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A selective peroxisome proliferator-activated receptor γ modulator with distinct fat cell regulation properties

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Abbreviations: PPARγ, peroxisomal proliferator-activated receptor γ; SPPARM, selective PPARγ modulator; CBP, CREB-binding protein; SRC-1, steroid receptor coactivator-1; PBP, PPAR binding protein; PRIP, PPAR interacting protein; aP2, adipose fatty acid binding protein; PDE, phosphodiesterase; NHR, nuclear hormone receptor; TZD, thiazolidinedione; TRAP, thyroid hormone receptor-associated protein; DRIP, vitamin D3 receptor-interacting protein; ARC, activator-recruited cofactor; PKA, cAMP-dependent protein kinase A; HSL, hormone sensitive lipase; FFA, free fatty acid; FK614, 3-(2,4-dichlorobenzyl)-2-methyl-N-(pentylsulfonyl)-3-H-benzimidazole-5-carboxamide; D-MEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT, reverse transcriptase; PCR, polymerase chain reaction; DBD, DNA binding domain; CPB, cyclophilin B, TNF, tumor necrosis factor; SAS, Statistical Analysis System; ERα, estrogen receptor α.

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ABSTRACT

Adipogenesis is an important process for the improvement of insulin resistance by peroxisome proliferator-activated receptor γ (PPARγ) agonists, such as rosiglitazone and pioglitazone. FK614 is a structurally novel class of PPARγ agonist that improves insulin sensitivity in animal models of type 2 diabetes. Herein, we characterize FK614, a selective PPARγ modulator (SPPARM) with differential properties affecting the regulation of fat cell function. 3-(2,4-Dichlorobenzyl)-2-methyl-N-(pentylsulfonyl)-3-H-benzimidazole-5carboxamide (FK614) behaves as a partial agonist in inducing the interaction of PPARγ with both transcriptional coactivators, CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1), but as a full agonist with both PPAR binding protein (PBP) and PPAR interacting protein (PRIP), which are required for PPARγ-mediated adipogenesis. In the differentiating 3T3-L1 adipocytes, the levels of adipose fatty acid binding protein (aP2) mRNA expression and triglyceride accumulation induced by FK614 were as efficacious as those of rosiglitazone and pioglitazone. In contrast, the effect of FK614 on aP2 gene expression in mature adipocytes was less than that of the other PPARγ agonists. Furthermore, the long-term treatment of mature adipocytes with rosiglitazone and pioglitazone reduced the expression of phosphodiesterase (PDE) 3B, the down-regulation of which has an important role in the development of insulin resistance; however, FK614 had no such effect in mature adipocytes. Thus FK614 behaves as a SPPARM with differential effects on the activation of PPARγ at each stage of adipocyte differentiation. The stage-dependent selectivity of FK614 may contribute to its enhanced insulin sensitization in differentiating adipocytes and to reduced insulin resistance at the stage of adipocyte hypertrophy.

INTRODUCTION

Peroxisome proliferator-activated receptor (PPAR) γ is a ligand-activated transcription factor and a member of the nuclear hormone receptor (NHR) superfamily (Tontonoz et al., 1994). PPARγ agonists, such as rosiglitazone and pioglitazone, which are thiazolidinedione (TZD) compounds, are clinically used to improve insulin resistance in type 2 diabetes (Olefsky, 2000). However, since weight gain and edema have been reported as side effects of these drugs (O'Moore-Sullivan and Prins, 2002), improvement of PPARγ agonists as anti-diabetic agents is still required. Adipocyte differentiation can be thought as a shift in the gene expression patterns that defines cellular phenotype, including alterations in cell shape and lipid accumulation accompanied by adipogenesis and adipocyte hypertrophy (Gregoire et al., 1998). PPARγ agonists are known to induce the differentiation of pre-adipocytes into mature adipocytes (Kletzien et al., 1992), suggesting that the adipogenic activity of PPARγ agonists contributes to insulin sensitization. Previous gene targeting studies demonstrated that PPARγ also participates in the regulation of adipocyte hypertrophy, since heterozygous PPARγ deficiency prevented adipocyte hypertrophy and the development of obesity-induced insulin resistance (Kubota et al., 1999; Miles et al., 2000). It seems likely that PPARγ plays dual roles in the regulation of insulin sensitivity, one in adipocyte differentiation that contributes to insulin sensitization and another in adipocyte hypertrophy which leads to insulin resistance. Accordingly, the level of PPARγ activation stimulated by PPARγ agonists could play a crucial role in modulating fat cell functions.

The transcriptional activity of PPARγ is regulated by ligand binding and by the recruitment of transcriptional coactivators (Spiegelman and Heinrich, 2004). PPAR binding protein (PBP), which was initially cloned as a PPARγ-binding transcriptional coactivator, functions as an anchor protein for the thyroid hormone receptor-associated protein (TRAP)/vitamin D_3

receptor-interacting protein (DRIP)/activator-recruited cofactor (ARC) complex (Zhu et al., 1997; Rachez et al., 1999). PPAR interacting protein (PRIP) also binds to PPARγ and several other NHRs (Zhu et al., 2000). It can interact with p300/CREB-binding protein (CBP) and DRIP130, a component of TRAP/DRIP/ARC complex (Ko et al., 2000). Previous gene targeting studies demonstrated that mouse embryonic fibroblasts derived from PBP or PRIP null mutants fail to differentiate into adipocytes under PPARγ stimulation (Ge et al., 2002; Qi et al., 2003). Therefore, the interaction of PPARγ with PBP or PRIP induced by PPARγ agonists seems to be a crucial step in PPARγ-mediated adipogenesis.

Phosphodiesterase (PDE) 3B is prominently expressed in insulin-sensitive cells, including white adipocytes and hepatocytes, implicated in regulating anti-glycogenesis and anti-lipolysis (Degerman et al., 1997; Zhao et al., 1997). The activity of PDE decreases in the adipose tissue of type 2 diabetic patients (Engfeldt et al., 1982), and mRNA and protein levels of PDE3B are lower in white adipose tissue of several rodent models of type 2 diabetes (Nagaoka et al., 1998; Tang et al., 1999). Decreases in PDE3B activity are accompanied by increases in intracellular cAMP, that in turn lead to the activation of cAMP-dependent protein kinase A (PKA). This then leads to the activation of hormone sensitive lipase (HSL), subsequently to an increase in the stored triglyceride hydrolysis rate, and finally to increases in the release of free fatty acid (FFA) from adipocytes (Degerman et al., 1997; Carey). Because of these factors, the down-regulation of PDE3B expression in fat cells is thought to be involved in the development of obesity-induced insulin resistance.

3-(2,4-Dichlorobenzyl)-2-methyl-N-(pentylsulfonyl)-3-H-benzimidazole-5-carboxamide (FK614) is a novel class of non-TZD insulin-sensitizing compound, incorporating a sulfonylcarbamoyl moiety as a key pharmacophore (Minoura et al., 2004). This compound is known to activate PPARγ-dependent transcription and to improve hyperglycemia,

hypertriglyceridemia, and glucose intolerance in animal models of type 2 diabetes (Minoura et al., 2004). Recently we have demonstrated that FK614 alters the conformation of PPARγ in a different manner to TZDs, that it promotes differential interactions with coactivators and that the magnitude of PPARγ activation by FK614 varies depending on the affinity of FK614-PPARγ complex for individual coactivators(Fujimura et al., 2005; Fujimura et al., 2006). Given this, FK614 is presumed to be a PPARγ ligand that may have different PPARγ activation properties in different tissues, cells, or at different stages of differentiation depending on the type or the content of coactivators in the target cells. Here, we examined whether FK614 behaves as a selective PPARγ modulator (SPPARM) with differential effects on the recruitment with coactivators to PPARγ and the activation of PPARγ at each stage of adipocyte differentiation. We also investigated the unique properties of FK614 as they relate to the regulation of fat cell function and the development of insulin resistance related to obesity.

MATERIALS and METHODS

Materials Materials were obtained from the following sources. CV-1 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and 3T3-L1 cells from the JCRB Cell Bank (Tokyo, Japan). Dulbecco's modified Eagle's medium (D-MEM) was from Nikken Bio Medical Laboratory (Kyoto, Japan), fetal bovine serum (FBS) from Moregate (Bulimba, Australia), and charcoal-stripped FBS from Hyclone (Logan, UT). FK614, rosiglitazone, and pioglitazone were synthesized at Astellas Pharma Inc. (Tokyo, Japan). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA).

Plasmids The human PPARγ1 (GenBank accession number NM_005037) was cloned from human liver cDNA using reverse transcriptase-polymerase chain reaction (RT-PCR). A VP16-human PPARγ1 fusion expression plasmid (pACT-hPPARγ1) was generated by inserting the full-length hPPARγ1 cDNA fused to the VP16 viral activation domain into the pACT expression vector (Promega, Madison, WI). To generate cDNAs encoding human coactivators fused to the DNA binding domain (DBD) of GAL4, short fragments of cDNAs for human CBP (GenBank accession number NM_004380), human steroid receptor coactivator-1 (SRC-1) (GenBank accession number NM_003743), human PBP (GenBank accession number NM_004774), and human PRIP (GenBank accession number NM_014071) encoding amino acids 1-115, 592-782, 501-738, and 819-1096, respectively, were amplified by RT-PCR using human lung cDNA for CBP and SRC-1, human kidney cDNA for PBP, and HeLa cell line cDNA for PRIP as templates, then individually cloned into the pBIND expression vector (Promega). The resulting GAL4-coactivator fusion expression plasmids pBIND-CBP, pBIND-SRC-1, pBIND-PBP, and pBIND-PRIP were used in the mammalian two-hybrid assay. The reporter plasmid, pGL3(R2.1)-G5 was generated by inserting a short DNA fragment containing five copies of the GAL4 response element upstream of a minimal TATA box from

pG5Luc (Promega) into pGL3(R2.1)-Basic vector (Promega).

Mammalian two-hybrid assay The African green monkey fibroblast cell line CV-1 was maintained in D-MEM supplemented with 10% FBS. Transfection of CV-1 cells was performed in 100 mm diameter dishes (1.2 x 10^6 cells per dish) using LipofectAMINE PLUS (Invitrogen) with 2.4 μg of pACT-hPPARγ1 and 4.8 μg of pGL3(R2.1)-G5, together with 1.2 μg of GAL4-coactivator fusion expression plasmid. After transfection, the cells were plated in 96-well plates in D-MEM supplemented with 10% charcoal-stripped FBS for 20 h, then treated with various concentrations of test compounds for 5 h. Cell extracts were prepared and assessed for luciferase activity, measured using the Dual-Luciferase Reporter Assay System (Promega) and the Mithras LB940 multilabel plate reader (Berthold, Bad Wildbad, Germany) in the luminometer mode, according to the manufacturers' instructions. Firefly luciferase activities were normalized for transfection efficiency against the activity of the internal control, *Renilla reniformis* luciferase. All experiments were carried out three times in duplicate.

Preparation of differentiating and mature adipocytes for quantitative analysis of mRNA The mouse 3T3-L1 fibroblast cell line was maintained in culture medium (D-MEM supplemented with 10% FBS). For the preparation of differentiating adipocytes, the 3T3-L1 cells were cultured in differentiation-inducing medium (D-MEM supplemented with 10% FBS, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/ml insulin) for 2 days in a 6-well plate. The cells were cultured for an additional day after changing to the culture medium. The differentiating 3T3-L1 adipocytes were then treated with various concentrations of test compounds for 48 h and harvested for mRNA analysis.

For the preparation of mature adipocytes, the 3T3-L1 cells were cultured in the differentiation-inducing medium for 3 days, then the cells were cultured in maintaining medium (D-MEM supplemented with 10% FBS, and 10 μg/ml insulin) for 2 days. The cells were

cultured for another 6 days after changing to the culture medium. The mature 3T3-L1 adipocytes were then treated with various concentrations of the test compounds for 48 h and harvested for mRNA analysis.

Quantitative real-time RT-PCR analyses Total RNA was isolated from 3T3-L1 adipocytes using TRIZOL (Invitrogen). Total RNA was treated with DNase RT Grade (Nippon Gene, Toyama, Japan) to remove residual genomic DNA. RT product was synthesized from total RNA using Taq Man Reverse Transcription Reagent (Applied Biosystems, Foster City, CA) and Random Hexamer as primers. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems). The GenBank accession number and the designed primers were as follows: mouse adipose fatty acid binding protein (aP2) (GenBank accession number K02109, forward primer 5'-ACTGGGCGTGGAATTCGAT-3', and reverse primer 5'-CGCCATCTAGGGTTATGATGCT-3'), mouse CBP (GenBank accession number S66385, forward primer 5'-TGCTTAGCTGTGACCTCATGGA-3', and reverse primer 5'-GCACAGAGTGGACCATTTGGA-3'), mouse SRC-1 (GenBank accession number NM_010881, forward primer 5'-GCAGATGGAAACCAGGACAAG-3', and reverse primer 5'-GGACGTCAGCAAACACCTGAA-3'), mouse PBP (GenBank accession number NM_013634, forward primer 5'-CCACTTCCAGAGTACAGCACTGA-3', and reverse primer 5'-GTTCTCTGGCTTCATGCTGTGA-3'), mouse PRIP (GenBank accession number NM_019825, forward primer 5'-GCTGACCAAGGCTTGTAAGAAAG-3', and reverse primer 5'-TTAGCCCAGGAGTTGTGATCTCT-3'), mouse PDE3B (GenBank accession number AJ132271, forward primer 5'-CCAGGTGTGCATCAAATTAGCA-3', and reverse primer 5'-CAATGCCTTCTGTCCATCTCAA-3'), and mouse cyclophilin B (CPB) (GenBank accession number M60456, forward primer 5'-CAGGAGAGAAAGGATTTGGCTACA-3', and reverse primer 5'-TCCACCCTGGATCATGAAGTC-3').

The expression levels of each mRNA were determined with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using a SYBR Green PCR Master Mix (Applied Biosystems). RT-PCR products were also analyzed on ethidium bromide-stained agarose gel to ensure that single amplicon of the expected size was actually obtained. The expression levels of mouse aP2, CBP, SRC-1, PBP, PRIP, and PDE3B mRNA were normalized to the expression level of mouse CPB mRNA, a house keeping gene. All experiments were performed three times.

Triglyceride measurement in 3T3-L1 adipocytes The 3T3-L1 cells were cultured in a 6-well plate for 2 days. The medium was replaced with the differentiation-inducing medium, and the cells were cultured for 2 days. The medium was then replaced by the culture medium and the cells were treated with various concentrations of the test compounds for 5 days. The intracellular triglyceride in the 3T3-L1 adipocytes was extracted by isopropanol. The amount of triglyceride was measured with a triglyceride assay kit (Wako, Osaka, Japan) using the acetylacetone method. The concentration of cellular protein was determined using a DC Protein Assay (Bio-Rad, Hercules, CA), after solubilizing with 0.1 N NaOH. Intracellular triglyceride content was calculated as per mg protein. All experiments were performed three times.

Three-dimensional collagen gel culture of 3T3-L1 adipocytes Three solutions for three-dimensional collagen gel culture were prepared: acid-soluble type I collagen solution (A solution; Nitta Gelatin, Osaka, Japan), 5-fold concentrated D-MEM without $NaHCO_3$ (B solution), and reconstruction buffer (C solution; 2.2% $NaHCO_3$, 200 mM HEPES, and 0.05 N NaOH). On day one, 7 volumes of A solution, 2 volumes of B solution, and 1 volume of C solution were mixed sufficiently and kept on ice. The mixture was then mixed with the evenly dispersed 3T3-L1 cells (2.5×10^5 cells/ml). The mixture containing the 3T3-L1 cells (800 μL)

was placed in a type I collagen-coated 12-well plate, then incubated at 37°C for 30 min. After gelation, the mixture was overlaid with 2.2 ml of the culture medium, then the cells were cultured for 2 days. On day 3, the medium was replaced with the differentiation-inducing medium, and on day 6 with the maintaining medium. On day 8, the medium was again replaced with the culture medium, and the cells were then treated with various concentrations of the test compounds. The cells were treated with the test compounds for 16 days, during which time the medium was replaced with fresh culture medium containing the test compound every 2 or 3 days. On day 24, the gel containing the adipocyte was scraped off and then minced in a test tube. Then the gel was digested using 10 mg/ml Collagenase S-1 (Nitta Gelatin) for 30 min. After centrifugation, the supernatant was removed and then the adipocytes were harvested for RNA analysis. For the preparation of tumor necrosis factor (TNF) α-treated cells, the 3T3-L1 cells embedded in the gel were differentiated and cultured from day 8 to day 23 in the culture medium without PPARγ agonists, as described above. On day 23, the adipocytes were treated with various concentrations of TNFα (Sigma) overnight. On day 24, the TNFα-treated adipocytes were obtained as described above.

Statistical analysis The IC_{50} value of TNFα for decreasing PDE3B gene expression was determined by nonlinear curve fitting using a Statistical Analysis System (SAS) (SAS Institute, Cary, NC). Significant difference of PDE3B expression level between non-treated control group and agonist-treated groups was assessed by Dunnett's multiple comparisons test using SAS. A p value of < 0.05 was considered significant.

RESULTS

FK614 induces unique ligand-specific interactions of PPARγ with different coactivators in a mammalian two-hybrid assay. Transcriptional coactivator recruitment to PPARγ is believed to be a crucial step for generating a diversity of biological properties (Olefsky, 2000). In the transfection study, we demonstrated that FK614 behaves as a partial or full agonist for transcriptional activation, depending on the amount of specific coactivators (Fujimura et al., 2005). PBP and PRIP are known to be coactivators that are required for PPARγ-mediated adipogenesis (Ge et al., 2002; Qi et al., 2003). To examine whether FK614 induces a differential interaction with transcriptional coactivators required for adipogenesis, when compared with rosiglitazone and pioglitazone, a mammalian two-hybrid assay was performed with CBP, SRC-1, PBP, and PRIP. To prevent interference with the activity of PPARγ and coactivator interaction by the transactivation of internal control induced by the high concentration of test compounds, we established a new mammalian two-hybrid assay using a rapid response luciferase vector. To study these interactions, full-length human PPARγ1 was fused to the VP16 activation domain, and the NHR interaction domain of the coactivators (Heery et al., 1997; Zhu et al., 1997; Zhu et al., 2000) was fused to the DBD of GAL4. Concentration-dependent increases in the association of CBP, SRC-1, PBP, or PRIP with PPARγ were observed in response to FK614, rosiglitazone, and pioglitazone (Fig. 1). The induction of recruitment of all coactivators by FK614, rosiglitazone, and pioglitazone was initiated at concentrations in excess of 1×10^{-6}, 1×10^{-7}, and 1×10^{-6} M, respectively. However, the magnitude of CBP and SRC-1 recruitment induced by 1×10^{-5} M FK614 was less than that promoted by rosiglitazone and pioglitazone. The magnitude of the recruitment induction above basal levels produced by 1×10^{-5} M FK614, rosiglitazone and pioglitazone was 2.5-, 4.2-, and 3.2-fold for CBP, and 5.1-, 19-, and 9.0-fold for SRC-1, respectively. In contrast, the

magnitude of PBP and PRIP recruitment induced by 1 x 10^{-5} M FK614 was similar to that of 1 x 10^{-5} M rosiglitazone and pioglitazone. At 1 x 10^{-5} M of FK614, rosiglitazone, and pioglitazone, the degree to which PPARγ bound to PBP was 2.8-, 2.8-, and 2.5-fold above basal levels, respectively. Likewise the degree to which PRIP bound to PPARγ was 2.4-, 2.4-, and 2.1-fold above basal levels at the same drug concentration. These results suggest that FK614 behaves as a partial agonist in inducing the interaction of PPARγ with CBP and SRC-1, but as a full agonist with PBP and PRIP. These findings then suggest that FK614 may function as a full agonist in PPARγ-mediated adipogenesis.

Stage-specific differential effects of FK614 on the activation of aP2 gene expression in differentiating and mature 3T3-L1 adipocytes. To investigate whether FK614 functions as a SPPARM in adipocytes, we examined the ability of FK614, rosiglitazone, and pioglitazone to promote PPARγ-mediated transactivation, as measured by aP2 gene expression in differentiating and mature 3T3-L1 adipocytes. In the differentiating adipocytes that had entered the growth arrest phase, but not yet accumulated fat droplets, concentration-dependent increases in aP2 gene expression were observed in response to FK614, rosiglitazone, and pioglitazone (Fig. 2A). The induction of aP2 gene expression was initiated at concentrations of 1 x 10^{-7}, 1 x 10^{-8}, and 1 x 10^{-7} M for FK614, rosiglitazone, and pioglitazone, respectively. The magnitude of aP2 gene expression above basal levels induced by 1 x 10^{-5} M FK614, rosiglitazone, and pioglitazone was 2.2-, 2.3- and 2.0-fold, respectively. Therefore, these results indicate that FK614 is a potent agonist that activates PPARγ in differentiating adipocytes as efficaciously as the other PPARγ agonists.

In the fully differentiated mature adipocytes that had become terminally differentiated and completed fat droplet accumulation, the concentration response analysis demonstrated that the induction of aP2 gene expression was initiated at concentrations of 1 x 10^{-7} M FK614, 1 x 10^{-8} M

rosiglitazone, and 1 x 10^{-7} M pioglitazone, respectively (Fig. 2B). The induction of aP2 expression caused by 1 x 10^{-5} M FK614, rosiglitazone, and pioglitazone in mature adipocytes was 2.9-, 6.5-, and 4.4-fold increases above the basal levels, respectively. In contrast to the aP2 gene expression in differentiating 3T3-L1 cells, FK614 is less efficacious in fully differentiated mature adipocytes than the other PPARγ agonists. These results indicate that FK614 exerts the stage-specific differential effects on the activation of the aP2 gene expression in differentiating and mature 3T3-L1 adipocytes. Therefore FK614 behaves as a SPPARM with differential effects on the activation of PPARγ at each stage of adipocyte differentiation.

Comparison of the expression levels of coactivators between differentiating and mature 3T3-L1 adipocytes. The different PPARγ ligands produce distinct alterations in PPARγ conformation, which then elicit a differential interaction with coactivators and thereby produce unique biological properties. (Olefsky, 2000). We demonstrated that FK614 behaves as a SPPARM at each stage of adipocyte differentiation. To explore the mechanism of the stage-specific differential effects of FK614, we measured the mRNA levels of the coactivators in differentiating and mature 3T3-L1 adipocytes. The expression levels of CBP and SRC-1 in differentiating adipocytes are similar to those in fully differentiated mature adipocytes (Fig. 3, A and B). In contrast, PBP and PRIP expression levels in differentiating adipocytes are 1.7- and 1.4-fold greater than those in fully differentiated mature adipocytes (Fig. 3, C and D).

Effect of FK614 on 3T3-L1 adipocyte differentiation. To determine whether FK614 can functionally promote adipocyte differentiation as well as activate the aP2 gene expression, we examined the ability of FK614, rosiglitazone and pioglitazone to promote the differentiation of 3T3-L1 pre-adipocytes into adipocytes by measuring the accumulation of triglyceride. Concentration-dependent increases in triglyceride content were observed in response to FK614, rosiglitazone, and pioglitazone (Fig. 4). The increases were initiated at concentrations of 1 x

10^{-7}, 1 x 10^{-8}, and 1 x 10^{-7} M for FK614, rosiglitazone, and pioglitazone, respectively. The minimum concentrations of PPARγ agonists for promoting adipocyte differentiation of 3T3-L1 cells are consistent with those required for inducing aP2 gene expression. This result indicates that the effect of PPARγ agonists on adipocyte differentiation in 3T3-L1 cells is exerted by the activation of the adipocyte-specific gene expression regulated by PPARγ. At 1 x 10^{-5} M of FK614, rosiglitazone, and pioglitazone, the levels of triglyceride content were similar for all three agonists, and the amount of storage was a 2.5-fold increase above the basal levels. Accordingly, these results indicate that FK614 is a potent PPARγ agonist that promotes adipocyte differentiation as efficaciously as the other PPARγ agonists.

Long-term effect of FK614 on insulin resistance in differentiated mature adipocytes. Adipogenesis seems to be an important process for the improvement of insulin resistance by PPARγ agonists, such as rosiglitazone and pioglitazone (Hallakou et al., 1997). However, PPARγ is known to participate in the adipocyte hypertrophy that leads to obesity-induced insulin resistance (Kubota et al., 1999). To investigate the long-term effect of PPARγ agonists on insulin resistance, we established a three-dimensional collagen gel culture assay method with 3T3-L1 adipocytes. It has been reported that many molecular markers, such as TNFα and FFA, indicate insulin resistance in adipocytes (Smith et al., 1999; Ruan and Lodish, 2003). For this reason, we determined the expression level of TNFα mRNA in fully differentiated mature 3T3-L1 adipocytes using real-time RT-PCR, for which the minimum detection level of TNFα mRNA was 0.5 attomol/1μg of total RNA, and found it to be near the minimum detection limit (data not shown). Thus the effect of PPARγ agonists on TNFα expression could not be investigated. The amount of secreted TNFα and FFA, the insulin-stimulated glucose uptake, and GLUT4 translocation could not be measured because the 3T3-L1 adipocytes were cultured in collagen gel. Adiponectin is secreted by adipocytes and its down-regulation leads to insulin

resistance (Tsao et al., 2002). However, PPARγ directly regulates adiponectin gene expression in adipocytes (Iwaki et al., 2003). Therefore, it seems that adiponectin is not a suitable molecular marker for the development of insulin resistance in our experiments. Decreases in PDE3B activity are accompanied by increases in cAMP levels, the induction of PKA activity, the phosphorylation and activation of HSL, and an increase in the release of FFA from adipocytes (Degerman et al., 1997; Carey). Thus, the down-regulation of PDE3B expression is thought to be involved in the development of insulin resistance in adipocytes. It was reported that the down-regulation of PDE3B mRNA closely correlated with the decrease in PDE3B protein levels and PDE3 activity in white adipose tissue of rodent models for type 2 diabetes (Nagaoka et al., 1998; Tang et al., 1999). Therefore, we investigated the expression levels of PDE3B mRNA in mature 3T3-L1 adipocytes. First, we examined the TNFα-induced down-regulation of PDE3B gene expression in this cell system. The expression level of PDE3B mRNA in mature adipocytes treated with TNFα was assessed quantitatively by real-time RT-PCR. The expression level of PDE3B mRNA decreased in a concentration-dependent manner in response to TNFα (Fig. 5A). The IC_{50} value of TNFα for decrease in PDE3B gene expression was 1.5 ng/ml. This concentration is consistent with those obtained in other *in vitro* experiments that demonstrated the effect of TNFα on the development of insulin resistance (Szalkowski et al., 1995). This result indicates that the decrease in PDE3B mRNA expression in this cell system may represent the development of insulin resistance in adipocytes.

Next, we performed comparative studies of the long-term effect of FK614, rosiglitazone, and pioglitazone on the expression level of PDE3B mRNA in mature 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes embedded in collagen gel were cultured in the presence of PPARγ agonists for 16 days, then the expression level of PDE3B mRNA was assessed. Concentration-dependent decreases in PDE3B gene expression were observed in response to the

presence of rosiglitazone and pioglitazone (Fig. 5B). Rosiglitazone and pioglitazone significantly down-regulated PDE3B gene expression at 1×10^{-7} or higher and 1×10^{-6} or higher concentrations, respectively. At the concentration of 1×10^{-5} M, both rosiglitazone and pioglitazone reduced the expression level of PDE3B mRNA to 42.0% and 28.3% of control levels, respectively. In contrast, FK614 did not induce a concentration-dependent down-regulation of PDE3B gene expression. Thus, the long-term treatment of mature adipocytes with rosiglitazone and pioglitazone induces the down-regulation of PDE3B mRNA; however, FK614 has no effect on PDE3B gene expression in fully differentiated mature adipocytes.

DISCUSSION

The present experiments provide evidence that FK614, a novel non-TZD insulin sensitizer, functions as a SPPARM that induces differential interactions with coactivators and has differential effects on the activation of PPARγ at each stage of adipocyte differentiation, resulting in distinct fat cell regulation properties that might be dependent upon the cellular context of coactivators. Previous gene targeting studies demonstrated that embryonic mouse fibroblasts derived from PBP or PRIP null mutants fail to differentiate into adipocytes under PPARγ stimulation (Ge et al., 2002; Qi et al., 2003). Therefore, PPARγ interaction with PBP or PRIP induced by PPARγ agonists seems to be a crucial step in PPARγ-mediated adipogenesis. Here, we first examined the effect of PPARγ agonists on the recruitment of key coactivators. The effect of FK614 on the recruitment of the coactivators CBP and SRC-1 was lower than that of the other two agonists. In contrast, the levels of PBP and PRIP recruitment to PPARγ induced by FK614 were similar to that of rosiglitazone and pioglitazone. These results suggest that FK614 behaves as a SPPARM whose selectivity depends on the affinity of the FK614-PPARγ complex for coactivators. This is presumably due to the induction of alternative receptor conformations induced by FK614 that is structurally different from any TZDs (Fujimura et al., 2006). We have previously demonstrated that the anti-diabetic activity of FK614 is as efficacious as rosiglitazone and pioglitazone, despite the fact that FK614 behaving as a partial agonist in a reporter gene using CV-1 cells (Minoura et al., 2004). However, we here show that FK614 behaves as a full agonist in the induction of the interaction of PPARγ with PBP and PRIP, which is required for adipogenesis. These results suggest that FK614 functions as an insulin sensitizer as efficaciously as other PPARγ agonists, because FK614 might behave as a full agonist in differentiating adipocytes.

To test this hypothesis, we next focused on the relationship between the transcriptional

activation of PPARγ and adipocyte differentiation, and examined the effects of FK614 on aP2 gene expression and the accumulation of triglyceride in differentiating adipocytes. PPARγ regulates the expression of adipogenic genes, such as aP2 and lipoprotein lipase, whose promoters contain regulatory elements of PPARγ (Tontonoz et al., 1994; Schoonjans et al., 1996). During adipocyte differentiation, PPARγ induces adipogenic genes to establish the mature adipocyte phenotype in response to agonist stimulation (Spiegelman, 1997). Therefore, the effect of PPARγ agonists on the expression of adipocyte-specific genes such as aP2, should correlate with morphological changes such as, cessation of cell growth and extensive lipid accumulation. Here we demonstrated that FK614, as well as other PPARγ agonists, induce aP2 gene expression in differentiating 3T3-L1 adipocytes. The expression level of aP2 gene induced by FK614 was similar to that of pioglitazone and rosiglitazone. We also found that FK614, as well as rosiglitazone and pioglitazone, increased triglyceride storage in differentiating 3T3-L1 adipocytes, which is consistent with aP2 gene expression data. These results indicate that FK614 behaves as a full PPARγ agonist in differentiating adipocytes as efficaciously as rosiglitazone and pioglitazone. FK614 induces the adipose specific gene expression with reflecting adipocyte differentiation.

The excessive activation of PPARγ-mediated transcription is known to promote adipocyte hypertrophy and the development of obesity-induced insulin resistance (Kubota et al., 1999). In addition, it has been reported that the level of aP2 gene expression under the condition of a high fat diet correlated with the development of insulin resistance in a study using aP2-deficient mice (Hotamisligil et al., 1996). Accordingly, the effect of PPARγ agonists on the induction of aP2 gene expression in mature adipocytes seems to be relevant for the development of insulin resistance. Here, we demonstrated that FK614 can also induce aP2 gene expression in mature 3T3-L1 adipocytes; however, the effect of FK614 is less than that of the other PPARγ agonists.

These results indicate that FK614 behaves as a full PPARγ agonist of in differentiating adipocytes, but does not activate PPARγ excessively in mature adipocytes. Specifically, FK614 functions as a SPPARM in a stage-dependent manner during adipocyte differentiation.

The conformation of the ligand-NHR complex is different with different ligands; a given ligand-NHR complex may recruit a somewhat different set of coactivators or corepressors, or it may do so with altered kinetics. These differences in the assembly of the transcriptional complex will be specific to the context of a particular promoter and its environment, such as cell cycle and type of tissue (Spiegelman and Heinrich, 2004). Several lines of evidence have been provided for the role of PBP and PRIP in the regulation of NHR-mediated gene expression. The overexpression of PBP or PRIP enhances ligand-dependent transactivation by several NHRs, such as PPARγ and estrogen receptor α (ERα) (Zhu et al., 1997; Ko et al., 2000; Zhu et al., 2000). Interestingly, high levels of mRNA and protein expression of PBP and PRIP have been observed in breast cancers; therefore, PBP and PRIP, by their ability to function as ERα coactivator, might play a role in mammary epithelial differentiation and in breast carcinogenesis (Lee et al., 1999; Zhu et al., 1999). These findings raise the possibility that the amount of coactivators regulates the biological processes by modulating the transactivation of NHRs. To understand the molecular mechanisms underlying behavior of FK614 as a SPPARM in stage-dependent manner during adipocyte differentiation, we examined the expression levels of coactivators in differentiating and mature 3T3-L1 adipocytes. The difference in the expression levels of PBP and PRIP in these cells was observed. It was noted that in each adipocyte stage, the expression levels of coactivators correlated well with the levels of aP2 expression induced by FK614. The exact molecular mechanism of stage-specific differential biological responses induced by FK614 remains to be elucidated. However, FK614 may induce a distinct alteration of PPARγ conformation that elicits a more favorable interaction with PBP and PRIP than with other

coactivators, such as CBP and SRC-1. This could lead to the differing biological responses for adipocytes between FK614 and other PPARγ agonists.

Although the exact mechanisms are not known, PDE3B gene expression in adipocytes is reduced in rodent models of types 2 diabetes. The changes of this gene expression as evidenced by mRNA, protein levels, and PDE activities are correlated with serum FFA concentrations and insulin resistance (Nagaoka et al., 1998; Tang et al., 1999). Furthermore, pioglitazone restores this altered gene expression with a parallel improvement in insulin resistance (Tang et al., 1999). These findings suggest that the down-regulation of PDE3B mRNA expression in adipose tissue plays a role in the development of insulin resistance. Here, we demonstrated that TNFα induced the down-regulation of PDE3B mRNA in 3T3-L1 mature adipocytes cultured over a long period of time. A previous study revealed that a mechanism for TNFα-mediated lipolysis in 3T3-L1 adipocytes, that includes the down-regulation of PDE3B gene expression, resulted in increased cAMP levels, the induction of PKA activity, and the phosphorylation and activation of HSL (Rahn Landström et al., 2000). Thus, the decreases in PDE3B mRNA in our cell system could represent the development of insulin resistance in adipocytes. The excess stimulation of PPARγ-mediated transactivation in mature adipocytes found to correlate with the hypertrophy of mature adipocytes and the development of obesity-induced insulin resistance (Kubota et al., 1999). To ensure that FK614 activated PPARγ to a lesser degree in mature adipocytes, we examined the long-term effect of PPARγ agonists on the regulation of PDE3B gene expression in mature 3T3-L1 adipocytes. Here we demonstrated that the long-term treatment of mature adipocytes with rosiglitazone and pioglitazone induced the down-regulation of PDE3B expression; however, FK614 had no such effect in mature adipocytes. These results suggest that FK614 has a low possibility for the development of obesity-induced insulin resistance when compared to the other PPARγ agonists. Although further studies are needed using a more

validated biomarker in our cell system and animal models to generate the data to support our finding, FK614 may contribute very weakly to the adipocyte hypertrophy that leads to obesity-induced insulin resistance, which results from the excessive stimulation of PPARγ. In relation to diabetes mellitus, it has been reported that heterozygous PPARγ-deficient mice are protected from high-fat diet-induced insulin resistance (Kubota et al., 1999; Miles et al., 2000). Consistent with this observation, Pro12Ala polymorphism in human PPARγ2 that moderately reduces the transcriptional activity of PPARγ has been shown to confer resistance to type 2 diabetes (Deeb et al., 1998). Recently, the appropriate functional antagonism of PPARγ accomplished by administration of a PPARγ antagonist was demonstrated to be an effective approach to prevent insulin resistance (Yamauchi et al., 2001). Therefore, the maintenance of PPARγ activation at appropriate levels by SPPARMs like FK614 may lead to therapeutic benefits in the control of obesity-induced insulin resistance. Several PPARγ agonists, such as F-L-Leu and nTZDpa, have been reported to improve insulin sensitivity, yet they have a lower adipogenic activity (Rocchi et al., 2001; Berger et al., 2003). Although the precise mechanism of action is unclear, F-L-Leu and nTZDpa may inhibit adipogenesis to prevent adipocyte hypertrophy. In contrast to these compounds, FK614 stimulates adipocyte differentiation as well as rosiglitazone or pioglitazone, however, FK614 is less efficacious in mature adipocytes to prevent adipocyte hypertrophy. Therefore FK614 is believed to be a unique SPPARM with fat cell regulation properties.

In summary, FK614 behaves as a SPPARM, with differential effects on the activation of PPARγ at each stage of adipocyte differentiation. FK614 may contribute to insulin sensitization in differentiating adipocytes effectively, but weakly to adipocyte hypertrophy that leads to insulin resistance in mature adipocytes. Despite the amount of PPARγ activation known to be crucial for the regulation of fat cell function, a novel class of SPPARM such as

FK614 might be beneficial, improving insulin sensitivity effectively, yet lacking activity where excessive activation of PPARγ is less desirable.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. FK614 induces unique ligand-specific interactions of PPARγ with different coactivators. CV-1 cells were transiently transfected with the reporter plasmid [pGL3(R2.1)-G5] and the VP16-human PPARγ1 fusion expression plasmid (pACT-hPPARγ1), in addition to GAL4-fusion expression plasmids for CBP (A), SRC-1 (B), PBP (C), or PRIP (D). The transfected cells were incubated with the indicated concentrations of FK614, rosiglitazone, or pioglitazone for 5 h. The cell extracts were then assessed for luciferase activity. Results are presented as the fold increase in the basal levels. Values are the mean ± S.E. of three independent experiments performed in duplicate.

Fig. 2. Stage-specific differential effects of FK614 on the activation of aP2 gene expression in differentiating and mature 3T3-L1 adipocytes. Differentiating (A) or fully differentiated mature (B) 3T3-L1 adipocytes were cultured in the presence or absence of the indicated concentrations of FK614, rosiglitazone, or pioglitazone. At 48 h after addition of compounds, the cells were harvested for RNA analysis. Both aP2 mRNA and CPB mRNA were measured using RT-PCR. All aP2 mRNA values were normalized to CPB expression in the same sample. The 3T3-L1 cells were processed for adipocyte differentiation as described in *Materials and Methods*. Results are presented as the fold increase in the basal levels. Values are the mean ± S.E. of three independent experiments.

Fig. 3. Comparison of the expression levels of coactivators in differentiating and mature 3T3-L1 adipocytes. Differentiating or fully differentiated mature 3T3-L1 adipocytes were harvested for RNA analysis. CBP (A), SRC-1 (B), PBP (C), PRIP (D), and CPB mRNA were measured

using RT-PCR. All coactivator mRNA values were normalized to CPB expression level in the same sample. The 3T3-L1 cells were processed for adipocyte differentiation as described in *Materials and Methods*. Values are the mean ± S.E. of three independent experiments.

Fig. 4. Effect of FK614 on the accumulation of triglyceride in differentiating 3T3-L1 cells. Differentiating 3T3-L1 adipocytes were cultured in the presence or absence of the indicated concentrations of FK614, rosiglitazone, or pioglitazone. After 5 days of adding compounds, the cells were harvested for the measurement of intracellular triglyceride content. The 3T3-L1 cells were processed for adipocyte differentiation as described in *Materials and Methods*. The intracellular triglyceride content was measured using the acetylacetone method. The triglyceride levels were normalized by the concentration of cellular protein. Values are the mean ± S.E. of three independent experiments.

Fig. 5. The long-term effect of FK614 on phosphodiesterase 3B gene expression in mature 3T3-L1 adipocytes. Gel-embedded mature 3T3-L1 adipocytes were treated with the indicated concentrations of TNFα (A) overnight or PPARγ agonists (B) for 16 days. The treated cells were harvested for mRNA analysis. Both PDE3B mRNA and CPB mRNA were measured using RT-PCR. PDE3B mRNA values were normalized to CPB expression in the same sample. The 3T3-L1 cells were processed for adipocyte differentiation, as described in *Materials and Methods*. Results are presented as the % of control. Values are the mean ± S.E. of three independent experiments. **, $P<0.01$, *, $P<0.05$ versus non-treated control.

Figure 1

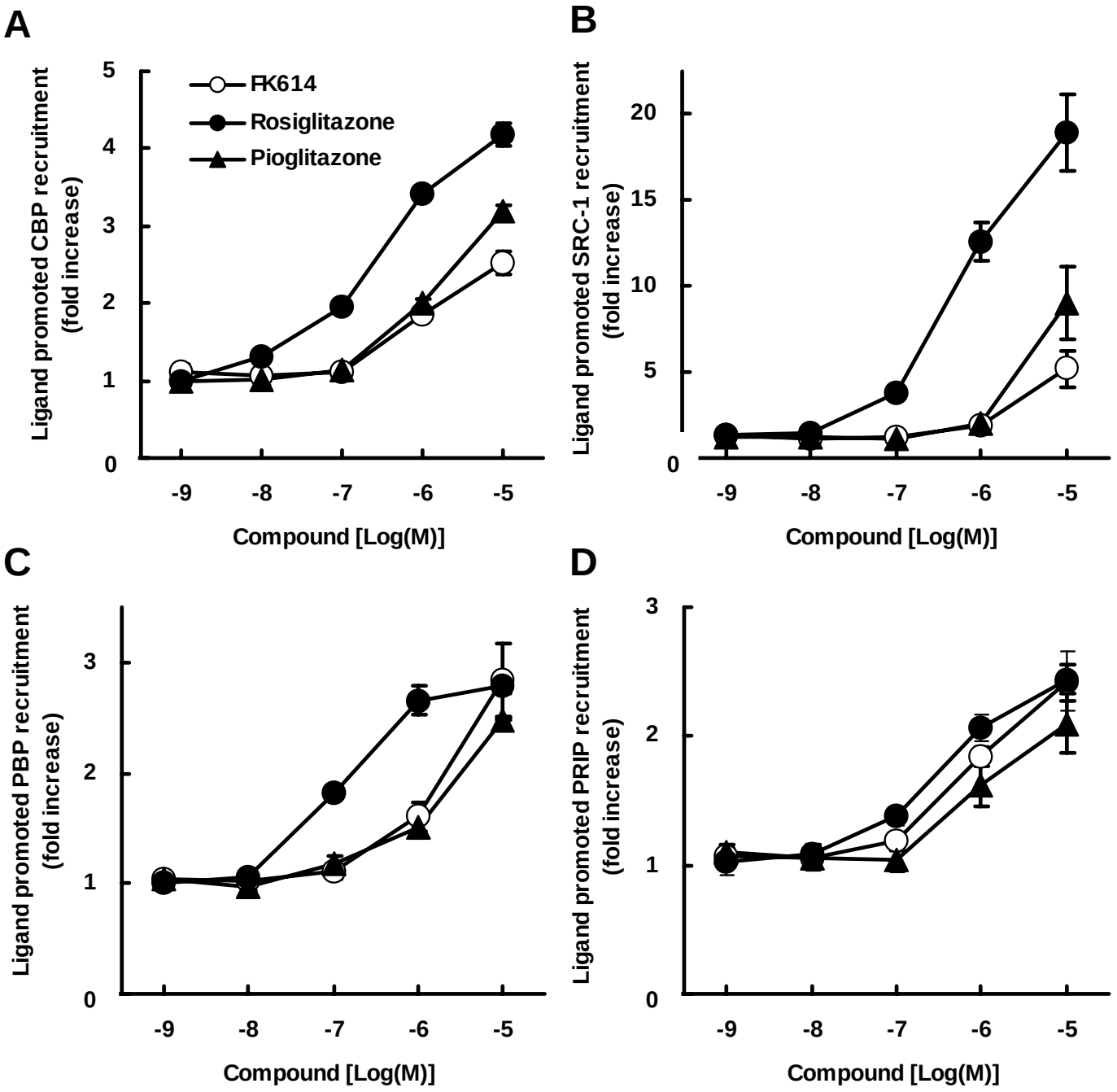
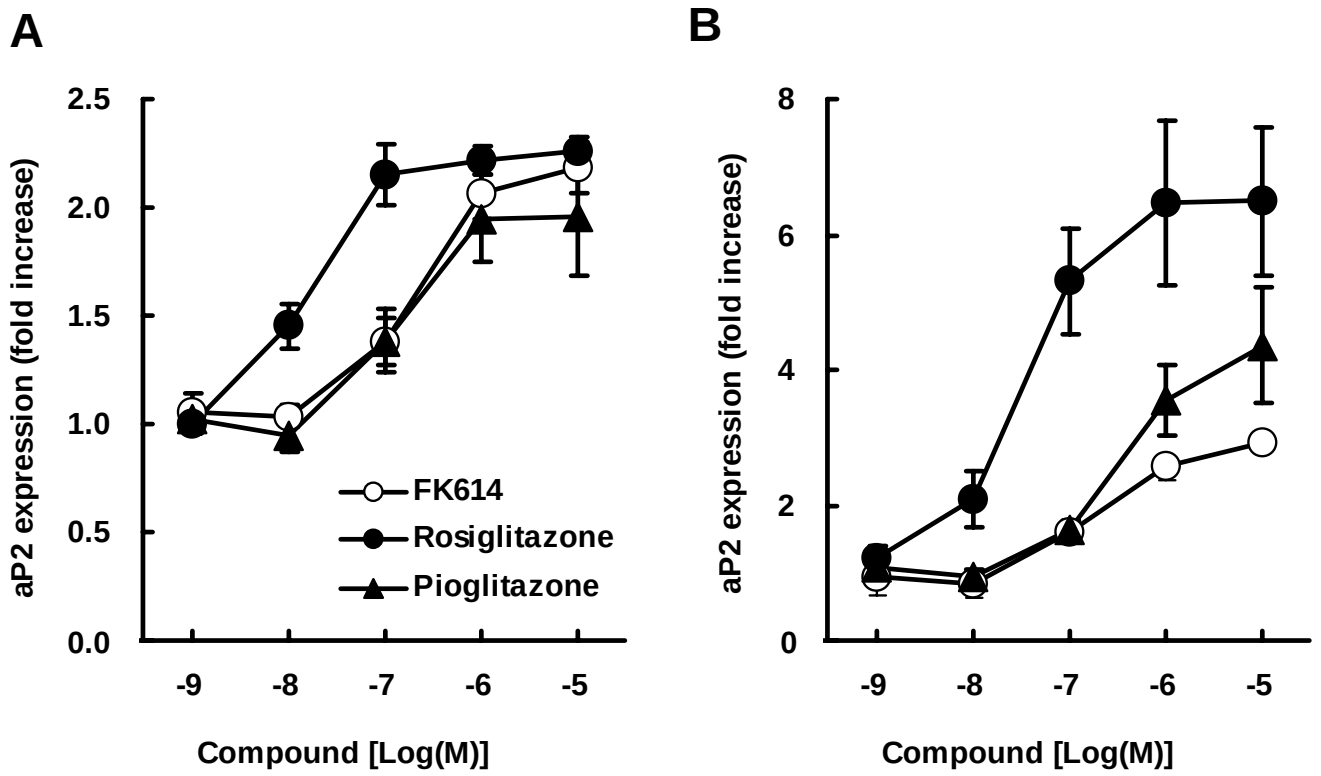


Figure 2



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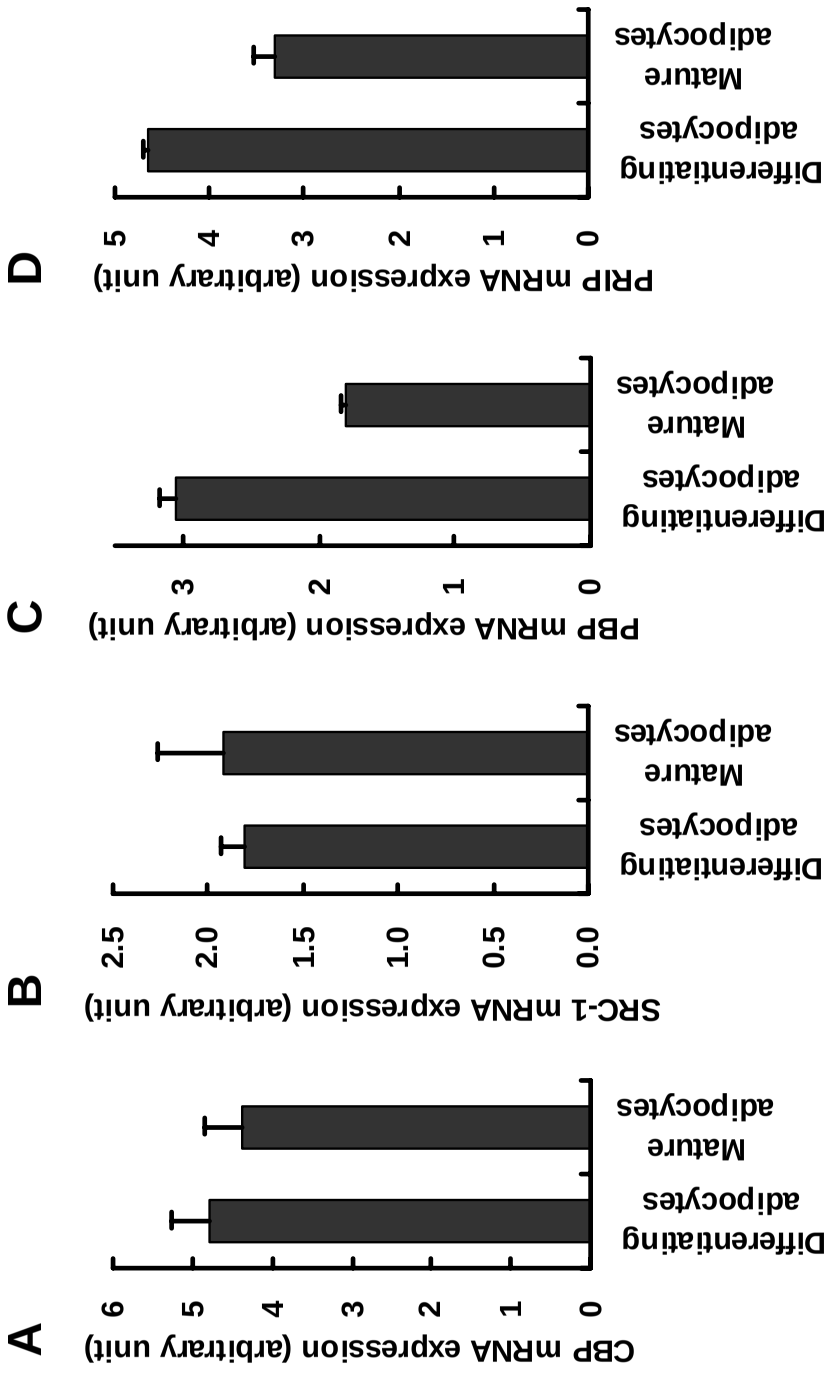

Figure 3

Figure 4

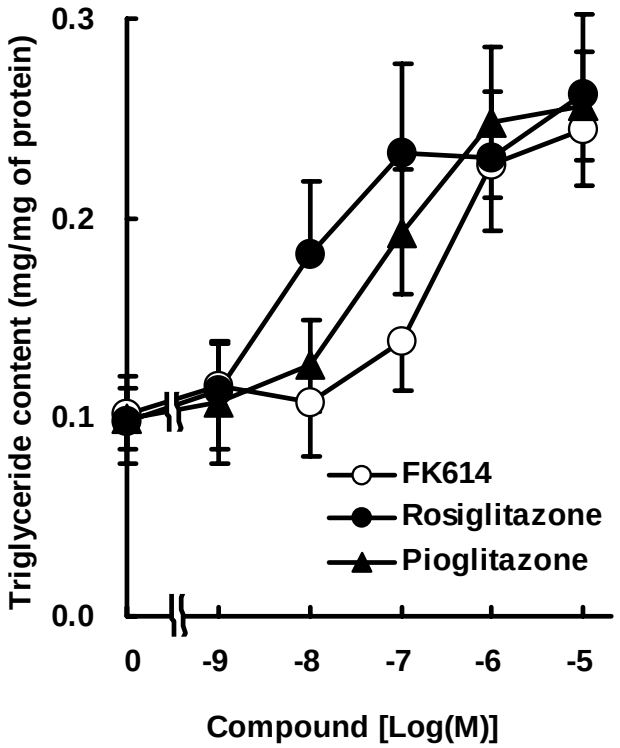

Figure 5