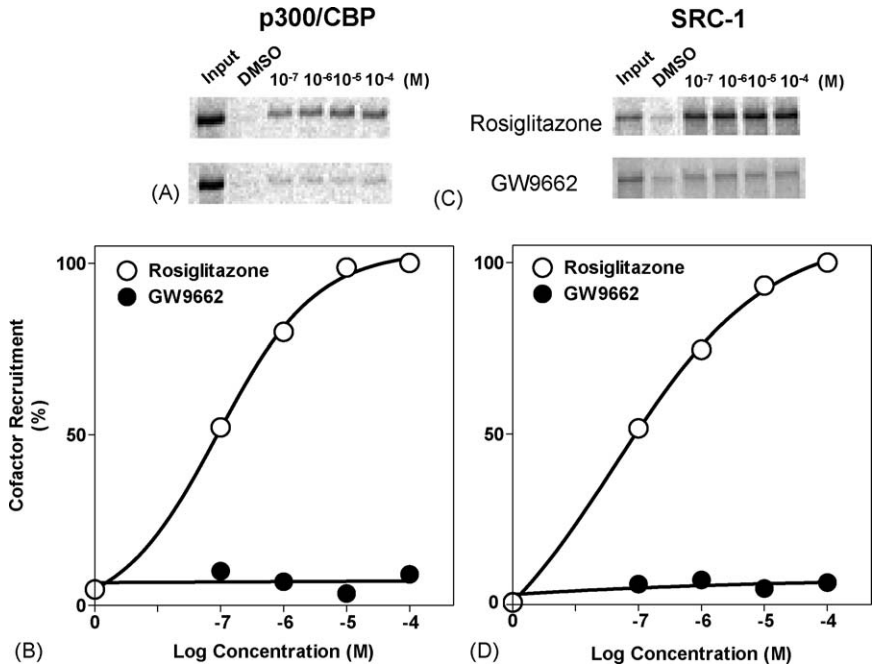

Fig. 4 – Comparison of GW9662- and rosiglitazone-induced recruitment of transcriptional PPARγ cofactors. GST–PPARγ2 fusion protein bound to glutathione–sepharose beads was incubated with increasingly higher concentrations of GW9662 or rosiglitazone in the presence or absence of either [^{35}S]p300 or [^{35}S]SRC-1. (A and C) Autoradiograms of SDS-polyacrylamide gels showing the amount of coactivator bound to PPARγ2 in the presence of either GW9662 or rosiglitazone. The concentration (*M*) of GW9662 or rosiglitazone is shown at the top of each lane. (B and D) Gel quantification results are shown. The experiment was repeated two times with similar results.

3.4. *GW9662 prevents HF diet-induced obesity*

PPARγ appears to play an important role in adipose tissue accumulation, since treatment of diabetic rodent models with PPARγ agonists affects body weight and fat mass, presumably by activating endogenous PPARγ [45,46]. GW9662 showed adipogenic antagonist activity in vitro. We therefore examined the effects of GW9662 on body weight and fat composition in HF diet mice. The body weights of the HF diet mice increased significantly 6 weeks into the study (Fig. 6A). The total body weight gain, visceral fat (epididymal and retroperitoneal fat pads) weight, and calorie intake of HF diet mice was significantly higher than that of HC mice (Fig. 6B–D). The calorie intake during the first week was similar to that during the final week in each experimental group (data not shown). HF diet mice treated with GW9662 for 8 weeks gained body weight at about the same rate as control diet (HC diet) mice, which was significantly less than the untreated HF diet mice (Fig. 6A and B). Consistent with this result, treatment of HF diet mice with GW9662 completely protected them from HF diet-induced increases in visceral adipose tissue mass (Fig. 6C). Although the calorie intake of GW9662-treated HF diet mice was similar to that of untreated mice, body weight and visceral fat mass decreased dramatically in GW9662-treated HF diet mice (Fig. 6D). Mice fed a HF-diet for 8 weeks had a similar liver weight to HC mice, with no appreciable fatty acid accumulation (data not shown). GW9662 did not affect liver weight or liver appearance (Table 1).

3.5. *GW9662 does not change HF diet-induced glucose intolerance*

To determine the effect of GW9662 treatment on glucose metabolism, oral glucose tolerance tests were performed on HC diet mice fasted overnight and GW9662-treated HF diet mice. The HF diet mice had significantly elevated base line fasted blood glucose values compared to the HC diet mice

Table 1 – Final body weight, liver weight, and non-fasted blood glucose, insulin, triglyceride, total cholesterol, and NEFA levels

Parameter	HC	HF	HF + GW9662
Body weight (g)	19.5 ± 0.3	24.1 ± 1.0***	20.7 ± 0.5**
Liver weight (g)	0.96 ± 0.06	1.1 ± 0.04	1.1 ± 0.02
Blood glucose (mg/dL)	105.2 ± 3.7	131.9 ± 2.9***	118.2 ± 3.6*
Insulin (ng/mL)	1.6 ± 0.6	1.3 ± 0.2	2.6 ± 0.5*
TG (mg/dL)	60.3 ± 4.0	51.4 ± 2.7	36.6 ± 2.4**
TC (mg/dL)	102.3 ± 3.5	97.5 ± 1.8	94.7 ± 2.4
NEFA (mEq/L)	0.53 ± 0.06	0.42 ± 0.02	0.38 ± 0.04

Note: Each value represents the mean ± S.E.M. of eight mice.
* $P < 0.05$ vs. HF by the one-way ANOVA followed by the Fisher's least significant difference test.
** $P < 0.01$ vs. HF by the one-way ANOVA followed by the Fisher's least significant difference test.
*** $P < 0.001$ vs. HC by the one-way ANOVA followed by the Fisher's least significant difference test.

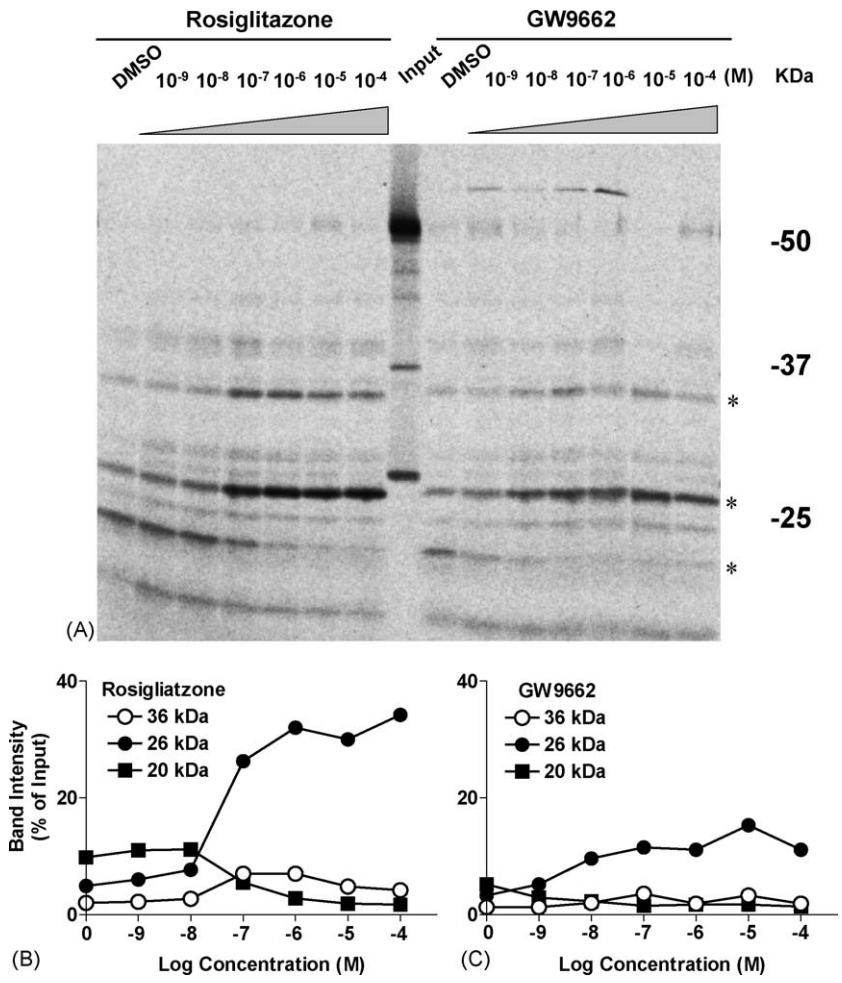

Fig. 5 – Difference in protease sensitivity of GW9662- and rosiglitazone-bound PPARγ. (A) Autoradiogram of an SDS-polyacrylamide gel showing [^{35}S]methionine-labeled full-length PPARγ2 digested with trypsin in the presence or absence of increasingly higher concentrations of either GW9662 or rosiglitazone. The migration of protein size markers (kDa) is indicated on the right. To measure the relative levels of the bands protected by each test compound on the gel, the intensity of the band of full-length human PPARγ2 treated without trypsin, around 50 kDa, was assumed to be 100%. (B and C) Gel quantification results are shown (B: rosiglitazone, C: GW9662). The experiment was repeated two times with similar results. Asterisk (*) denotes trypsin-resistant protein fragments.

(HF diet mice 75.9 ± 1.8 mg/dL, HC diet mice, 61.7 ± 3.8 mg/dL), and the glucose levels rose higher and remained elevated for longer than those of the HC diet mice. Treatment of HF diet mice with GW9662 did not affect the increase of fasted blood glucose levels (77.1 ± 2.7 mg/dL) or glucose intolerance (Fig. 7).

GW9662 treatment of non-fasted HF diet mice caused blood glucose levels to decrease slightly and plasma insulin levels to increase. Although GW9662 reduced plasma triglycerides levels, the plasma NEFA and total cholesterol levels of HF diet mice did not change significantly (Table 1).

4. Discussion

The purpose of this study was to clarify the effects of PPARγ antagonism on excess adiposity observed in HF-diet mice using a synthetic full PPARγ antagonist.

Leesnitzer et al. reported that GW9662 was an antagonist of both PPARγ with an IC_{50} of 3.3 nM and PPARδ with an IC_{50} of 4.1 nM, as well as a partial agonist of PPARα with an EC_{50} of 22 nM (the maximal activity was 42% of that of the PPARα agonist, GW7647) in the experiments using isolated ligand binding domains (LBDs). However, when full-length receptors were used in the cell-based assays, GW9662 showed no agonist or antagonist activity against PPARδ. The lack of effects of GW9662 on PPARα and PPARδ may reflect a decreased reactivity of the binding site Cys relative to the LBDs. From these results, they summarized that GW9662 was a potent selective PPARγ antagonist [27].

In a binding assay performed in this study, GW9662 bound to PPARγ LBD and replaced [^{3}H]rosiglitazone with a K_i value of 13 nM. This data suggested that GW9662 was high affinity ligand of PPARγ. However, GW9662 neither increased PPARγ transactivation nor accelerated 3T3-L1 adipocyte differentiation in vitro. GW9662 also suppressed 3T3-L1 adipocyte

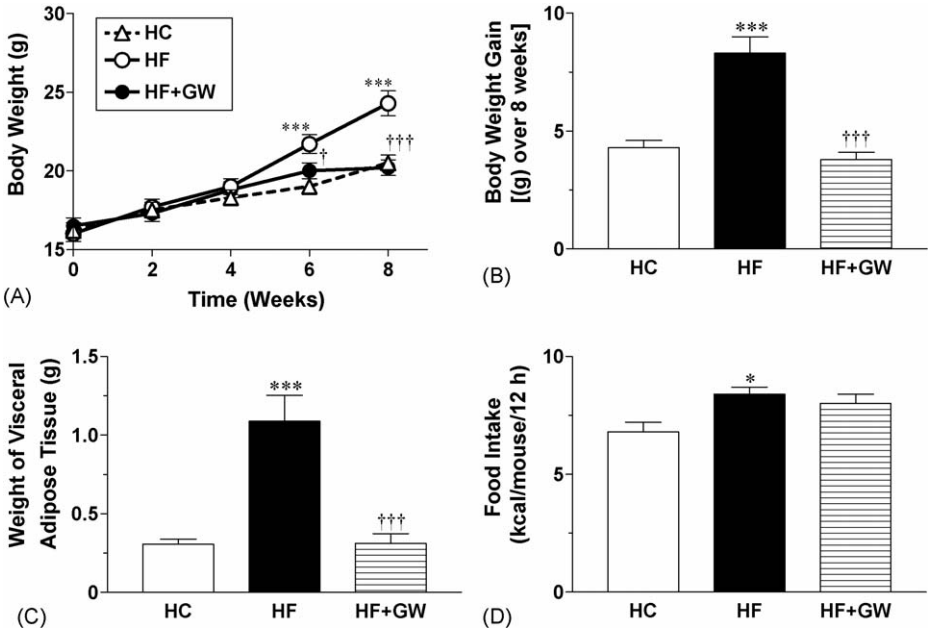

Fig. 6 – Body weight gain and increases in adipose tissue mass while being fed a HF diet were prevented in GW9662-treated mice. (A and B) Body weight gain during an 8-week period of consuming a HF diet in vehicle-treated and GW9662-treated mice compared to those consuming a HC diet. (C) Total weight of visceral fat (epididymal and retroperitoneal fat pads) from HF or HC diet mice. (D) Calorie intake in each experimental group was measured for 12 h (from 8 p.m. to 8 a.m.) in separate 3 days at 8 weeks. GW9662 treatment did not affect food intake. Values are expressed as the mean ± S.E.M. ($N = 8$). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. HC. $^{\dagger\dagger\dagger}P < 0.001$ vs. HF.

differentiation that had been dose dependently induced by 1 μM rosiglitazone. Based on this experimental evidence, we concluded that GW9662 was a full PPARγ antagonist.

The trypsin digestion assay showed that the conformational change in PPARγ induced by GW9662 was different from that induced by rosiglitazone. Since cofactor recruitment is induced by changes in protein conformation, treatment with GW9662 did not recruit the same PPARγ cofactors as those recruited by rosiglitazone. The cofactors used in this study, such as p300/CBP, SRC-1, and TIF-II, are known basal transcriptional factors and important PPARγ transactivation factors [41–43]. Berger et al. [47] analyzed the in vivo and in vitro effects of troglitazone, pioglitazone, and rosiglitazone on blood glucose levels, PPARγ binding, transactivation, and conformational changes in the receptor. They concluded that the hypoglycemic activity of the TZDs was directly mediated through PPARγ binding and the resulting active conformation of the receptor. Although GW9662 was a high affinity PPARγ ligand, it did not induce the preferred conformational change required for cofactor recruitment. Therefore, GW9662 did not induce PPARγ transactivation or adipocyte differentiation. In this study, we used HepG2 hepatoma cells for the PPARγ activation assay, and 3T3-L1 adipocytes for analysis of adipocyte differentiation. It is possible that the transcriptional mechanism could behave differently in hepatic cell lines versus adipocytes. The data generated in this study support the relationships between cofactor recruitment and transactivation/adipocyte differentiation posed in this paper. The suggested hypotheses are reasonable since each cofactor used in the cofactor recruitment assay expressed in both hepatic and adipose cells and is a basal transcriptional factor.

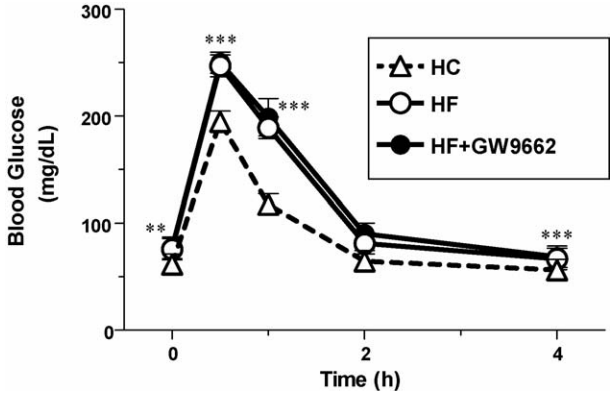

Fig. 7 – OGTT after 8 weeks under either a HF or HC diet. Blood glucose levels were measured at the indicated time points. Values are expressed as the mean ± S.E.M. ($N = 8$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HC.

HF diet mice have been used to construct a model of obesity-induced insulin resistance [15,40]. The effect of this type of diet was evident in the increased amount of visceral adipose tissue present in the mice. Rosiglitazone, a PPARγ agonist, has also been shown to increase adipose tissue mass in Wistar rats fed a HF diet [44,45]. However, this increase in adiposity was not observed in PPARγ Hetero KO (PPARγ HT) mice [15] or fat-selective PPARγ-KO (FKOγ) mice fed a HF diet [20]. These data suggest that PPARγ plays an important role in the hyperformation of adipose tissue induced by a HF diet. The data generated in this study indicate that PPARγ-specific antagonists suppress HF diet-induced increases in visceral

adipose tissue. This, in turn, supports our hypothesis about the role PPARγ plays in increased adiposity.

Treatment with GW9662 failed to ameliorate the glucose intolerance induced by a HF diet. The PPARγ-deficient models suggest insulin resistance since the mice exhibited hyperinsulinemia. The GW9662-treated mice in this study exhibited insulin levels twice as high as those of the control HF mice, whereas their blood glucose levels were only 10% less (Table 1). These results suggest that PPARγ antagonism induces slight insulin resistance. This insulin resistance seems to cause the inability of GW9662 to improve glucose intolerance. Two theories as to why PPARγ antagonism would induce insulin resistance have been suggested. The first is that the lack of adequate adipose tissue mass itself contributes directly to impaired glucose metabolism. Although human studies have established that more than 80% of insulin-mediated glucose uptake occurs in skeletal muscle [48], research using mice suggests that adipose tissue may also be a significant target. For example, mice with a muscle-specific knockout of the insulin receptor gene still exhibited normal glucose tolerance, demonstrating that tissues other than muscle, most notably adipose, play a major role in glucose metabolism in mice [49]. The second theory is that the decrease in visceral fat resulting from treatment with GW9662 caused insulin resistance via impairment of the leptin pathway. The second theory is based on the evidence that adipose tissue helps control energy homeostasis via leptin, a hormone it is known to secrete, and possibly other hormones as well [50]. Treatment of HF diet-fed PPARγ HT mice with a retinoid X receptor (RXR) antagonist, HX531, induced lipoatrophy, a decrease in the serum leptin level, and insulin resistance [24]. These results obtained by Yamauchi et al. suggest that decreases in PPARγ/RXR activity may be associated with decreased serum leptin levels due to adipose tissue depletion. This may then lead to impairment of the leptin pathway resulting in insulin resistance [24]. It is likely that the serum leptin level decreased in GW9662-treated mice since GW9662 decreased the weight of visceral adipose tissue in the HF diet mice.

In contrast, GW9662 treatment did not exaggerate glucose intolerance in HF diet mice. Several groups have conducted analyses utilizing mouse models of reduced amounts of adipose tissue, such as those using aP2/DTA mice [51,52], A-ZIP/F-1 mice [50,53], and fld mice [54]. These adipose-deficient models demonstrated severe glucose intolerance and illustrated that adipose tissue is necessary for the normal regulation of glucose metabolism. The results of this study showed that even though the amount of visceral adipose tissue in GW9662-treated mice was reduced, their glucose intolerance was similar to that of control-HF diet mice. GW9662-treated mice did not completely lose their visceral adipose tissue as the model mice did. GW9662-treated mice retained the same amount of visceral adipose tissue as the HC diet mice (i.e. normal level). This normal level of visceral adipose tissue would prevent severe glucose intolerance by secreting leptin and/or controlling energy homeostasis directly. The fact that the food intake of GW9662-treated HF diet mice was similar to that of non-treated HF diet mice (Fig. 6D) supports this idea. Further studies will be required to determine how GW9662 selectively suppresses the increased adiposity induced by a HF diet only.

PPARγ2 KO mice and FKOγ mice exhibited normal glucose tolerance when fed regular chow [8,20]. Muscle-specific PPARγ KO (MKOγ) mice on a 2-week HF diet exhibited impaired glucose tolerance indistinguishable from that of the wild type (WT) [17]. The development of glucose intolerance after 15 weeks on a HF diet in WT mice was only somewhat inhibited in PPARγ HT mice [15]. These reports on gene targeting methods, along with our current data generated using a synthetic ligand, show that reduced PPARγ function will not cause severe glucose intolerance.

A recent study documented that fat mass reduction accompanied by hyperlipidemia and hepatomegaly was observed in 14-month-old FKOγ mice, whereas liver weight and gross morphology in 6-month-old FKOγ mice were similar to those of WT mice [20]. Similar results were observed in MKOγ mice [17,19]. Six-month-old PPARγ2 KO mice also had a normal liver appearance and no appreciable fatty acid accumulation in the liver [8]. The liver weight and appearance of the mice treated with GW9662 for 8 weeks did not change (Table 1). These results suggest that a fatty liver is formed as a result of crosstalking between adipose tissue, liver, and muscle over a long period of time. It is likely that the 8-week feeding period used in this study was too short to precipitate detectable alterations of the liver.

In conclusion, results obtained using the synthetic PPARγ antagonist suggest that (1) the synthetic PPARγ antagonist suppresses the excess adiposity observed in HF diet-induced obesity similar to the results of knock-out mice studies, (2) antagonism of PPARγ does not cause severe glucose intolerance, and (3) it's possible that a PPARγ antagonists could be used as anti-obesity drugs.

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