Unique Properties of Coactivator Recruitment Caused by Differential Binding of FK614, an Anti-diabetic Agent, to Peroxisome Proliferator-Activated Receptor γ

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FK614 is a structurally novel class of peroxisome proliferator-activated receptor γ (PPARγ) agonist, with the mechanism of its insulin-sensitizing action most likely due to activation of PPARγ. In this study, properties of FK614 for PPARγ binding, ability to induce conformational change, and coactivator recruitment were investigated. FK614, rosiglitazone, and pioglitazone competed specific binding of [3H]rosiglitazone to PPARγ with Ki values of 11 nM, 47 nM, and 1.3 μM, respectively. Limited tryptic digestion of PPARγ with FK614 or rosiglitazone produced distinct patterns of digested polypeptides, suggesting that FK614 directly binds to PPARγ but induces specific alterations in receptor conformation. FK614 induced interaction of PPARγ with nuclear receptor coactivator CBP but of lower magnitude than rosiglitazone and pioglitazone. The estimated Ki values of FK614-, rosiglitazone-, and pioglitazone-PPARγ complex to CBP peptide were 1.8, 0.64, and 0.72 μM, respectively, indicating FK614-PPARγ complex exhibits a lower affinity for CBP peptide compared to other agonist-PPARγ complexes. When tested the effect of FK614 on CBP recruitment induced by 9(S)-hydroxyoctadecadienoic acid, an endogenous ligand, FK614 negatively modulated PPARγ activation. The unique properties of FK614 may underlie the molecular basis of ligand-dependent transcriptional modulation mediated by PPARγ.

Key words FK614; coactivator recruitment; non-thiazolidinedione; peroxisome proliferator-activated receptor γ (PPARγ) modulator; anti-diabetes

Thiazolidinedione (TZD) compounds, such as rosiglitazone and pioglitazone, are anti-diabetic agents that improve insulin resistance. The action of TZDs is due to activation of peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor (NHR) family of ligand-activated transcription factors. However, since weight gain and edema have been reported as side effects of these drugs, improvement of PPARγ agonists as anti-diabetic agents is still required. Furthermore, to offer a promising therapeutic approach to not only diabetes but also metabolic syndrome, new types of PPARγ agonists, that can improve dyslipidemia, are likely to be beneficial.

Under physiological conditions, activity of PPARγ is controlled by endogenous ligands. 9(S)-Hydroxyoctadecadienoic acid (HODE), which is one of the most potent endogenous ligands, is an oxidation product of linoleic acid and a major oxidized lipid component of oxidized low density lipoprotein (LDL), present at high levels in atherosclerotic lesions. FK614 is a structurally novel class of peroxisome proliferator-activated receptor γ (PPARγ) agonist, with the mechanism of its insulin-sensitizing action most likely due to activation of PPARγ. In this study, properties of FK614 for PPARγ binding, ability to induce conformational change, and coactivator recruitment were investigated. FK614, rosiglitazone, and pioglitazone competed specific binding of [3H]rosiglitazone to PPARγ with Ki values of 11 nM, 47 nM, and 1.3 μM, respectively. Limited tryptic digestion of PPARγ with FK614 or rosiglitazone produced distinct patterns of digested polypeptides, suggesting that FK614 directly binds to PPARγ but induces specific alterations in receptor conformation. FK614 induced interaction of PPARγ with nuclear receptor coactivator CBP but of lower magnitude than rosiglitazone and pioglitazone. The estimated Ki values of FK614-, rosiglitazone-, and pioglitazone-PPARγ complex to CBP peptide were 1.8, 0.64, and 0.72 μM, respectively, indicating FK614-PPARγ complex exhibits a lower affinity for CBP peptide compared to other agonist-PPARγ complexes. When tested the effect of FK614 on CBP recruitment induced by 9(S)-hydroxyoctadecadienoic acid, an endogenous ligand, FK614 negatively modulated PPARγ activation. The unique properties of FK614 may underlie the molecular basis of ligand-dependent transcriptional modulation mediated by PPARγ.

Recently, we reported that the novel non-TZD compound [3-(2,4-dichlorobenzyl)-2-methyl-N-(pentylsulfonyl)-3-H-benzoimidazole-5-carboxamide] (FK614) substantially improves hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and glucose intolerance in genetically obese and diabetic animal models. FK614 has been shown to promote PPARγ-mediated transcription in a cell-based reporter gene assay, suggesting that FK614 exerts its pharmacological effects through PPARγ activation. Despite FK614 producing a robust anti-diabetic effect in vitro, this compound appears to be a partial agonist of PPARγ mediated transactivation in vivo. Considering that FK614 behaves as a partial agonist of PPARγ, it is likely that antagonistic cross talk exists between FK614 and endogenous ligands. In this study, whether FK614 directly induces conformational changes in PPARγ, produces specific alterations in PPARγ conformation, and induces differential interaction with coactivators compared to...
other PPARγ ligands was examined. Furthermore, whether FK614 negatively regulates the biological responses evoked by endogenous ligands was also investigated.

MATERIALS AND METHODS

Materials Materials were obtained from the following sources: *Escherichia coli* BL21 and BL21(DE3)pLysS was purchased from Stratagene (La Jolla, CA, U.S.A.). [3H]-rosiglitazone, [35S]methionine, and glutathione-Sepharose 4B beads were from Amersham Bioscience (Piscataway, NJ, U.S.A.). Trypsin was purchased from Sigma (St. Louis, MO, U.S.A.). FK614, rosiglitazone, and pioglitazone were synthesized by Astellas Pharma Inc. (Tokyo, Japan). 9(S)-HODE was purchased from Laradan Fine Chemicals (Malmö, Sweden). Streptavidin-conjugated allophycocyanin (SA-APC) and europium-conjugated anti-glutathione S-transferase (GST) antibody (Eu-anti-GST antibody) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, U.S.A.), respectively. All other chemicals and reagents were purchased from Sigma or Nacalai Tesque (Kyoto, Japan).

Plasmids Human PPARγ1 and PPARγ2 were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) with human adipose tissue and liver cDNA as templates, respectively. Full length cDNA for human PPARγ1 was cloned into pCDM8 (Invitrogen, Carlsbad, CA, U.S.A.). The resultant plasmid, pCDM8-hPPARγ1, was used for *in vitro* translation of human PPARγ1. Expression plasmids for GST fusion protein were generated by insertion of full-length human PPARγ2 or the ligand-binding domain (LBD) of human PPARγ into pGEX-4T-3 or pGEX-4T-2 vector (Amersham Bioscience, respectively). Full length cDNA for human CBP was amplified by RT-PCR with human lung cDNA as template and cloned into pcDNA3.1 (Invitrogen). The resultant plasmid, pcDNA3.1-hCBP, was used for *in vitro* translation of human CBP.

Ligand Binding Assay LBD of human PPARγ was expressed as a GST fusion protein (GST-hPPARγLBD) in *Escherichia coli* strain BL21. GST-hPPARγ2 was purified using glutathione-Sepharose 4B beads. [35S]methionine-labeled human CBP was synthesized in a coupled transcription/translation system, using pcDNA3.1-hCBP as template. The obtained [35S]-labeled human CBP was incubated with GST-hPPARγ2 and glutathione-Sepharose 4B beads in the presence or absence of compounds in binding buffer (8 mM Tris–HCl, pH 7.6, 120 mM KCl, 8% glycerol, 0.5% 3-(3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate, 1 mg/ml BSA, 1 mM DTT, 10 µg/ml aprotinin, 10 µM/ml leupeptin, 10 µg/ml pepstatin). These mixtures were incubated overnight at 4 °C, and beads were washed 4 times to remove unbound coactivators. Bound proteins were extracted into SDS sample loading buffer, then separated by SDS-PAGE. Bound CBP was visualized by autorigraphy and quantified by counting radioactivity using a BAS2000. Experiments were performed 3 or 4 times. EC50 values for human CBP recruitment were determined by nonlinear curve fitting using Statistical Analysis System (SAS) (SAS Institute, Cary, NC, U.S.A.). Each EC50 value was calculated under conditions where the maximum recruitment activity induced by each ligand was 100%. Significant difference of the values of CBP recruitment between non-treated control group and each ligand was assessed by Dunnett’s multiple comparisons using SAS. Statistical significance was assumed at *p*<0.05.

Homogeneous Time-Resolved Fluorescence (HTRF) Assay Amino acid sequences of cofactor peptides, tagged at the N termini with biotin, used in HTRF assays were as follows: human CBP (N-Biotin-SGNL-ADP ASNL-ELLRGGSGS-C),19 human NCoR (N-Biotin-ADPASN-GLEDIIRKALMGSF-C),18 GST-hPPARγ2 (50 nm) was incubated with 3 nM Eu-anti-GST antibody, 150 nM SA-APC, and 300 nM biotin-peptide in the absence or presence of compounds in 100 µl of assay buffer (10 mM HEPES, pH 7.6, 1 mM DTT, 100 mM NaCl, 0.05% Tween 20, 1 mg/ml BSA) for 16 h at 4 °C. The assay was performed in a 96-well black plate (Nalge Nunc International, Rochester, NY, U.S.A.) and fluorescence was measured using an ARVO HTS multilabel counter (PerkinElmer Life and Analytical Sciences). HTRF signal was expressed as the ratio of emission intensities at 665 : 615 nm multiplied by a factor of 1000. For saturation translation system (Promega, Madison, WI, U.S.A.). Transcription/translation reactions were equally aliquoted, then diluted test compounds were added. These mixtures were incubated for 30 min at room temperature, then distilled water or increasing concentrations of trypsin was added. Protease digestion was allowed to proceed for 20 min at 25 °C, then was terminated by addition of SDS sample loading buffer and boiled for 1 min at 100 °C. Resulting solutions were resolved by SDS-PAGE, then radio-labeled digestion products were visualized by autoradiography using a bio-imaging analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan). Autoradiograms were analyzed using image analyzing software (ATTO Densito Graph 4.0, ATTO, Tokyo, Japan), and molecular mass of protected fragments was measured. Experiments were performed 3 times. Measurement of molecular mass was performed in all 3 experiments, then the mean was calculated for 3 experiments.

**GST Pull-Down Assay** Full-length human PPARγ2 was expressed as a GST fusion protein (GST-hPPARγ2) in *Escherichia coli* strain BL21. GST-hPPARγ2 was purified using glutathione-Sepharose 4B beads. [35S]methionine-labeled human CBP was synthesized in a coupled transcription/translation system, using pcDNA3.1-hCBP as template. The obtained [35S]-labeled human CBP was incubated with GST-hPPARγ2 and glutathione-Sepharose 4B beads in the presence or absence of compounds in binding buffer (8 mM Tris–HCl, pH 7.6, 120 mM KCl, 8% glycerol, 0.5% 3-(3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate, 1 mg/ml BSA, 1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin). These mixtures were incubated overnight at 4 °C, and beads were washed 4 times to remove unbound coactivators. Bound proteins were extracted into SDS sample loading buffer, then separated by SDS-PAGE. Bound CBP was visualized by autoradiography and quantified by counting radioactivity using a BAS2000. Experiments were performed 3 or 4 times. EC50 values for human CBP recruitment were determined by nonlinear curve fitting using Statistical Analysis System (SAS) (SAS Institute, Cary, NC, U.S.A.). Each EC50 value was calculated under conditions where the maximum recruitment activity induced by each ligand was 100%. Significant difference of the values of CBP recruitment between non-treated control group and each ligand treated groups was assessed by Dunnett’s multiple comparisons using SAS. Statistical significance was assumed at *p*<0.05.
experiments, GST-hPPARγ2 (10 nM) was incubated with 5 nM Eu-anti-GST antibody, 600 nM SA-APC, in the presence of 10 μM FK614, 10 μM rosiglitazone, or 100 μM pioglitazone with various concentrations of non-labeled and biotin-labeled CBP peptides with a molar ratio of 50:1, in 100 μl of assay buffer for 16 h at 4 °C. Nonspecific binding was determined in the presence of 2 mM unlabeled CBP peptide. Specific binding of CBP peptide to human PPARγ2 is evaluated by subtracting the HTRF signal of nonspecific binding from the HTRF signal of total binding. Experiments were performed 3 times. EC50 values were determined by nonlinear curve fitting using SAS. Each EC50 value for coactivator recruitment was calculated under conditions where the maximum recruitment activity induced by each ligand is 100%. EC50 value for NCoR dissociation was calculated under conditions where the amount of NCoR binding to PPARγ in the absence of the ligand was 0% and HTRF signal in the absence of GST-hPPARγ2 was 100%. Estimated Ki values for CBP peptide binding to ligand-PPARγ complex were determined by linear regression analysis using GraphPad Prism.

**RESULTS**

**Ability of FK614 to Interact Directly with PPARγ** To determine whether activation of PPARγ by FK614 is due to direct binding of FK614 to PPARγ, a competition binding assay was performed using [3H]rosiglitazone and GST-hPPARγLBD. FK614, rosiglitazone, and pioglitazone displaced [3H]rosiglitazone binding to GST-hPPARγLBD in a concentration dependent manner (Fig. 1). This indicates that FK614 can bind directly to PPARγ, similar to TZDs such as rosiglitazone and pioglitazone. It appears that FK614 is a high-affinity PPARγ ligand with a Ki value of 11 nM. Binding affinity of FK614 was 4.3- and 120-fold higher than that of rosiglitazone (Ki: 47 nM) and pioglitazone (Ki: 1.3 μM), respectively (Table 1). FK614 shows a distinctly steeper slope of the displacement curve compared to rosiglitazone and pioglitazone (Fig. 1). The Hill coefficient for FK614, rosiglitazone, and pioglitazone was 3.2, 1.3, and 1.2, respectively. The distinct property of FK614 for binding to PPARγ is probably due to different conformational changes of the PPARγ in response to binding of alternative ligands.

**Alteration in Conformations of PPARγ Induced by FK614 and Rosiglitazone** To determine whether FK614 can directly induce conformational change in PPARγ and whether FK614 produces specific alterations in PPARγ conformation, the limited trypsin digestion of a full-length human PPARγ1 was performed. Human PPARγ1 labeled with [35S]methionine was pre-incubated with dimethyl sulfoxide (DMSO) (vehicle), 10 μM FK614, or 10 μM rosiglitazone, then digested with various concentrations of trypsin (Fig. 2). Incubation of the receptor with various concentrations of trypsin in the absence of ligand led to complete digestion of human PPARγ1. In contrast, rosiglitazone produced protease resistant fragments, a major 22 kDa and a minor 32 kDa fragment, following partial protease digestion. The protease protection pattern was similar to previous results reported by Elbrecht et al.25 FK614 also generated protease resistant fragments, however, a distinct digestion pattern was identified. FK614 induced stronger protection than rosiglitazone, and the 32 kDa fragment was detected more clearly than in the rosiglitazone-treated samples. In addition, a 25 kDa fragment found in FK614-bound receptor was faintly detected. Thus the results indicate that these two lig-

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**Table 1.** Ki and EC50 Values for PPARγ Ligands in Binding, GST Pull-Down, and HTRF Assays

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ki (nM) in binding assay</th>
<th>EC50 (nM) in pull-down assay</th>
<th>EC50 (nM) in HTRF assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK614</td>
<td>11</td>
<td>3.6</td>
<td>3.7</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>47</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>1300</td>
<td>&gt;1200</td>
<td>200</td>
</tr>
</tbody>
</table>

*The amount of full length CBP recruitment was not obtained at the saturating concentration for pioglitazone in GST pull-down assay, therefore EC50 value is estimated to be greater than 1200 nM for pioglitazone in that assay.*

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![Image](Image306x162 to 547x335)

**Fig. 1.** Displacement of [3H]Rosiglitazone from GST-hPPARγLBD by FK614, Rosiglitazone, and Pioglitazone

A competition binding assay was performed for 3 h with 15 nM [3H]rosiglitazone in the absence or presence of indicated concentrations of unlabeled FK614 (open circle), rosiglitazone (closed circle), or pioglitazone (closed triangle). Results are presented as % of control. Values are mean±S.E. of 3 independent experiments. Ki values are listed in Table 1.

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![Image](Image350x600 to 503x744)

**Fig. 2.** Difference in Protease Sensitivity of FK614- and Rosiglitazone-Bound PPARγ

Autoradiogram of a SDS-PAGE showing [35S]methionine-labeled full length human PPARγ1 digested with increasing concentrations of trypsin (0, 50, 100, 250 μg/ml) for 20 min at 25 °C. Receptor was pre-incubated with DMSO, 10 μM FK614, or 10 μM rosiglitazone. Experiments were performed 3 times with similar results. Asterisk denotes trypsin-resistant protein fragments.
and interact with PPARY directly and induce distinct PPARY conformations.

**Ligand Type-Specific Differential Interaction of PPARY with Cofactors** To determine whether FK614 directly promotes coactivator recruitment as an agonist and to address FK614-induced differential interaction with coactivators compared to other compounds, the effect of FK614, rosiglitazone, and pioglitazone on recruitment of CBP to PPARY by GST pull-down assay was studied. GST-hPPARY2 was incubated with [35S]methionine-labeled human CBP in the absence or presence of various concentrations of ligands (Fig. 3). FK614, rosiglitazone, pioglitazone, and 9(S)-HODE promoted the recruitment of CBP to PPARY in a concentration-dependent manner. In response to rosiglitazone, an obvious increase in recruitment of CBP was obtained at 10 nM, reaching a plateau at 1 μM. Maximal recruitment of CBP in the presence of 9(S)-HODE-bound receptor was approximately 6-fold above basal level. Induction of CBP recruitment for pioglitazone and 9(S)-HODE was initiated at 10 nM and 1 μM, respectively, and maximal response was achieved at 100 μM or higher. Magnitude of CBP binding to PPARY at the highest concentration was similar in the 3 ligands. In contrast, FK614 exhibited a mechanistically different property in the concentration response curve for CBP recruitment. FK614 significantly promoted CBP recruitment to PPARY at 10 nM and higher concentrations. Maximal level of CBP recruitment induced by FK614 was about 2-fold above basal level. These results indicate that FK614 can induce recruitment of CBP to PPARY as a potent agonist for PPARY, and shows differential properties in coactivator recruitment compared to other ligands, presumably due to the distinct conformational change of PPARY induced by FK614.

Transcriptional activity of PPARY is regulated by dissociation or association with corepressors and coactivators in a ligand dependent manner. To examine the effects of FK614 on coactivator or corepressor interaction, HTRF assays using peptides derived from the receptor interaction domain of corepressors or coactivators were performed. Figure 4A shows that HTRF signal was increased by recruitment of peptide from human CBP in the presence of various concentrations of FK614, rosiglitazone, and pioglitazone. However, the maximum response of FK614 was lower than that of the other 2 compounds. Results were similar to those observed in GST pull-down assays using full-length human CBP. In the absence of ligand, NCoR-PPARY interaction-dependent HTRF signal was observed, indicating that peptide from human NCoR was constitutively bound to PPARY (Fig. 4B). FK614, rosiglitazone, and pioglitazone promoted dissociation of NCoR peptide from PPARY in a concentration-dependent manner (Fig. 4B). At 10 μM FK614, 10 μM rosiglitazone, and 100 μM pioglitazone, the level of PPARY binding to NCoR peptide was 2.8, 5.0, and 5.9% of control levels, respectively. These results indicate that FK614 promotes release of the constitutive interaction between corepressor and non-ligand bound PPARY as efficaciously as rosiglitazone and pioglitazone. Using this assay, EC50 values of FK614, rosiglitazone, and pioglitazone for corepressor dissociation and coactivator recruitment were determined (Table 1). EC50 values of FK614 for CBP recruitment were 4.6- and 54-fold smaller than that of rosiglitazone and pioglitazone, respectively. FK614 was 6.1- and 120-fold more potent than rosiglitazone and pioglitazone, respectively, for NCoR dissociation. Interestingly, EC50 values of each compound for NCoR dissociation were higher than that for CBP recruitment. These results suggest that affinity of PPARY ligands for corepressor-unbound PPARY is higher than that for PPARY-NCoR complex and that this is probably due to the conformational
change of PPARγ induced by the interaction of NCoR peptide with PPARγ.

Affinity of Ligand-PPARγ Complex for Coactivator
To investigate whether FK614 induces differential affinity of PPARγ for coactivator compared with other TZDs, estimated $K_d$ values for the peptide from human CBP were determined using HTRF assays. Figure 5A shows saturation binding curves for CBP peptide in the presence of 10 μM FK614, 10 μM rosiglitazone, or 100 μM pioglitazone. At 20 μM CBP peptide, binding of CBP peptide to PPARγ was almost saturated in the presence of rosiglitazone. Since, it was assumed that HTRF signal indicates the amount of CBP peptide that binds to all PPARγ, in the presence of 20 μM CBP peptide and 10 μM rosiglitazone. Furthermore, it has been shown by X-ray crystal structure analysis that coactivators bind to PPARγ at a ratio of 1 mol coactivator:1 mol PPARγ in the presence of ligands. Therefore, it was also assumed that CBP binding at saturation approaches a ratio of 1 mol coactivator:1 mol PPARγ. Amounts of free and bound CBP peptide under the all conditions were then estimated. Linear regression analysis of bound/free versus bound Scatchard transformation data yielded estimated $K_d$ values of 1.9, 0.64, and 0.72 μM in the presence of FK614, rosiglitazone, and pioglitazone, respectively (Fig. 5B). The estimated $K_d$ value of FK614-PPARγ complex to CBP peptide was 3.0- and 2.7-fold larger than that of rosiglitazone- and pioglitazone-PPARγ complex, respectively. These results indicate that FK614-PPARγ complex exhibits low affinity compared to other TZD-PPARγ complexes in CBP peptide binding, presumably due to the different conformational changes of PPARγ induced by FK614.

Negative Modulation of 9(S)-HOE-Induced Coactivator Recruitment to PPARγ by FK614
To address the possibility that FK614 negatively regulates biological responses evoked by endogenous ligands, the effect of FK614 on coactivator recruitment promoted by 9(S)-HOE was examined by GST pull-down assay. As shown in Fig. 6, FK614 caused a concentration dependent decrease in magnitude of CBP binding to PPARγ induced by 9(S)-HOE, inhibiting to the level obtained by stimulation with FK614 alone. This inhibitory effect was observed even at 10 nm FK614, which is in good agreement with results obtained in ligand binding and FK614-promoted coactivator recruitment experiments. On the other hand, 10 μM rosiglitazone did not inhibit CBP peptide recruitment in PPARγ induced by 9(S)-HOE (data not shown). These results demonstrate that the biological effect of 9(S)-HOE, an efficacious endogenous agonist for PPARγ, can be modulated negatively by FK614.

DISCUSSION
FK614, a structurally novel class of PPARγ agonist incorporating a sulfonlcarbamoyl moiety as a key pharmacophore, improves hyperglycemia in diabetic db/db mice. FK614 has been shown to promote PPARγ-mediated transcription in cell-based reporter gene assay, suggesting that FK614 exerts its pharmacological effects through PPARγ activation, like the previously reported rosiglitazone and pioglitazone. In this study, FK614 behaved as a novel class of PPARγ ligand with unique properties in PPARγ activation. First, we focused on the binding of FK614 to PPARγ and the effect of FK614 on conformational change of PPARγ. Competitive binding analysis showed that FK614 competes
for binding of [3H]rosiglitazone to PPARγ. In protease protection assays, FK614 altered protease sensitivity of PPARγ and generated ligand-like protection patterns that can be observed in rosiglitazone-bound receptor. These results clearly indicate that FK614 can directly bind to PPARγ and induce a conformational change in PPARγ. In the binding assay, FK614 showed a distinctly steeper slope for the displacement curve. Furthermore, in limited protease sensitivity assay, FK614 induced a stronger protection of the 32 kDa fragment, also the 25 kDa fragment, that was not present in rosiglitazone-treated samples, faintly detected in FK614-bound receptor. These observed differences strongly suggest that the conformational changes induced by FK614 in PPARγ were slightly different from those by TZD compounds, presumably because FK614 interacts with PPARγ in a different manner. These results show that FK614 can alter the conformation of PPARγ upon direct binding and suggest that it could lead to differential biological responses compared to other TZDs, while those compounds act on PPARγ as a primary site of action. The coefficient of Hill plot for FK614 was higher than that for other TZD compounds. This observation suggests a positive cooperative interaction of FK614 with PPARγ. Although further investigation is required to characterize the mechanism of interaction of FK614 with PPARγ, it may be possible that dimerization of the receptor[20] exhibits a cooperative interaction, like interaction of estradiol with the estrogen receptor.[29] Binding of FK614 to one component of a possible PPARγ homodimer may lead to an allosteric conformational change enhancing the affinity of the other unoccupied component for FK614. Alternatively, similar to 4-hydroxytamoxifen binding that inhibits estradiol binding to estrogen receptor,[20] PPARγ may have different binding sites for FK614 and rosiglitazone, and binding of FK614 to PPARγ may reduce the affinity of rosiglitazone binding to PPARγ.

Second, we focused on the interaction between transcriptional cofactors and PPARγ, and examined the effects of FK614, rosiglitazone, and pioglitazone on the recruitment of coactivator to PPARγ in vitro. FK614, as well as other compounds, promotes coexpressor release and coactivator recruitment to PPARγ, behaving as a PPARγ agonist. In this study, the maximal effect of FK614 in coactivator recruitment was much lower than other compounds, as the FK614-PPARγ complex exhibits lower affinity for the CBP peptide than other ligand-PPARγ complexes. In previous studies, the maximal effect of FK614 on PPARγ mediated transcription in cell-based reporter gene assay was lower than other compounds, such as rosiglitazone and pioglitazone.[23] This strongly suggests that the partial-agonistic feature of FK614 observed in reporter gene assay is due to the lower magnitude of recruitment of coactivators to FK614-PPARγ complexes. The effect of rosiglitazone on CBP recruitment was initiated at a concentration of 10 nM, increasing gradually to reach a plateau at 1 μM. FK614-induced recruitment was also initiated at a concentration of the same order of magnitude as rosiglitazone, in the plateau. These observations are consistent with the rank order of potencies in the binding study. Interestingly, estimated Kd value of FK614-PPARγ complex was larger than that of rosiglitazone- and pioglitazone-PPARγ complex in CBP peptide binding. These results suggest that FK614 is a potent ligand for receptor occupancy, but produces receptor conformations with reduced affinity for coactivators. Thus, FK614 behaves as a high affinity ligand for PPARγ with low intrinsic activity. Therefore, at this point FK614 is clearly distinguishable from other TZD compounds.

Finally, we focused on the possibility that FK614 negatively modulates PPARγ activity induced by endogenous ligand, and examined the effect of FK614 on 9(S)-HODE-induced coactivator recruitment using GST pull-down assay. 9(S)-HODE promoted coactivator recruitment to PPARγ and behaved as a PPARγ agonist. Magnitude of coactivator recruitment achieved by 9(S)-HODE was greater than that of the maximal response of FK614, indicating that 9(S)-HODE is more efficacious than FK614. FK614 caused a concentration-dependent decrease in the magnitude of CBP binding to PPARγ induced by 9(S)-HODE and inhibited the level obtained when stimulated by FK614 alone. This inhibitory effect was observed even at low concentrations of FK614, which is in good agreement with results obtained in ligand binding and FK614-promoted coactivator recruitment experiments. As FK614 is a potent ligand in receptor occupancy, FK614 competes effectively with 100 μM 9(S)-HODE even at low concentrations. The results described above indicate that, unlike the full-agonists, FK614 negatively modulates PPARγ activity induced by 9(S)-HODE. High concentrations of 9(S)-HODE were required to obtain activation of coactivator recruitment. In tissues containing high amounts of 9(S)-HODE, such as macrophage, biological effects mediated by such endogenous ligands may be negatively regulated by FK614. Thus, FK614 could behave as a tissue- and stage-specific PPARγ modulator that activates or represses PPARγ-mediated pathways, depending on the cell context of target tissues. 9(S)-HODE is an oxidative metabolite of linoleic acid, a major oxidized lipid component of oxidized LDL,[4,5] and is present at high levels in atherosclerotic lesions.[6] 9(S)-HODE-mediated PPARγ activation is involved in many cellular events, including fatty acid influx,[6] foam cell formation,[4] VEGF expression,[7] and cholesterol efflux,[8,9] thus is believed to exert both pro- and anti-atherogenic effects on macrophages.[31] In atherosclerotic lesion development, high concentration of 9(S)-HODE may exert its atherogenic effect on macrophages by inducing the expression of CD36, a scavenger receptor, which mediates oxidized LDL uptake into macrophages and induces foam cell formation.[3] Namely, scavenger receptor substrate, such as oxidized LDL initiates and promotes its own uptake by increasing the level of receptor expression. It is expected that selective PPARγ modulators like FK614 may negatively modulate CD36 expression induced by endogenous ligands and exert an anti-atherogenic effect. As PPARγ agonists are used in type 2 diabetic patients at high risk of developing complications of atherosclerosis, these observations have important clinical implications. Therapeutic benefits of generations of PPARγ ligands in patients with type 2 diabetes could presumably be greatly enhanced by selecting compounds that retain insulin-sensitizing activities and are optimized for their anti-atherogenic activities,[31] such as compounds like FK614. FK614 has selective PPARγ partial agonist activity as do previously reported partial agonists, such as troglitazone,[13] MCC-555,[32] GW0072,[32] PAT5A,[14] and nTZDpa.[15] Many of these compounds have potent hypoglycemic and hypotriglyceridemic effects.

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activities in animal models of type 2 diabetes.\textsuperscript{33–35} However, previous studies have not reported whether these compounds negatively modulate PPARY-activity promoted by endogenous ligands, such as 9(S)-HODE. Therefore, FK614 may differ from other selective PPARY partial agonists, and may have better efficacy as therapy for arteriosclerosis as well as type 2 diabetes.

In relation to diabetes mellitus, it has been reported that heterozygous PPARY deficient mice are protected from high-fat diet-induced insulin resistance.\textsuperscript{36,37} Consistent with this observation, Pro12Ala polymorphism in human PPARY has been shown to confer resistance to type 2 diabetes.\textsuperscript{38} Recently, appropriate functional antagonism of PPARY/RXR by administration of PPARY-antagonist was demonstrated to be a beneficial approach against insulin resistance.\textsuperscript{39} Therefore maintenance of PPARY activation in appropriate levels by a PPARY modulator like FK614 may lead to therapeutic benefits for the control of insulin resistance.

In conclusion, FK614 can bind to PPARY as an agonist that shows differential properties in coactivator recruitment compared to other TZD ligands, due to distinct conformational changes of PPARY. These distinct properties of FK614 may lead to different biological responses compared to other insulin-sensitizing compounds.

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