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Structural basis for differential activities of enantiomeric PPARγ agonists: Binding of S35 to the alternate site

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ABSTRACT

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily. It functions as a ligand-activated transcription factor and plays important roles in the regulation of adipocyte differentiation, type 2 diabetes mellitus, and inflammation. Many PPARγ agonists bind to the canonical ligand-binding pocket near the activation function-2 (AF-2) helix (i.e., helix H12) of the ligand-binding domain (LBD). More recently, an alternate ligand-binding site was identified in PPARγ LBD; it is located beside the Ω loop between the helices H2′ and H3. We reported previously that the chirality of two optimized enantiomeric PPARγ ligands (S35 and R35) differentiates their PPARγ transcriptional activity, binding affinity, and inhibitory activity toward Cdk5 (cyclin-dependent kinase 5)-mediated phosphorylation of PPARγ at Ser245 (in PPARγ1 numbering; Ser273 in PPARγ2 numbering). S35 is a PPARγ phosphorylation inhibitor with promising glucose uptake potential, whereas R35 behaves as a potent conventional PPARγ agonist. To provide a structural basis for understanding the differential activities of these enantiomeric ligands, we have determined crystal structures of the PPARγ LBD in complex with either S35 or R35. S35 and R35 bind to the PPARγ LBD in significantly different manners. The partial agonist S35 occupies the alternate site near the Ω loop, whereas the full agonist R35 binds entirely to the canonical LBP. Alternate site binding of S35 affects the PPARγ transactivation and the inhibitory effect on PPARγ Ser245 phosphorylation. This study provides a useful platform for the development of a new generation of PPARγ ligands as anti-diabetic drug candidates.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors that belongs to the thyroid hormone receptor-like nuclear receptor subfamily 1 [1]. PPARs bind to cognate peroxisome proliferator response elements (PPREs) through hetero-dimerization with retinoid X receptors (RXRs) and regulate the transcription of target genes [2]. Among three known mammalian PPAR subtypes (PPARα, PPARγ, and PPARδ/β) that show distinct ligand specificity and tissue distribution, PPARγ is highly expressed in adipocytes and macrophages [3,4]. It plays essential roles in adipocyte differentiation, glucose homeostasis, lipid metabolism, insulin sensitization, and inflammatory action [5]. Thus, PPARγ is a potential therapeutic target for metabolic syndrome and inflammatory diseases such as type 2 diabetes and atherosclerosis [6,7].

Like other nuclear receptors, PPARγ contains an N-terminal domain, a DNA-binding domain, a hinge region, and a ligand-binding domain (LBD). The LBD of PPARγ is comprised of 12 α-helices in a three-layer sandwich and a small four-stranded β-sheet. The canonical ligand-binding pocket (LBP) of PPARγ is a Y-shaped hydrophobic cavity within the 12 helix-bundle of LBD. Its volume is in the range of 1300–1440 Å^3 [8,9], which is much larger than those of most other nuclear receptors [1]. In the apo form of the PPARγ LBD, the activation function-2 (AF-2) helix [i.e., helix 12 (H12)], a highly flexible switch element, is in a state of equilibrium among many different conformations varying from active to inactive [10]. Binding of PPARγ agonists in the canonical LBP induces conformational changes in the LBD via the formation of a hydrogen bond network that stabilizes the AF-2 helix [8]. Binding of

Abbreviations: PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; LBP, ligand-binding pocket; TZD, thiazolidinedione; Cdk5, cyclin-dependent kinase 5; SRC-1, steroid receptor coactivator-1; r.m.s., root-mean-square

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agonists also affects the recruitment of coactivators [8] or corepressors [11], which is a key mechanism for modulating target gene expression. In addition to the canonical LBP near the AF-2 helix, an NMR study identified an alternate ligand-binding site in the LBD of PPARγ adjacent to the Ω loop between helices H2′ and H3 [7,12].

Synthetic ligands of PPARγ include a class of insulin-sensitizing anti-diabetic drugs referred to as thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone [13], and a series of L-tyrosine-based compounds [14]. Despite the clinical benefit of TZDs, their use has been associated with adverse effects, including weight gain, increased adipogenesis, renal fluid retention, plasma volume expansion and possible increased incidences of cardiovascular events [15–17]. Rosiglitazone and pioglitazone, PPARγ TZD full agonists, are structurally similar but afford different clinical adverse events, indicating that subtle changes in the ligand-receptor interaction can lead to differences in the pharmacology of the TZD drugs [17]. This highlights the importance of the need for a more complete understanding of the mechanism of PPARγ modulation by synthetic ligands [17].

It was reported that synthetic PPARγ ligands exert another biochemical function by blocking the phosphorylation of PPARγ at Ser245 (in PPARγ1 numbering; Ser273 in PPARγ2 numbering) by cyclin-dependent kinase 5 (Cdk5) [18]. In this paper, all the residues of PPARγ LBD follow the PPARγ1 numbering. The phosphorylation of PPARγ does not alter its adipogenic capacity but leads to dysregulation of the genes whose expression is altered in obesity, including lowered expression of the insulin-sensitizing adipokine, adiponectin [18]. The Cdk5-mediated phosphorylation of PPARγ is blocked by anti-diabetic PPARγ ligands, such as rosiglitazone (a full agonist) and MRL24 (a partial agonist) [18]. Inhibition of PPARγ phosphorylation in obese patients by rosiglitazone is very tightly associated with the anti-diabetic effects of this drug [18]. We previously identified two optimized enantiomeric PPARγ ligands (R35 and S35) that have different effects on gene expression, adipogenesis, and PPARγ phosphorylation [19]. We also reported that the chirality of these enantiomeric ligands differentiates their PPARγ transcriptional activity, binding affinity, and inhibitory activity toward Cdk5-mediated phosphorylation of PPARγ at Ser245. S35, a partial agonist of PPARγ, shows an inhibitory activity toward Cdk5-mediated phosphorylation of PPARγ at Ser245 with promising glucose uptake potential, whereas its enantiomer R35 is a highly potent conventional PPARγ agonist [19].

To provide a structural basis for understanding their differential activities, we have determined crystal structures of the PPARγ LBD in complex with either S35 or R35. Unexpectedly, these enantiomeric ligands bind to the PPARγ LBD in significantly different manners. The partial agonist S35 occupies the alternate site near the Ω loop and does not interact with the AF-2 helix, whereas the full agonist R35 binds entirely to the canonical LBP and interacts with the AF-2 helix. The flexible Ω loop region is stabilized by the binding of S35 in the alternate site. The S35-bound PPARγ LBD simultaneously accommodates a fatty acid (modeled here as myristate) in the canonical LBP. In contrast, the fatty acid binding is prohibited by the binding of R35 to the PPARγ LBD. This study shows that different binding modes of S35 and R35 in the PPARγ LBD determine their differential behaviors as PPARγ agonists. This finding may lead to the development of a new generation of anti-diabetic drugs that target PPARγ.

2. Material and methods

2.1. Protein expression and purification

The gene encoding the human PPARγ LBD construct (residues 195–477 in PPARγ1 numbering) was PCR-amplified using a human cDNA clone encoding PPARγ (clone ID: hMU000317) as the template, which was provided by the Korea Human Gene Bank, Medical Genomics Research Center, Korea Research Institute of Bioscience & Biotechnology. It was cloned into the expression vector pET-28b(+) (Novagen). This construct of the recombinant protein adds a 21-residue N-terminal fusion tag (MGSSHHHHHH SSGLVPRGSH M) containing a His_6 tag and a thrombin cleavage site in front of the starting residue Ala195. The recombinant human PPARγ LBD was overexpressed in *Escherichia coli* Rosetta 2(DE3) cells using the Luria Broth culture medium. Protein expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside and the cells were incubated for additional 24 h at 18 °C following growth to mid-log phase at 37 °C. The cells were lysed by sonication in buffer A (20 mM Tris-HCl at pH 8.5, 150 mM NaCl, 10% (v/v) glycerol and 0.1 mM tris(2-carboxyethyl) phosphine hydrochloride) containing 5 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. The crude lysate was centrifuged at 36,000*g* for 1 h. The supernatant was applied to a HiTrap Chelating HP affinity chromatography column (GE Healthcare), which was previously equilibrated with buffer A containing 5 mM imidazole. Upon eluting with a gradient of imidazole in the same buffer, the human PPARγ LBD was eluted at 45–100 mM imidazole concentration. The eluted protein was desalted in buffer A using a HiPrep 26/10 desalting column (GE Healthcare) to remove imidazole, and the protein was cleaved with 2 units of thrombin (Sigma Aldrich) per mg of the PPARγ LBD at 4 °C overnight. Both the N-terminal fusion tag and the uncleaved protein were removed by affinity chromatography on a HiTrap Chelating HP affinity chromatography column. The flow-through was applied to a HiLoad XK-16 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with buffer A. Fractions containing the human PPARγ LBD were pooled and concentrated to 15.4 mg ml^{-1} using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore).

2.2. Crystallization

Before crystallization, the purified PPARγ LBD and the LXXLL motif-containing peptide (ERHKILHRLLQEGSPS corresponding to residues 685–700 of the human SRC-1) were mixed in a molar ratio of 1:2, in the presence or absence of a 10-fold molar excess of the PPARγ ligand S35 or R35. After overnight incubation, the protein-ligand complexes were crystallized by the sitting-drop vapor diffusion method using the Mosquito robotic system (TTP Labtech) at 23 °C by mixing 0.2 μl of the protein solution and 0.2 μl of the reservoir solution. PPARγ·R35·SRC-1 crystals were obtained with a reservoir solution of 57.5% (v/v) Tacsimate at pH 7.0. Single crystals grew to dimensions of approximately 0.2 × 0.1 × 0.1 mm within a few days. PPARγ·S35·SRC-1 crystals were obtained with a reservoir solution of 2.2 M sodium malonate at pH 7.0. The initial PPARγ·S35·SRC-1 crystals appeared as multiple crystals that were not suitable for diffraction data collection. Therefore, microseeding technique was used to obtain single crystals. Several pieces of the initial crystals were transferred into an Eppendorf tube containing a Seed Bead (Hampton Research) and 50 μl stabilization solution of 2.2 M sodium malonate at pH 7.0, and were vortexed to produce microseeds. The stock solution of microseeds was then briefly centrifuged and diluted serially by a factor of 100–1000 in the same stabilization solution. Each sitting drop was prepared by mixing the protein solution, the reservoir solution, and the microseed solution in a volume ratio of 1:0.7:0.3. Single crystals grew reproducibly to dimensions of approximately 0.35 × 0.2 × 0.1 mm within a few days. PPARγ·SRC-1 crystals were also grown using the microseeding technique under the same reservoir condition. Single crystals grew to dimensions of approximately 0.2 × 0.2 × 0.1 mm within a few days.

2.3. X-ray data collection, structure determination, and refinement

X-ray diffraction data for the R35-bound and agonist-free PPARγ LBD were collected at 100 K using a Quantum Q270 CCD detector system (Area Detector Systems Corporation, Poway, California) at the BL-7A experimental station of Pohang Light Source, Korea. The X-ray data from the crystal of S35-bound PPARγ LBD were collected at 100 K using a Quantum 315r CCD detector system (Area Detector Systems