

Activation of peroxisome proliferator-activated receptor- α stimulates both differentiation and fatty acid oxidation in adipocytes^S

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Abstract Peroxisome proliferator-activated receptor- α (PPAR α) is a dietary lipid sensor, whose activation results in hypolipidemic effects. In this study, we investigated whether PPAR α activation affects energy metabolism in white adipose tissue (WAT). Activation of PPAR α by its agonist (bezafibrate) markedly reduced adiposity in KK mice fed a high-fat diet. In 3T3-L1 adipocytes, addition of GW7647, a highly specific PPAR α agonist, during adipocyte differentiation enhanced glycerol-3-phosphate dehydrogenase activity, insulin-stimulated glucose uptake, and adipogenic gene expression. However, triglyceride accumulation was not increased by PPAR α activation. PPAR α activation induced expression of target genes involved in FA oxidation and stimulated FA oxidation. In WAT of KK mice treated with bezafibrate, both adipogenic and FA oxidation-related genes were significantly upregulated. These changes in mRNA expression were not observed in PPAR α -deficient mice. Bezafibrate treatment enhanced FA oxidation in isolated adipocytes, suppressing adipocyte hypertrophy. Chromatin immunoprecipitation (ChIP) assay revealed that PPAR α was recruited to promoter regions of both adipogenic and FA oxidation-related genes in the presence of GW7647 in 3T3-L1 adipocytes. **These findings indicate that the activation of PPAR α affects energy metabolism in adipocytes, and PPAR α activation in WAT may contribute to the clinical effects of fibrate drugs.**—Goto, T., J.-Y. Lee, A. Teraminami, Y.-I. Kim, S. Hirai, T. Uemura, H. Inoue, N. Takahashi, and T. Kawada. **Activation of peroxisome proliferator-activated receptor- α stimulates both differentiation and fatty acid oxidation in adipocytes.** *J. Lipid Res.* 2011. 52: 873–884.

Supplementary key words PPAR α • adipocyte differentiation • insulin resistance • metabolic syndrome

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The prevalence of obesity has markedly increased over the last few decades not only in wealthy industrialized countries, but also in poor underdeveloped nations (1). Obesity and overweight have adverse health effects and affect the risk and prognosis of many serious medical conditions, such as type 2 diabetes, coronary heart disease, high blood pressure, and some forms of cancer. Obesity causes excess fat accumulation not only in various tissues, particularly adipose tissues, but also in other insulin-responsive organs such as the skeletal muscle and liver, predisposing one to the development of insulin resistance (2–5). However, the molecular mechanisms underlying insulin resistance and obesity have not been fully clarified, and effective therapeutic approaches are currently of general interest.

Peroxisome proliferator-activated receptors (PPAR) α , γ , and δ are FA-activated nuclear transcription factors that control mRNA expression of numerous genes involved in energy metabolism (6–8). In particular, PPAR α is expressed mostly in tissues with high rates of FA oxidation and peroxisomal metabolism (9), such as the liver, brown fat, and heart (10–12). In these tissues, PPAR α regulates mRNA expression of genes involved in FA oxidation, and synthetic PPAR α agonists, such as fibrates,

Abbreviations: 2-DG, 2-deoxy-d-glucose; ACO, acyl-CoA oxidase; ap2, adipocyte fatty-acid-binding protein; BAT, brown adipose tissue; ChIP, chromatin immunoprecipitation; CPT1b, carnitine-palmitoyl transferase-1b; GPDH, glycerol-3-phosphate dehydrogenase; HFD, high-fat diet; IL-6, interleukin-6; ITT, insulin tolerance test; MCP-1, monocyte chemoattractant protein-1; OCR, oxygen consumption rate; OGTT, oral glucose tolerance test; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-response element; RER, respiratory exchange ratio; SV, stromal vascular; TG, triglyceride; TNF α , tumor necrosis factor- α ; TZD, thiazolidinedione; UCP3, uncoupling protein 3; WAT, white adipose tissue; WT, wild-type.

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decrease circulating lipid levels and are commonly used to treat hyperlipidemia and other dyslipidemic states (9, 13). PPAR α -deficient (PPAR $\alpha^{-/-}$) mice exhibit higher levels of serum triglyceride (TG) (14). Moreover, these mice show extensive hepatic lipid accumulation and increased gonadal adipose storage and plasma FFA levels (15). In addition, the presence of severe hypoketonemia, hypoglycemia, and hypothermia in PPAR $\alpha^{-/-}$ mice clearly supports a function of PPAR α in metabolic regulation.

Previous studies showed that adipose tissue dysfunction plays an important role in the development of obesity and obesity-associated diseases (16, 17). Adipose tissue also participates in the regulation of energy homeostasis as an important endocrine organ that secretes many biologically active molecules called adipocytokines (16–19). The secretion pattern of adipocytokines is closely related to adipocyte condition (18). Small adipocytes secrete insulin-sensitizing hormones such as adiponectin, although hypertrophied adipocytes exhibit low expression levels of these factors but increased expression levels of hormones related to insulin resistance, such as tumor necrosis factor- α (TNF α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6), resulting in insulin resistance observed in obesity (19). Indeed, thiazolidinediones (TZD), insulin sensitizers, promote differentiation of preadipocytes by PPAR γ activation to increase the number of small adipocytes and to decrease the number of hypertrophied adipocytes by promoting apoptosis (20).

Besides their hypolipidemic effects, PPAR α activators attenuate adiposity in animal models of obesity and type-2 diabetes mellitus (21, 22). Recently, several studies have shown that PPAR α is expressed in the adipose tissue of humans and rodents, indicating that adipose tissue might also be a target organ of PPAR α activators (22–25). Hiuge et al. demonstrated that fibrates directly and transcriptionally increase adiponectin expression level via adipose PPAR α (23). Furthermore, it was suggested that PPAR α ligands directly stimulate lipolysis in wild-type (WT) adipocytes, but this effect is not observed in PPAR α -deficient adipocytes (25). These previous findings indicate that PPAR α functions in adipocytes. However, the role of PPAR α in adipose tissue is not fully understood.

In this study, we investigated whether PPAR α activation affects the energy metabolism in white adipose tissue (WAT). Treatment with bezafibrate, a PPAR α agonist, markedly attenuated adiposity in WT mice but not in PPAR $\alpha^{-/-}$ mice fed a high-fat diet (HFD). Both genetic and HFD-induced obesity decreased PPAR α mRNA expression level, and fasting increased the expression level in WAT. PPAR α activation promoted both adipocyte differentiation and FA oxidation in 3T3-L1 adipocytes. These effects were also observed in vivo, which resulted in the enhancement of whole-body oxygen consumption and suppression of adipocyte hypertrophy. These findings indicate that the pharmacological activation of PPAR α affects energy metabolism in adipocytes and that the PPAR α activation in WAT may contribute to the clinical effects of fibrate drugs.

Animal experiments

All the mice were maintained in a temperature-controlled (23°C) facility with a constant 12 h light/dark cycle and free access to water. For the analysis of organ PPAR α expression levels, 8-week-old male C57BL/6 mice (CLEA Japan, Tokyo, Japan) were fed a standard diet (CRF-1, Charles River Japan, Tokyo, Japan). Non-fasted, fasted (24 h), and fasted (24 h)/refed (24 h) C57BL/6 mice were used before euthanization. After euthanization, their tissues were harvested for RNA isolation. The harvested tissues were immediately frozen in liquid nitrogen and stored at -80°C until use. We used male C57BL/6 mice fed either the standard diet or 60% HFD (D12492 Research Diet, MO) for 12 months for the diet-induced obese model mice. Male ob/ob mice and lean control mice (age 12 weeks from Charles River Japan) received a standard diet for genetically obese mice.

To investigate the effect of the PPAR α agonist on adipose tissue metabolism, we used KK mice or WT and PPAR $\alpha^{-/-}$ mice with a Sv/129 genetic background. Five-week-old male KK (CLEA Japan) mice were maintained for five to eight weeks either on HFD or on HFD containing 0.2% bezafibrate. The energy intake of all mice was adjusted by pair-feeding. Five to eight weeks after feeding, the mice were subjected to analyses of their oxygen consumption, locomotor activity, oral glucose tolerance test (OGTT), insulin tolerance test (ITT), mRNA expression, FA oxidation in isolated adipocytes, and histological features. Eight-week-old WT and PPAR $\alpha^{-/-}$ mice were fed HFD or HFD containing 0.2% bezafibrate for six weeks. The same amount of food was given to all mice. Ten h after fasting, tissues were harvested for RNA analysis. The animal care procedures and methods were approved by the Animal Care Committee of Kyoto University.

To analyze the oxygen consumption of mice, male KK mice fed HFD or HFD containing 0.2% bezafibrate for five weeks were used. The oxygen consumption rate (OCR) of mice under the fed condition was measured using an indirect calorimetric system (Oxymax, Columbus Instruments, OH) every 9 min for 20 h. The measurements started at 9:00 PM and ended at 6:00 PM (both the dark and light phases were 10 h).

Male KK mice were maintained for five weeks either on HFD or HFD containing 0.2% bezafibrate. For OGTT, d-glucose (2 g/kg body weight) was administered through a gastric feeding tube after overnight fasting. For ITT, human insulin (Eli Lilly Japan, Kobe, Japan) was injected intraperitoneally (0.6 units/kg body weight) into nonfasted animals. Blood samples were collected from the tail vein before and 30, 60, 90, and 120 min after injection. Plasma glucose level was determined by the glucose C-test Wako (Wako Pure Chemicals, Osaka, Japan) in accordance with the manufacturer's protocols.

Epididymal WAT was removed from each animal, fixed in 10% formaldehyde/PBS, and maintained at 4°C until use. The fixed samples were embedded in paraffin. They were cut into 12 μm sections using a microtome and mounted on silanized slides. Adipocyte size distribution was determined using National Institutes of Health (NIH) Image J software.

Cell culture and retrovirus-mediated PPAR α expression

To prepare stromal-vascular (SV) cells and adipocytes from WAT, epididymal WAT from male mice was removed under sterile conditions and washed in KRH buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO $_4$, 1 mM CaCl $_2$; pH 7.4). Minced tissue was transferred to a sterile polypropylene tube containing collagenase (1 mg/ml, type II, Sigma) and 1% FA-free BSA (BSA). After incubation at 37°C for 45 min with shaking, the digested tissue was filtered through a sterile 250 μm nylon mesh.

The digested tissue was centrifuged at 200 *g* for 2 min, and mature adipocytes and SV cells were collected. Both cell types were washed twice by centrifugation using KRH buffer containing 1% FA-free BSA. After washing, cells were subjected to each assay.

3T3-L1 murine preadipocytes (from American Type Culture Collection, Manassas, VA) were cultured as previously described (26). Human multipotent adipose tissue-derived stem cells were cultured as previously reported (27). Cells 14 days after the induction of differentiation were used as mature adipocytes.

A PCR fragment coding mouse PPAR α obtained by RT-PCR using total liver RNA from C57BL/6 mice was inserted into the site between BamHI and NotI of pMX-puro (a kind gift from Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) (pMX-PPAR α). The PCR primers used were as follows: the 5'-primer, TTTGATCCATGGTGGACACAGAGAGCCCCATC; and the 3'-primer, TTTGCGGCCGCTCAGTACATGTCTCTGTAGATCTCTTGC. Plat-E cells (kindly provided by Dr. Naohito Aoki, Mie University, Tsu, Japan) were cultured in a growth medium at 37°C in 5% CO₂. The pMX-PPAR α was transfected into Plat-E packaging cells using Lipofectamine2000 (Invitrogen, CA) in accordance with the manufacturer's instructions. Viral supernatants were harvested 48 h after transfection. Viral supernatants were applied to 3T3-L1 cells in the growth medium supplemented with 5 μ g/ml polybrene. To eliminate uninfected cells, the medium was replaced with a growth medium containing 2.5 μ g/ml puromycin.

Insulin-stimulated 2-deoxy-d-glucose uptake assay

The level of uptake of 2-deoxy-d-[1,2-³H]glucose (2-DG) was measured as previously described (26). Briefly, 10 days after differentiation induction, 3T3-L1 cells were deprived of serum by incubation for 5 h in 12-well plates containing DMEM. The cells were then incubated with 100 nM insulin for 20 min in 450 μ l of KRH buffer containing 1% FA-free BSA. Glucose uptake was initiated by adding 50 μ l KRH buffer containing 1 mM 2-deoxy-d-[1,2-³H]glucose (1 μ Ci) (American Radiolabeled Chemicals, MO) to each well, and after 10 min, uptake was terminated by washing the cells three times with ice-cold PBS buffer. The cells were solubilized with 0.1N NaOH, and incorporated radioactivity was measured by liquid scintillation counting. The radioactivity was normalized to protein content determined by the method of Bradford (Bio-Rad Laboratories, CA).

FA oxidation assay

Ten days after differentiation induction, 3T3-L1 cells overexpressing PPAR α (L1-PPAR α) or control cells (L1-Mock) were incubated in DMEM containing 0.2 mM palmitic acid, 2.5% FA-free BSA, 200 μ M l-carnitine, and [¹⁴C]palmitic acid (1 μ Ci/ml) (American Radiolabeled Chemicals) for 16 h. FA oxidation products were assessed as previously described (28) with modification. Briefly, the labeling medium was collected and centrifuged, and the supernatant was transferred to a 50-ml polypropylene tube. An uncapped Eppendorf tube containing a piece of filter paper soaked in benzethonium hydroxide was placed inside a 50 ml tube. After the tube was sealed, 200 μ l of 70% perchloric acid was added to the medium sample to release [¹⁴C]CO₂. The tube was then shaken at 37°C for 1 h. The saturated filter paper containing trapped [¹⁴C]CO₂ was assessed for radioactivity in a liquid scintillation counter. The acidified medium was centrifuged twice to remove particulate matter, and 200 μ l of supernatant was assessed for the amount of [¹⁴C]labeled acid-soluble FA metabolites (ASM).

FA oxidation with isolated adipocytes was analyzed as previously described (29). In brief, isolated adipocytes were prepared from epididymal WAT and washed in KRH with 1% BSA. A fresh buffer was added to the cells at a volume twice the packed cell

volume. Tubes containing cells and buffer were inverted several times to obtain a homogenous mixture, and equal aliquots of each sample were distributed into 50 ml polystyrene tubes. [¹⁴C] palmitic acid was added at a final concentration of 1 μ Ci/ml. The tubes with a piece of filter paper soaked in benzethonium hydroxide were then sealed and incubated at 37°C for 2 h. The tubes were gently shaken every 30 min during the incubation. After 2 h, 12 M HCl was added to the cells to release [¹⁴C]CO₂, and each tube was resealed and incubated at 37°C overnight. The amount of [¹⁴C]CO₂ released was measured by scintillation counting of the filter paper. Radioactivity was normalized to genomic DNA content in each packed cell. Packed cells were incubated overnight with proteinase K (150 μ g/ml). Genomic DNA was purified using phenol/chloroform, and its content was quantified by measuring the absorbance at 260 nm.

Measurement of OCR in 3T3-L1 cells

Cellular OCR was measured using a Seahorse Bioscience XF24 analyzer (30) in 24-well plates at 37°C, with correction for positional temperature variations adjusted from 4 empty wells evenly distributed within the plate. L1-Mock or L1-PPAR α cells were induced to differentiate and were maintained for 10 days with or without the PPAR α agonist GW7647. Immediately before the measurement, cells were washed, and 675 μ l of nonbuffered (sodium-carbonate-free) pH 7.4 DMEM medium supplemented with 0.2 mM palmitic acid, 0.2 mM l-carnitine, and 2% FA-free BSA was added to each well. After a 15 min equilibration period, four successive 1.5 min measurements were performed at 7 min intervals with intermeasurement mixing to homogenize the oxygen concentration in the medium. Rotenone (final concentration of 100 nM) was injected to each well using the internal injectors of the cartridge, and four successive 1.5 min measurements were performed at 7 min intervals with intermeasurement mixing.

Measurements of TG content and GPDH activity

To determine intracellular TG amount, 3T3-L1 cells grown in 12-well plates were washed with PBS, and lipids were extracted by hexane-isopropyl alcohol (3:2, v/v) for 1 h at room temperature. Aliquots were transferred and evaporated under a decreased pressure. Samples were resuspended in 10% TritonX-100 in isopropyl alcohol, and TG content was measured enzymatically using triglyceride G test Wako (Wako Pure Chemicals). For Oil Red O staining, 3T3-L1 cells 10 days after the induction of differentiation were fixed with 10% formaldehyde/PBS and stained with Oil Red O solution [0.5% Oil Red O-isopropyl alcohol/H₂O (3:2, v/v)].

Cells were washed twice with PBS and collected with a cell scraper into 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The harvested cells were sonicated for 5 s at 40 watts with a microson ULTRASONIC CELL DISRUPTOR (Misonix, Inc., NY). After centrifugation at 12,800 *g* for 5 min at 4°C, the supernatants were assayed for glycerol-3-phosphate dehydrogenase (GPDH) activity as described elsewhere (31).

RNA analysis

Total RNA was prepared from mouse tissues or cultured 3T3-L1 and human multipotent adipose tissue-derived stem adipocytes using Qiazol lysis reagent (QIAGEN, CA) or Sepasol(R)-RNA I Super (Nacalai Tesuque) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, WI) in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP, Takara, Shiga, Japan). To visualize and compare the expression levels of PPAR α and 36B4, semiquantitative

PCR was performed. PCR amplification was performed with denaturation at 94°C for 20 s, annealing at 60°C for 40 s, and polymerization at 72°C for 20 s. PCR was performed for 35 cycles. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. To quantify mRNA expression, real-time RT-PCR was performed with a LightCycler System (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence signals as described previously (31–34). The oligonucleotide primers of mouse and human 36B4 and PPAR α , mouse adipogenic marker genes, and PPAR α target genes were designed using a PCR primer selection program in the website of the Virtual Genomic Center from the GenBank database. All oligonucleotide primer sets used to measure the expression levels of 36B4, adipocyte fatty-acid-binding protein (aP2), PPAR γ , and adiponectin were previously described (31–34). The primers used for measurements of the mRNA expression levels of other genes (upstream and downstream, respectively) were TCAGGG-TACCACTACGGAGT and CTTGGCATTCTTCCAAAGCG for mouse PPAR α mRNA; ACTCCACCTGCAGAGCAACCA and TAGATCTCCTGCAGTAGCGGG for human PPAR α mRNA; GCACCATTGCCATTTCGATACA and CCACTGCTGTGAGAAT-AGCCGT for acyl-CoA oxidase (ACO) mRNA; CTGTTAGGCCT-CAACACCGAAC and CTGTGATGGCTAGGCTGTACAT for carnitine-palmitoyl transferase-1b (CPT1b) mRNA; and TAC-CCAACCTTGGCTAGACCTC and GCAACTTCTCCTTGATGATGTCGTA for uncoupling protein 3 (UCP3) mRNA. To compare mRNA expression level among the samples, the copy numbers of all transcripts were divided by that of mouse 36B4 showing a constant expression level in adipocytes. All mRNA expression levels

were represented as a ratio relative to that of the control in each experiment.

Immunoblotting

Total cellular proteins were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS; pH 7.4) and a protease inhibitor cocktail). The protein concentration of samples was determined using a protein assay kit (Bio-Rad Laboratories). Protein samples (30 μ g) were subjected to SDS-PAGE on a 10% gel. Separated proteins were transferred electrophoretically to PVDF membranes (Millipore, MA), which were blocked with 5% nonfat dried milk in phosphate-buffered saline. The membranes were incubated with antibodies to PPAR α (Perseus Proteomics, Tokyo, Japan) and β -actin (Cell Signaling Technology, MA), and then with peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Santa Cruz, CA), respectively. Proteins were detected using an ECL Western blotting detection system (GE Healthcare, NJ).

Chromatin immunoprecipitation (ChIP) assay

3T3-L1 cells 14 days after differentiation induction were fixed with 1% formaldehyde in PBS for 10 min. The cells were then rinsed twice with ice-cold PBS containing 2% bovine serum and 0.05% Na $_3$ N, centrifuged for 5 min at 3,000 rpm, and resuspended in lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and a protease inhibitor cocktail]. Following incubation for 20 min on ice, samples were sonicated on ice. The lysates were centrifuged at 15,000 rpm for 10 min, and the collected supernatant was diluted in buffer [1.1% Triton X-100, 0.11% so-

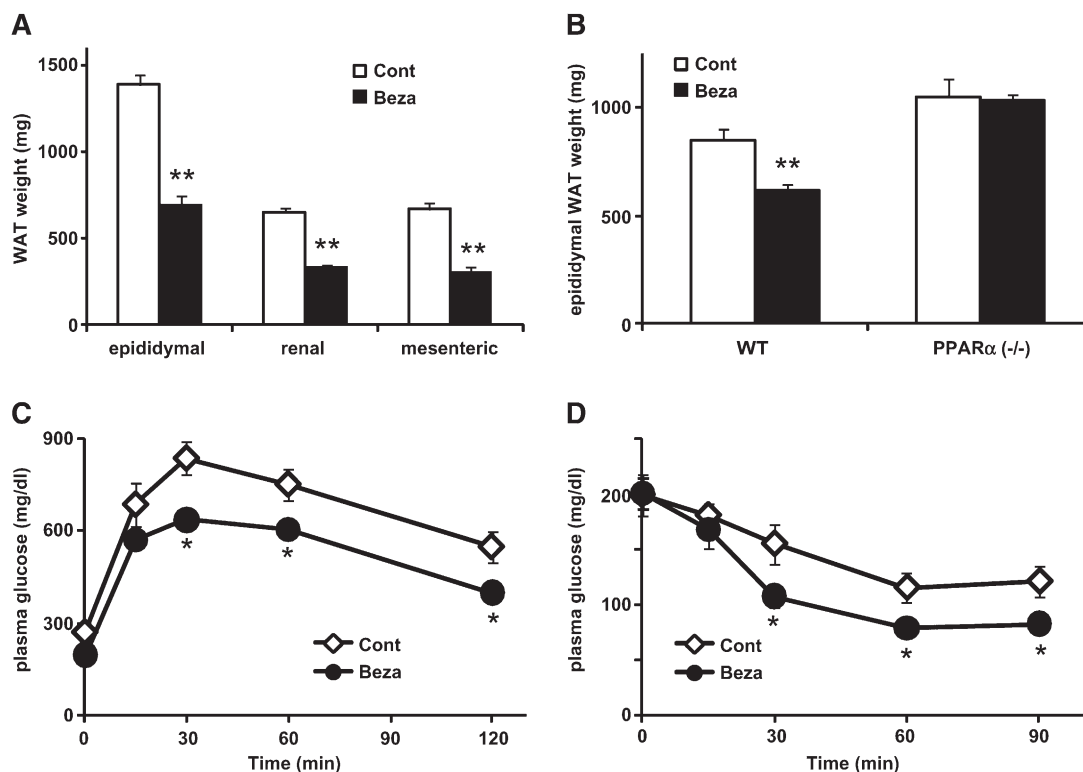


Fig. 1. Effects of PPAR α activator on adiposity and insulin resistance. A, B: White adipose tissue (WAT) weight from male mildly obese KK mice (A) and wild-type (WT) or PPAR α -deficient (PPAR α ^{-/-}) mice (B) fed high-fat diet control (Cont) or high-fat diet containing 0.2% bezafibrate (Beza) for four and six weeks. In each experiment, the mice were housed under pair-fed conditions. C, D: Plasma glucose levels during oral glucose tolerance test (GTT) (C) and insulin tolerance test (ITT) (D). Both GTT and ITT were performed on KK mice fed high-fat diet (Cont) or high-fat diet containing 0.2% bezafibrate (Beza) for five weeks under pair-fed condition. Plasma glucose level was measured by enzymatic colorimetric assay. Data are means \pm SEM of 4–6 animals per group. * P < 0.05, ** P < 0.01, compared with control diet group.

dium deoxycholate, 167 mM NaCl, 50 mM Tris-HCl; (pH 8.0) and a protease inhibitor cocktail]. Samples were precleared with 5 μ g of sheared salmon sperm DNA and 50 μ l of protein A/G-Sepharose beads (Santa Cruz) for 2 h. Immunoprecipitation with the antibody to PPAR α or normal mouse IgG (Santa Cruz) was performed overnight. Samples were then incubated with 20 μ l of protein A/G-Sepharose beads for 3 h followed by 10 min sequential washes in RIPA I (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, 50 mM Tris-HCl, and 150 mM NaCl; pH 8.0), RIPA II (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, 50 mM Tris-HCl, 500 mM NaCl; pH 8.0), buffer III (0.25M LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl; pH8.0), and Tris-EDTA buffer. Precipitates were then extracted by incubating with elution buffer (0.5% SDS, 5 mM EDTA, 300 mM NaCl, 10 mM Tris-HCl; pH8.0) at 65°C overnight. After the RNase A and proteinase K treatment, DNA fragments were purified with phenol/chloroform. Purified samples were used as templates for PCR performed for 35 cycles. Oligonucleotide primers composed of the following sequences (upstream and downstream) were used for PCR: aP2, GAATTCCAGCAGGAATCAGG and GCCAAAGAGACACAGAGGGCG (300 bp); CPT1b, CCTGT-GCTGGTCCCCAACTCACAGC and CTCCTGGTGACCTTTTC-CCTACAT T (279 bp).

Statistical analysis

The data were expressed as means \pm S.E.M. Statistical significance was evaluated using unpaired Student's *t*-test for the two groups. The results were considered significant at *P* < 0.05.

RESULTS

Administration of PPAR α agonist attenuated adiposity and improved insulin resistance in mice fed HFD

To study the effect of PPAR α activation on energy metabolism in WAT, we treated mice fed HFD with bezafibrate, a PPAR α agonist. Since PPAR α agonists have been shown to reduce food intake in rodents (35), all mice were housed in pair-fed conditions in each experiment. Administration of bezafibrate (0.2%) in mildly obese diabetic KK mice fed HFD for four weeks significantly decreased adiposity (50, 49, and 54% decrease in epididymal, perirenal, and mesenteric WAT, respectively) (Fig. 1A and supplementary Table I). Because bezafibrate has been reported to activate other PPAR subtypes (36), we used PPAR α ^{-/-} and WT (control) mice, and a similar experiment was performed. For a six-week treatment, bezafibrate significantly reduced adiposity in WT mice similarly to KK mice (Fig. 1B and supplementary Table II). However, this effect was not observed in PPAR α ^{-/-} mice. Although the WAT weight was slightly higher in PPAR α ^{-/-} mice than in WT mice, the difference was not statistically significant. These results indicate that pharmacological PPAR α activation reduces adiposity independently of its anorexic effect. Next, to examine the effect of a PPAR α agonist on insulin sensitivity, we carried out a GTT and ITT. Plasma glucose levels in both tests in the bezafibrate-treated mice were significantly lower than those in the pair-fed control mice (Fig. 1C, D), indicating that a PPAR α agonist ameliorates HFD-induced insulin resistance in KK mice.

PPAR α expression level increased during adipocyte differentiation in 3T3-L1 cells and changed depending on energy condition in WAT

We next examined the expression of PPAR α in cultured adipocytes and WAT. As previously reported (22, 23), PPAR α mRNA was highly expressed in the liver, brown adipose tissue, and kidney (supplementary Fig. IA, B). The PPAR α mRNA expression level in WAT was higher than that in the skeletal muscle, although that in WAT was lower than that in tissues with high mRNA expression level of PPAR α (supplementary Fig. IA, B). In WAT, PPAR α mRNA was expressed at a higher level in the adipocyte fraction than in the SV cell fraction (supplementary Fig. IC). In cultured 3T3-L1 cells, the mRNA expression level of PPAR α increased in parallel with the progression of adipocyte differentiation and in a pattern similar to that of adiponectin mRNA expression (Fig. 2A). In human multipotent adipose tissue-derived stem cells characterized by an extensive proliferative potential and the ability to undergo multilineage differentiation, including adipogenic differentiation (27), adipogenic induc-

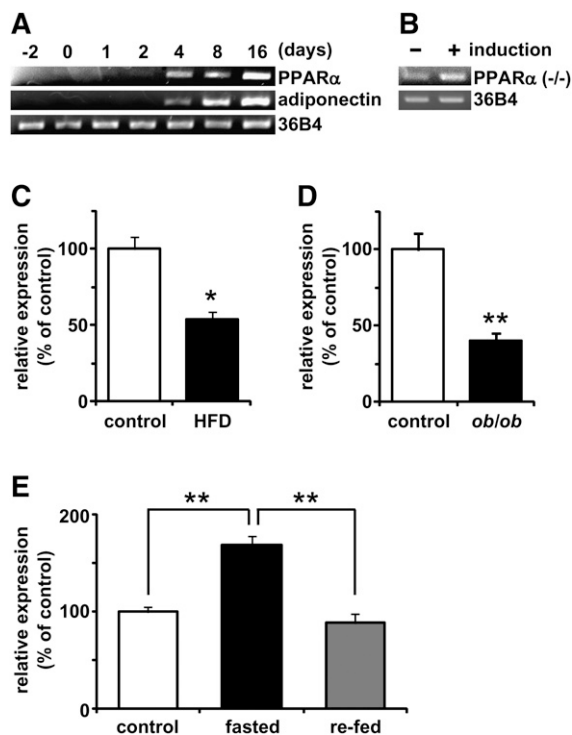


Fig. 2. mRNA expression levels of PPAR α in cultured cells and WAT. A, B: mRNA expression levels of murine PPAR α and adiponectin or human PPAR α during adipocyte differentiation. 3T3-L1 cells and human multipotent adipose tissue-derived stem cells were harvested at the indicated times after differentiation induction. Semiquantitative PCR conditions are described in "Materials and Methods." C, D: mRNA expression levels of PPAR α in obese model mice and lean control mice. PPAR α mRNA expression levels in C57BL/6 mice fed high-fat diet (HFD) or normal diet (control) for 12 months (C) and in ob/ob mice or lean control mice (D) were quantified by a real-time PCR as described in "Materials and Methods." E: The mRNA expression levels of PPAR α in WAT in C57BL/6 under fed (control), fasted (24 h), and fasted/refed (24 h after refeeding) conditions were quantified by real-time PCR. Data are means \pm SEM of 4-6 animals per group. **P* < 0.05, ***P* < 0.01.

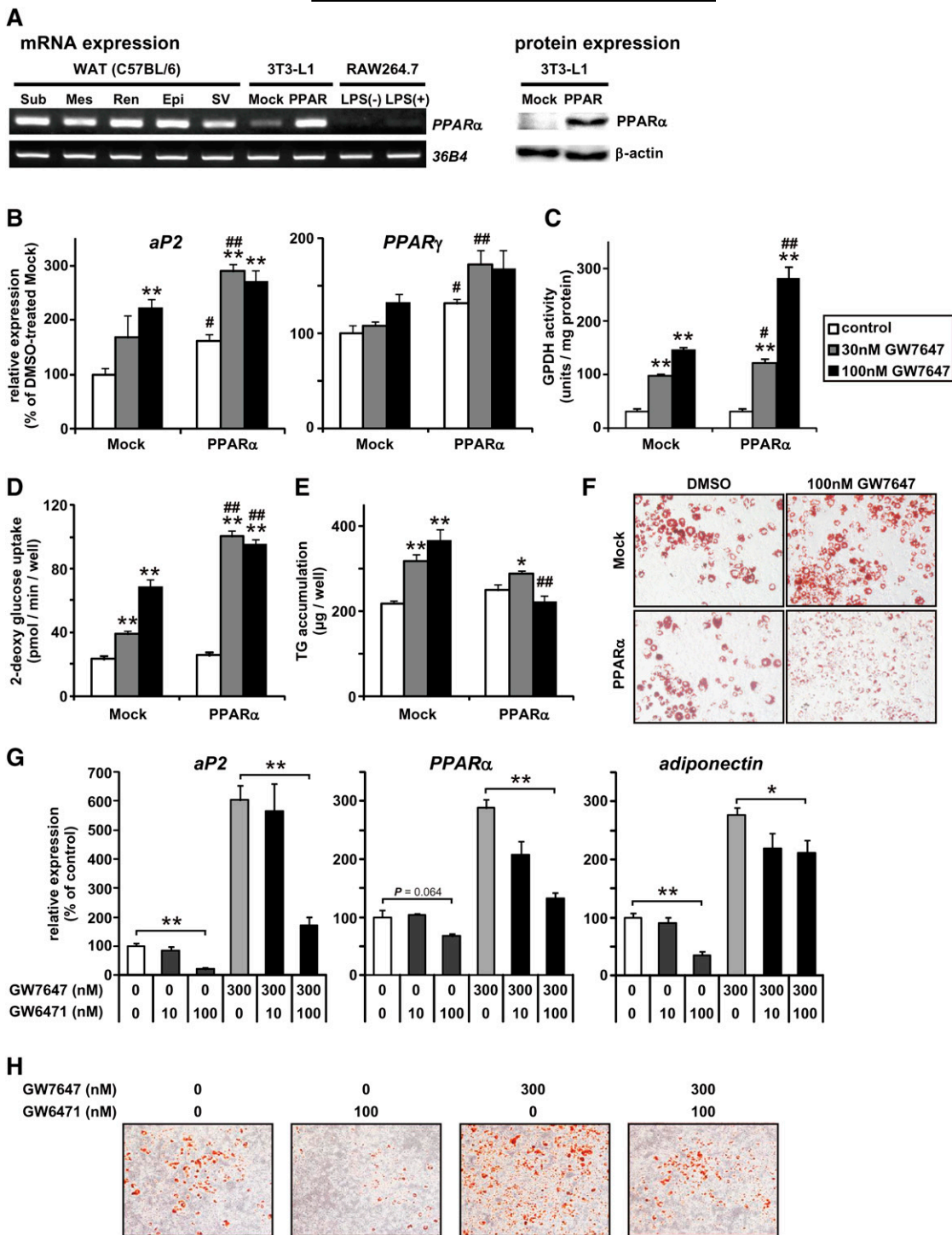


Fig. 3. PPAR α activation increased mRNA expression levels of marker factors for adipocyte differentiation but not TG content. **A:** PPAR α expressions in WAT and cultured cells. PPAR α mRNA levels in several WATs [subcutaneous (Sub), mesenteric (Mes), perirenal (Rea), and epididymal (Epi) WAT from C57BL/6 mice], stromal-vascular (SV) cells from epididymal WAT in C57BL/6 mice, and cultured cells [3T3-L1 cells overexpressing PPAR α (L1-PPAR α), mock control (L1-Mock), RAW264.7 macrophages, and LPS-stimulated RAW264.7 macrophages] were visualized by semiquantitative PCR (left). PPAR α protein levels in L1-Mock and L1-PPAR α were visualized by Western blotting (right). **B-F:** L1-Mock and L1-PPAR α cells were induced to differentiate and cultured with or without GW7647 (30 or 100 nM) for 6-10 days. **B-E:** The mRNA expression levels of adipogenic marker genes (*aP2* and *PPAR γ*) (on day 10) (**B**), glycerol-3-phosphate dehydrogenase (GPDH) activity (on day 6) (**C**), the capacity of insulin-stimulated 2-DG transport (on day 10) (**D**), and intracellular triglyceride (TG) amounts (**E**) in L1-Mock and L1-PPAR α were determined as described in "Materials and Methods." **F:** Microscopy views of representative L1-Mock and L1-PPAR α cells treated with or without 100 nM GW7647, fixed with formalin, and stained with Oil Red O. The original magnification is 100 \times . Data are means \pm SEM; n = 4-6. * P < 0.05, ** P < 0.01 compared with DMSO-treated L1-Mock cells. # P < 0.05, ## P < 0.01 compared with L1-Mock cells treated with the same compounds. **G, H:** 3T3-L1 cells were induced to differentiate and cultured with or without GW7647 (300 nM) and GW6471 (100 nM) for 10 days. The mRNA expression levels of *aP2* and *PPAR γ* (**G**) and microscopy views of representative cells stained with Oil Red O (**H**). The original magnification is 100 \times . Data are means \pm SEM; n = 4. * P < 0.05, ** P < 0.01.

tion also induced PPAR α mRNA expression (Fig. 2B). Because diet-induced obesity and fasting affects the hepatic expression of PPAR α (37, 38), we assessed whether PPAR α mRNA expression level in WAT changes depending on energy condition. The PPAR α mRNA expression in WAT was significantly decreased in both HFD-induced obese mice (12 months) (Fig. 2C) and the genetically obese mouse model (ob/ob) (Fig. 2D) compared with the lean control mice. In the HFD-fed and ob/ob mice, the PPAR α mRNA expression level in WAT decreased by 54 and 40%, respectively. Conversely, 24 h fasting increased PPAR α mRNA expression level by 1.5-fold (Fig. 2E). The extent of increase was diminished by refeeding after 24 h (Fig. 2E). These findings indicate that PPAR α mRNA expression in WAT is changed depending on the energy condition, suggesting that PPAR α physiologically and pharmacologically plays an important role in the control of energy balance.

PPAR α activation entirely correlated with increases in adipogenic gene expression level, GPDH activity, and insulin-stimulated glucose uptake level but not with lipid accumulation level in 3T3-L1 cells

To investigate the direct effect of PPAR α activation on adipocyte metabolism, we examined PPAR α -agonist-treated 3T3-L1 adipocytes. Because the PPAR α mRNA expression level was much lower in 3T3-L1 cells before differentiation induction than in murine SV cells included with preadipocytes (Fig. 3A), we established 3T3-L1 cells overexpressing PPAR α mRNA (L1-PPAR α) and mock control cells (L1-Mock). The mRNA and protein expression levels of PPAR α in L1-PPAR α cells were slightly higher than those in SV cells (Fig. 3A). First, we examined the effects of PPAR α activation on adipocyte differentiation. GW7647, highly a specific PPAR α activator, was added at the time of differentiation induction and kept in the me-

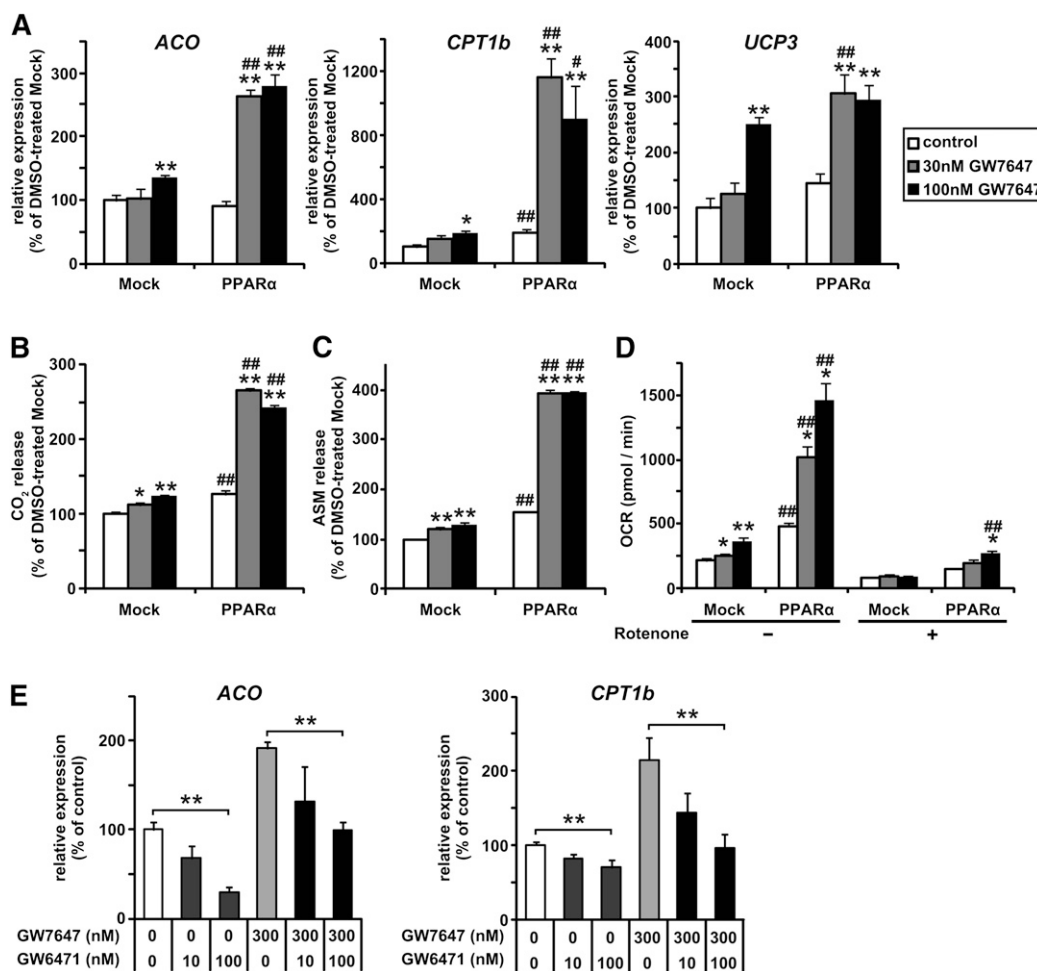


Fig. 4. Effects of PPAR α activation on FA oxidation in cultured adipocytes. A-D: 3T3-L1 cells overexpressing PPAR α (L1-PPAR α) and mock control (L1-Mock) cells were induced to differentiate and cultured with or without GW7647 (30 or 100 nM) for 10 days. The mRNA expression levels of genes involved in FA oxidation (*ACO*, *CPT1b*, and *UCP3*) (A), oxidation of [¹⁴C]palmitic acid to CO₂ (B), and acid-soluble FA metabolites (ASM) (C) for 16 h, and oxygen consumption rate (OCR) (D) in L1-Mock and L1-PPAR α was determined as described in "Materials and Methods." Data are means \pm SEM; n = 3-6. **P* < 0.05, ***P* < 0.01 compared with DMSO-treated L1-Mock. #*P* < 0.05, ##*P* < 0.01 compared with L1-Mock treated with the same compounds. E: 3T3-L1 cells were induced to differentiate and cultured with or without GW7647 (300 nM) and GW6471 (100 nM) for 10 days. The mRNA expression levels of *ACO* and *CPT1b* was measured by real-time PCR. Data are means \pm SEM; n = 4. ***P* < 0.01.

dium throughout the differentiation period. As shown in Fig. 3B, the addition of GW7647 induced the mRNA expression of adipogenic marker genes, such as those encoding aP2 and PPAR γ . Furthermore, the GW7647 treatment increased both lipogenic enzyme GPDH activity (on day 6) and insulin-stimulated 2-DG uptake capacity (on day 10) (Fig. 3C, D). The effects of GW7647 depended on GW7647 concentration and exogenous PPAR α expression. These results indicate that PPAR α activation promotes adipocyte differentiation. However, surprisingly, the TG content was markedly lower in L1-PPAR α than in L1-Mock in the presence of 100 nM GW7647 (Fig. 3E, F), although the lipogenic GPDH activity and insulin-stimulated 2-DG uptake capacity were higher in L1-PPAR α than in L1-Mock, as shown above. Even in unmanipulated 3T3-L1 cells, GW7647 induced aP2, PPAR γ , and adiponectin expression and lipid accumulation during adipocyte differentia-

tion (Fig. 3G, H). In contrast, the addition of the PPAR α antagonist GW6471 inhibited them (Fig. 3G, H). Thus, in 3T3-L1 cells, PPAR α activation may be associated with adipocyte differentiation physiologically.

PPAR α activation enhanced fatty acid oxidation and OCR in 3T3-L1 cells

To clarify the inhibitory effect of PPAR α activation on lipid accumulation, we examined GW7647-induced FA oxidation in L1-PPAR α . Transient GW7647 treatment did not affect the amount of glycerol release or FFA release under our experimental conditions (data not shown). Furthermore, the mRNA expression levels of lipases (hormone-sensitive lipase and adipose triglyceride lipase) related to lipolysis in adipocytes were not changed by the GW7647 treatment (data not shown). On the other hand, chronic GW7647 treatment during adipocyte differentia-

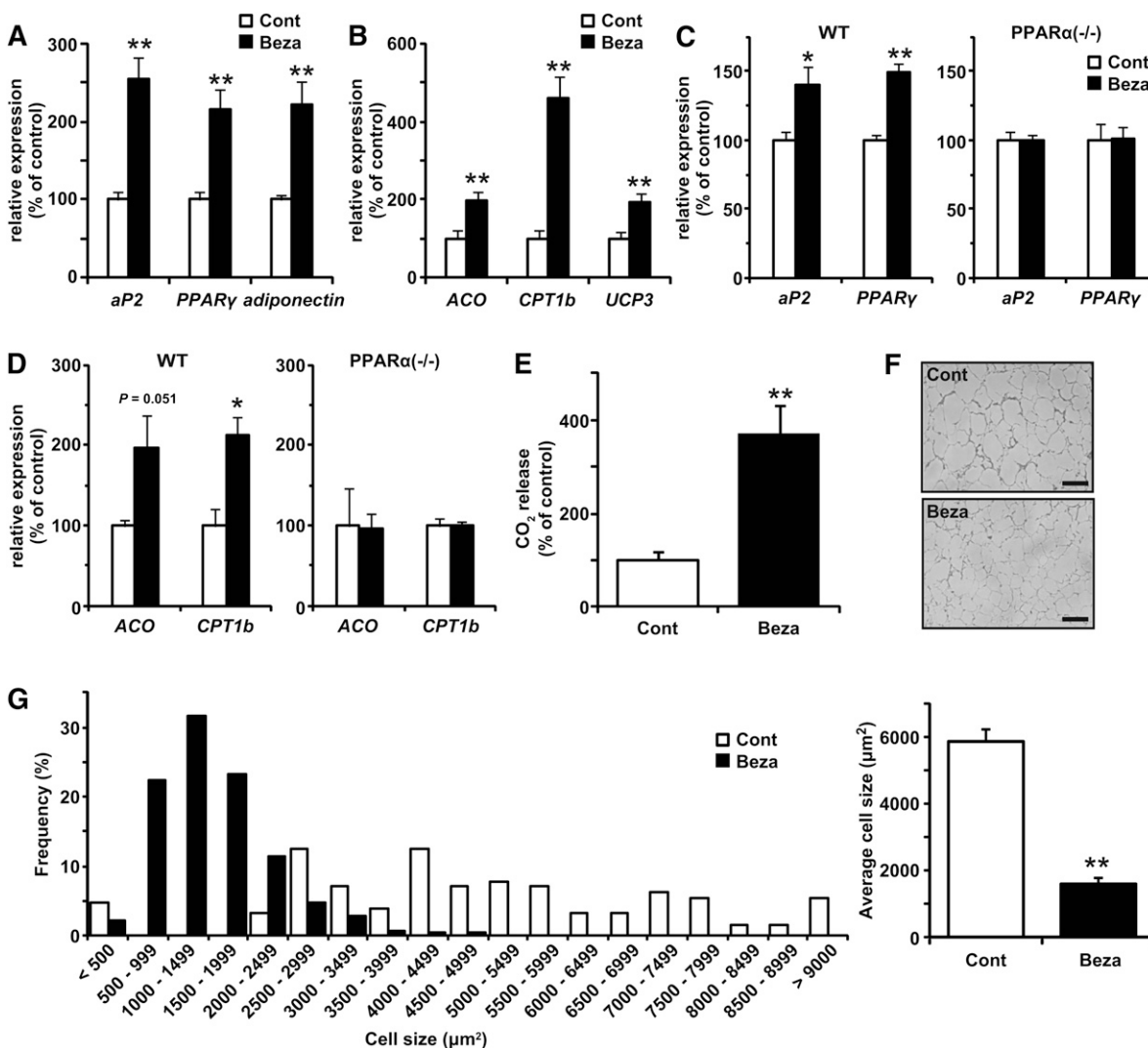


Fig. 5. Effects of PPAR α activator on adipocyte differentiation and FA oxidation in WAT. KK mice (A, B, E-G) and wild-type (WT) or PPAR α -deficient (PPAR α ^{-/-}) mice (C, D) fed HFD (Cont) or HFD containing 0.2% bezafibrate (Beza) were housed under the pair-fed condition for 4-6 weeks. A-D: The mRNA expression levels of adipogenic marker genes (*aP2*, *PPAR γ* , and *adiponectin*) (A, C) and genes involved in FA oxidation (*ACO*, *CPT1b*, and *UCP3*) (B, D) in WAT were determined by real-time PCR. E-G: Oxidation of [¹⁴C]palmitic acid to CO₂ in isolated adipocytes (E), microscopy views of representative histological sections of epididymal WAT (F), and adipocyte size distribution in epididymal WAT (G) from mice fed each experimental diet for 6 weeks were examined. Data are means \pm SEM; n = 3-8. *P < 0.05, **P < 0.01 compared with mice fed control diet.

tion induced the mRNA expression of genes involved in FA oxidation, such as those encoding ACO, CPT1b, and UCP3 (Fig. 4A). The induction was observed to be more marked in L1-PPAR α than in L1-Mock. When cells were incubated with [14 C]palmitic acid for 16 h, the GW7647 treatment enhanced the oxidation of [14 C]palmitic acid to CO $_2$ and acid-soluble FA metabolites (Fig. 4B, C). Moreover, OCR determined by extracellular flux analysis (30) was increased by the GW7647 treatment (Fig. 4D). Rotenone, an inhibitor of mitochondrial electron transport, almost completely diminished the increase in OCR. These findings indicate that PPAR α activation in adipocytes enhances FA oxidation and increases OCR via the induction of PPAR target genes related to FA oxidation, suggesting that the effects of PPAR α activation contribute to the discrepancy between adipogenic activity and lipid accumulation levels induced by PPAR α activation. Even in unmanipulated 3T3-L1 cells, GW7647 induced ACO and CPT1b expression (Fig. 4E). In contrast, the addition of a PPAR α antagonist GW6471 inhibited it (Fig. 4E). Therefore, PPAR α activation might play some physiological roles in fatty acid oxidation in 3T3-L1 cells.

PPAR α activation induced both adipogenic and FA-oxidation-related genes in WAT and enhanced FA oxidation in isolated adipocytes and the entire body

Next, we examined whether the effects of PPAR α activation observed in cultured adipocytes were observed in vivo. Treatment of KK mice fed HFD with bezafibrate for four weeks significantly increased the expression levels of both adipogenic marker genes (*aP2*, *PPAR γ* , and *adiponectin*) and genes involved in FA oxidation (*ACO*, *CPT1b*, and *UCP3*) (Fig. 5A, B). Similar mRNA inductions by bezafibrate administration for six weeks were observed in WT mice but not in PPAR α ^{-/-} mice (Fig. 5C, D), suggesting that PPAR α plays an essential role in the induction of genes involved in both adipogenesis and FA oxidation in WAT by the bezafibrate treatment. Using an indirect calorimetric system, we found that OCR was higher in the bezafibrate-treated mice than in the control KK mice (supplementary Fig. IIA), despite nearly the same locomotor activity (data not shown). Because these mice were fed HFD, the respiratory exchange ratio (RER) was always low during this measurement; however, the RER of the bezafibrate-treated mice was slightly but significantly lower in the light phase (supplementary Fig. IIB). Moreover, the capacity for the oxidation of [14 C] palmitic acid to CO $_2$ was higher in the isolated adipocytes of the bezafibrate-treated mice (Fig. 5E). The size of adipocytes was smaller in epididymal WAT from the bezafibrate-treated mice (Fig. 5F, G). These findings were similar to those in cultured cells and indicate that PPAR α activation induced adipogenesis and FA oxidation in WAT, resulting in suppression of adipocyte hypertrophy.

PPAR α seemed to be recruited to the promoter region of genes involved in both adipogenesis and FA oxidation in 3T3-L1 cells

Finally, to confirm whether PPAR α was recruited to the promoter region, which contained an identified PPAR-response element (PPRE), of genes related to adipogenesis

and FA oxidation in 3T3-L1 cells, we performed ChIP assay. We focused on *aP2* as an adipogenic gene and *CPT1b* as an FA oxidation gene. As shown in Fig. 6, in the presence of GW7647, ChIP assay revealed that PPAR α recruitment to the promoter regions of both *aP2* and *CPT1b* was enhanced in 3T3-L1 cells 14 days after differentiation induction. These regions have been shown to contain PPRE (39, 40); it was suggested that PPAR α activation by ligand binding enhanced the direct binding of PPAR α to PPRE.

DISCUSSION

Several recent studies have demonstrated that PPAR α is expressed in WAT of humans and rodents (22–24). WAT plays a key role in regulating whole-body energy metabolism. In this study, we attempted to clarify the functions of PPAR α in WAT. We found that the mRNA expression level of PPAR α in WAT was decreased by diet-induced and genetic obesity and, conversely, was increased by fasting. These findings suggest that the PPAR α activity in WAT plays a physiological role in energy metabolism. PPAR α ^{-/-} mice exhibited increased adiposity and adipocyte hypertrophy (41). Moreover, these phenotypes seemed to be independent of the hepatic PPAR α action (41). The findings in PPAR α ^{-/-} mice were in contrast to those in 3T3-L1 cells treated with a PPAR α agonist in this study.

A number of reports have shown that PPAR α is particularly important during fasting (11, 15, 38, 42). Fasted PPAR α ^{-/-} mice suffer from various metabolic defects, including hypoketonemia, hypothermia, and hypoglycemia. During the fasting period, lipolysis in WAT releases glycerol and FA into the blood. It has been reported that PPAR α activators stimulate lipolysis in WAT (25). Therefore, it is suggested that the increase in PPAR α mRNA expression level in WAT during fasting is important for adaptation to fasting.

Moreover, several studies have shown that the number of mitochondrial DNA copies and the expression levels of mitochondrial genes are decreased in WAT of obese animal models and humans (29, 43, 44). In the diabetic state, the mitochondrial β -oxidation of FA is attenuated. In addition, mitochondria show abnormal morphologies (44). In this study, the increase in OCR (Fig. 4D) was almost di-

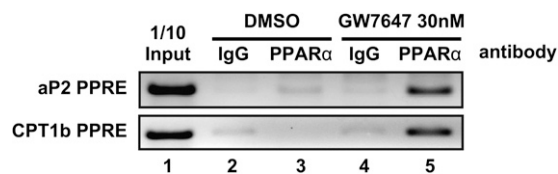


Fig. 6. ChIP assays of *aP2* and *CPT1b* promoter in 3T3-L1 adipocytes treated with or without GW7647. ChIP assays were performed as described in “Materials and Methods.” Soluble chromatin from 3T3-L1 cells, whose differentiation was induced and maintained for 14 days with or without of 30 nM GW7647, was immunoprecipitated with control mouse IgG (lanes 2 and 4) or antibodies against PPAR α (lanes 3 and 5). Immunoprecipitates were analyzed by PCR using specific primers for the mouse *aP2* or *CPT1b* promoter region containing potential PPRE. PCR was performed with total chromatin input (lane 1).

minished in the presence of rotenone, an inhibitor of mitochondrial electron transport, in the PPAR α -transfected 3T3-L1 adipocytes, suggesting that PPAR α activity is important for mitochondrial FA oxidation in adipocytes. Thus, it is suggested that the downregulation of PPAR α in obese WAT is involved in obesity-induced mitochondrial dysfunction and metabolic disorders.

WAT has not been considered as a main target tissue of PPAR α agonists thus far. However, we detected PPAR α mRNA expression at higher levels in WAT than in the skeletal muscle, where PPAR α agonists stimulate FA oxidation (45), although the PPAR α mRNA expression level in adipocytes was much lower than those in the liver and brown adipose tissue (BAT). In addition, several reports have recently indicated the effects of PPAR α agonists on adipocytes. For example, PPAR α agonists transcriptionally induce the mRNA expression of adiponectin and its receptors via PPAR α activation both in vivo and in vitro (23, 22). It has also been reported that Wy-14643 directly enhances lipolysis in isolated adipocytes (25). In this study, we showed that PPAR α agonists promoted FA oxidation and adipocyte differentiation in cultured adipocytes and WAT, both of which are closely related to the pathogenesis of metabolic disorders. Therefore, the data from our and other groups suggest that the PPAR α activation in adipocytes is pharmacologically significant for the regulation of lipid metabolism to maintain whole-body energy balance, although to clarify details of the significance of PPAR α activation in adipocytes, further investigation is necessary, such as experiments using PPAR α WAT-specific knockout mice.

Several studies have demonstrated that adipocytes contain large amounts of mitochondria in their tiny cytosolic compartment (46, 47). Several genetic and pharmacological studies indicate that an increase in FA oxidation capacity in adipocytes leads to the attenuation of adiposity and improvement of obesity-induced metabolic disorders (48–53). Moreover, intense hyperleptinemia induced by means of adenovirus-mediated transfer of the leptin gene causes a rapid disappearance of WAT, at least in part through mechanisms other than decreased food intake (54). Leptin induces the expression of genes involved in FA oxidation partly through a process dependent on PPAR α activation (55). The hyperleptinemia-induced WAT loss was not observed in PPAR α ^{-/-} mice (56). Because this WAT loss is not accompanied by any increase in plasma free FA and ketone body levels or ketonuria (57), it was suggested that FA derived from adipocyte TG is oxidized inside adipocytes via PPAR α activation. Indeed, in this study, the treatment with bezafibrate significantly attenuated adiposity and enhanced FA oxidation in adipocytes, accompanied by the upregulation of genes related to FA oxidation in WAT. These findings indicate that bezafibrate acts on PPAR α in WAT directly, suggesting that WAT is a target of pharmacological treatments with PPAR α agonists.

Interestingly, PPAR α activation promoted not only FA oxidation but also adipocyte differentiation. Adipocyte differentiation is closely related to obesity and obesity-induced metabolic disorders, such as insulin resistance (18–20). PPAR γ plays a central role in the regulation of

adipocyte differentiation, and its synthesized agonists, thiazolidinediones, potentially facilitate adipocyte differentiation to improve insulin resistance (18–20). In this study, the treatment with a PPAR α agonist induced the mRNA expression of adipogenic genes, such as *aP2* and *PPAR γ* , at higher levels in PPAR α -expressing 3T3-L1 cells (L1-PPAR α) than in control 3T3-L1 cells (L1-Mock). In addition, the induction of these genes was observed only in WT mice but not in PPAR α ^{-/-} mice. These observations suggest that PPAR α activation can promote adipocyte differentiation. The effect of PPAR α on adipocyte differentiation was confirmed by ChIP assay, showing that PPAR α was recruited to PPRE in each promoter region of adipogenic genes directly regulated by PPAR γ in WAT. A previous study has also shown that PPAR α activation induces the mRNA expression of glycerol kinase (58), which catalyzes the direct conversion of glycerol to glycerol-3-phosphate in human adipocytes. Glycerol-3-phosphate is an important material for FA esterification to form TG in adipocytes. Thus, it is suggested that a PPAR α -specific adipogenic pathway exists, although the effect of PPAR α seems to be partially shared with that of PPAR γ .

We provided experimental evidence that PPAR α activation in adipocytes leads to the promotion of both adipocyte differentiation and the FA oxidation pathway. These effects of PPAR α activation are caused by the direct binding of PPAR α to PPRE located in the promoter regions of genes involved in adipocyte differentiation and FA oxidation. We also demonstrated that PPAR α expression level in WAT is changed by several nutrient conditions, such as obesity and fasting. Because adipocytes are closely related to whole-body carbohydrate and lipid metabolism, PPAR α activity in WAT may play an important role in regulating whole-body energy metabolism. Furthermore, PPAR α in WAT may contribute to the improvement of obesity-induced metabolic disorders, such as insulin resistance, in combination with fibrate drugs. ■

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