Structural Basis for Iloprost as a Dual Peroxisome Proliferator-activated Receptor α/δ Agonist*

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Iloprost is a prostacyclin analog that has been used to treat many vascular conditions. Peroxisome proliferator-activated receptors (PPARs) are ligand-regulated transcription factors with various important biological effects such as metabolic and cardiovascular physiology. Here, we report the crystal structures of the PPARα ligand-binding domain and PPARδ ligand-binding domain bound to iloprost, thus providing unambiguous evidence for the direct interaction between iloprost and PPARα and a structural basis for the recognition of PPARα/δ by this prostacyclin analog. In addition to conserved contacts for all PPARα ligands, iloprost also initiates several specific interactions with PPARs using its unique structural groups. Structural and functional studies of receptor-ligand interactions reveal strong functional correlations of the iloprost-PPARα/δ interactions as well as the molecular basis of PPAR subtype selectivity toward iloprost ligand. As such, the structural mechanism may provide a more rational template for designing novel compounds targeting PPARs with more favorable pharmacologic impact based on existing iloprost drugs.

Prostacyclin (or PGI2)3 is a member of the eicosanoids family of lipid molecules produced in endothelial cells and plays key roles in cardiovascular homeostasis (1). Because this eicosanoid molecule undergoes rapid degradation and only has a half-life of 42 s, several stable synthetic PGI2 analogs have been developed to study the molecular mechanisms and clinical applications of this important eicosanoid (2–4). Iloprost is such a PGI2 analog that has been used to treat pulmonary arterial hypertension and several other vascular conditions by inhibiting platelet aggregation and inducing vasodilatation (5, 6).

Despite the importance of iloprost in human therapy, the identity of the receptors for this drug remains to be further elucidated. Although iloprost was originally identified to function through the cell surface prostacyclin receptor (7), several lines of evidence indicate that iloprost is also associated with the PPAR pathway (8,9). The three PPAR subtypes (α, β, δ, and γ) are ligand-regulated transcription factors that belong to a nuclear receptor family involved in many aspects of human physiology (10, 11). As such, PPARs are effective targets for many therapeutic uses. The pharmacologic actions of PPARs are mediated first through their LBDs, which bind ligands, then recruit nuclear receptor coactivators (or corepressors) to regulate expression of the downstream target genes. Interestingly, PPARs have been revealed as important regulators in angiogenesis (12, 13). Indeed, some effects of iloprost in vivo, like angiogenesis and up-regulation of vascular endothelial growth factor, function through PPARα-dependent mechanisms (14, 15). In addition, PPARδ is required for the antiapoptotic action of PGI2 in endothelial cells (16).

Several eicosanoids have been shown to function through the PPAR signaling pathway (9, 17–19). For example, 15-deoxy-D12,14-prostaglandin J2 was implicated as a natural ligand for PPARγ, whereas eicosapentaenoic acid is able to bind all three PPARs (20). However, it remains unclear whether iloprost binds directly to PPARs because the binding experiments failed to demonstrate the direct interaction between iloprost and PPARα (8). Moreover, the selectivity of iloprost toward PPAR subtypes has remained puzzling to date. Clearly, the molecular basis for the interaction between PPARs and iloprost needs to be defined further to develop a PGI2-based drug design strategy targeting PPARs with more efficacious and combinatorial therapeutic potentials.

To investigate the molecular mechanisms of iloprost-regulated PPAR activity, we first determined the cofactor-binding profiles for PPARγ in response to iloprost using biochemical peptide profiling. To uncover the molecular mechanism for the binding selectivity of PPARs to iloprost, the high resolution crystal structures of the PPARα LBD and PPARδ LBD complexed with iloprost were determined, respectively. Through a combination of mutagenesis, biochemical binding studies, and structural analysis, we revealed the molecular basis for the selectivity of PPARα/δ among all three PPARs for the binding of iloprost, thus providing critical perspective regarding the differential roles of three PPAR subtypes in prostacyclin signaling and cardiovascular therapeutic effects.

EXPERIMENTAL PROCEDURES

Protein Preparation—The human PPARα LBD (residues 196–468) and PPARδ LBD (residues 170–441) were expressed...
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as N-terminal His6 fusion protein from the expression vector pET24a (Novagen). BL21 (DE3) cells transformed with each expression plasmid were grown in LB broth at 25 °C to an A600 of 1.0 and induced with 0.1 mM isopropyl 1-thio-D-galactopyranoside at 16 °C. Cells were harvested, resuspended, and sonicated in 200 ml of extract buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, and 25 mM imidazole) per 6 liters of cells. The lysate was centrifuged at 20,000 rpm for 30 min, and the supernatant was loaded on a 5-ml NiSO4-loaded HisTrap HP column (GE Healthcare). The column was washed with extract buffer, and the protein was eluted with a gradient of 25–500 mM imidazole. The PPARα LBD and the PPARδ LBD were further purified with a Q-Sepharose column (Amerham Biosciences). To prepare the protein-ligand complex, we added a 5-fold excess of the iloprost ligand to the purified protein, followed by filter concentration to 10 mg/ml. The PPARα LBD was complexed with 2-fold of PGC-1α peptide (VDELSLLQKLHATS) before filter concentration.

Crystalization, Data Collection, and Structure Determination—The crystals of the PPARα-iloprost complex were grown at room temperature in hanging drops containing 1.0 ml of the above protein-peptide solutions and 1.0 ml of well buffer containing 0.1 M HEPES, pH 7.0, and 20% PEG 3350, whereas the crystals of PPARδ-iloprost complex were grown in the well buffer containing 20% PEG 5000, 2% tacsimate, pH 5.0, 5% glycerol, and 100 mM sodium citrate. The crystals were directly flash frozen in liquid nitrogen for data collection. Diffraction data were collected with a MAR225 CCD detector at the ID line of sector-21 at the Advanced Photon Source. The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package (21). The structures were determined by molecular replacement in the CCP4 suite.

FIGURE 1. Iloprost is a dual PPARα/δ agonist. A, receptor-specific transactivation by 1 μM iloprost. The COS-7 cells were cotransfected with the plasmids encoding various full-length nuclear receptors and their cognate luciferase reporters (see “Experimental Procedures”). After transfection, cells were treated with dimethyl sulfoxide (DMSO) or 1 μM iloprost. B, dose responses of iloprost in transactivating PPAR (α, δ, and γ). COS-7 cells were cotransfected with plasmids encoding full-length PPARs and a PPRE luciferase reporter. After transfection, cells were treated with dimethyl sulfoxide or iloprost ligand with various concentrations.

FIGURE 2. Iloprost promotes the interaction of coactivator LXXLL motifs with PPARα and PPARδ, respectively. Modulation of the interaction of PPARα, PPARδ, and PPARγ with various coactivator LXXLL motifs and corepressor motifs in response to 1 μM iloprost is shown by AlphaScreen assays. The peptide sequences are listed under “Experimental Procedures”.

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The search model for PPARα-ilocprof is the 1K7L, whereas the model for PPARδ-ilocprof is 3GWX in Protein Data Bank (20). Manual model building was carried out with COOT (22), followed by REFMAC refinement in the CCP4 suite.

**Cofactor Binding Assays**—The binding of the various peptide motifs to PPAR LBDs in response to ligands was determined by AlphaScreen™ assays using a hexahistidine detection kit from PerkinElmer Life Sciences as described before (23). The experiments were conducted with ~20–40 nM receptor LBD and 20 nM biotinylated cofactor peptides in the presence of 5 μg/ml donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 0.05 mM CHAPS, and 0.1 mg/ml BSA, all adjusted to pH 7.4.

The peptides with an N-terminal biotinylation were as follows: SRC1–2, SPSSHSSLTERHKILHRLLQEGSP; SRC2–3, QEPVSPKKKENALLRYLLDKDDTKD; PGC-1α, AEEPS-LKLLLAPA; CBP-1, SGNLVPDAASKHKQLSELLRGGS; and NCOR-2, GHSFADPASNLGLEDIIRKALMGSF.

**Transient Transfection Assay**—COS-7 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and were transiently transfected using Lipofectamine 2000 (Invitrogen) (24). All mutant PPAR plasmids were created using the QuikChange site-directed mutagenesis kit (Stratagene). 24-well plates were plated 24 h prior to transfection (5 × 10⁴ cells/well). For native promoter reporter assays, the cells were

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**TABLE 1**

| Parameters | PPARα-Ilo | PPARα-Ilo-PGC-1β |
|------------|-----------|-------------------|
| **Data collection** | | |
| Space group | P21 | P21 |
| Cell dimensions | 49.12, 111.90, 55.01 | 44.84, 60.97, 54.16 |
| β, γ (°) | 90.0, 93.15, 90.0 | 90.0, 108.9, 90.0 |
| Resolution (Å) | 50.00-2.23 (2.27-2.23) | 50.00-2.21 (2.25-2.21) |
| R_exp (°) | 0.115 (0.246) | 0.070 (0.199) |
| I/σ | 34.4 (5.9) | 53.5 (10.5) |
| Completeness (%) | 99.2 (96.6) | 99.6 (95.0) |
| Redundancy | 5.9 (5.1) | 4.9 (4.3) |

**Refinement**

| Resolution (Å) | 36.90-2.30 | 28.40-2.21 |
| No. reflections | 26,191 | 13,835 |
| R_work/R_free (%) | 19.1/25.9 | 17.6/23.9 |
| No. atoms | Protein | 4,331 | 2,221 |
| | Ligand/ion | 52 | 26 |
| | Water | 403 | 93 |
| B factors | Protein | 20.5 | 37.8 |
| | Ligand/ion | 16.8 | 42.9 |
| | Water | 23.6 | 40.8 |
| R.m.s.d. | Bond lengths (Å) | 0.008 | 0.008 |
| | Bond angles (°) | 1.2 | 1.1 |

* Values in parentheses are for highest resolution shell.
* R_exp = Σ|I_{obs} - I_{calc}|/ΣI_{obs}.
* R_factor = Σ|F_{o}-F_{c}|/ΣF_{c}, where F_{o} and F_{c} are observed and calculated structure factors, R_{m.s.d.} is calculated from a randomly chosen 8% of reflections excluded from refinement, and R_{m.s.d.} was calculated for the remaining 92% of reflections.
* R.m.s.d. is the root mean square deviation from ideal geometry.

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**FIGURE 3. Recognition of iloprost by PPARα and PPARδ.** A and B, structures of iloprost bound with PPARα (A) and PPARδ (B) in ribbon representation. PPARα and PPARδ are colored in green and blue, respectively. The bound iloprost is shown in stick representation with carbon and oxygen atoms depicted in yellow and red, respectively. C and D, 2F_{o} – F_{c}, electron density map (1.0σ) showing bound iloprost to the PPARα LBD (C) and PPARδ LBD (D). The bound iloprost is shown in stick representation with carbon and oxygen atoms depicted in yellow and red, respectively.
cotransfected with plasmids encoding full-length nuclear receptors and their cognate luciferase reporters as follows: human PPARs (α, δ, and γ) and PPAR response element-Luc; human RORα or RORγ and the Pcp2/ROR response element-Luc reporter; human glucocorticoid receptor or androgen receptor and the MMTV-Luc; human liver X receptors and TK-LXRE3-Luc; human farnesoid X receptor, and ecysone response element-Luc. Ligands were added 5 h after transfection. Cells were harvested 24 h later for the luciferase assays. Luciferase data were normalized to Renilla activity cotransfected as an internal control.

RESULTS

Iloprost Is a Dual PPARα/δ Agonist That Induces Their Transcriptional Activities—To probe the physiological roles of iloprost on PPAR signaling, we first characterize the transcriptional properties of PPARs in response to iloprost. COS-7 cells were cotransfected with plasmids encoding three PPARs and various other nuclear receptor full-length receptors, respectively, together with their cognate response reporters. Consistent with previous observations, iloprost showed strong agonist activity on PPARα and PPARδ, but not on PPARγ and a variety of other nuclear receptors tested, including farnesoid X receptor, liver X receptorα and β, retinoic acid-related receptorα and γ (RORα and RORγ), mineralocorticoid receptor, or glucocorticoid receptor (Fig. 1A). Further, full dose curves revealed that iloprost activated PPARα and PPARδ in a concentration-dependent manner with an approximate EC50 of 200 nM range, suggesting that iloprost is a potent PPARα/δ dual agonist (Fig. 1B).

Iloprost Promotes the Interaction of Coactivator LXXLL Motifs with PPARα and PPARδ, Respectively—To unravel the biochemical mechanism of PPARα/δ activation by iloprost, we determined the ability of iloprost to promote recruitment of coactivator LXXLL motifs by PPARα/δ using AlphaScreen biochemical assay, which is a widely used assay to detect ligand-dependent interaction between nuclear receptors and their coactivators(23). In this assay, the coactivator peptides and the PPAR LBDs, excitation with a laser beam causes the donor beads to emit single-oxygen molecules that activate the fluorophores in the acceptor beads, and light is recorded as coregulator binding activity. As shown in Fig. 2, the treatment of iloprost strongly enhanced the interaction of PPARα/δ with various coactivator LXXLL motifs from the family of steroid receptor coactivators (SRC1–2 and SRC2–3), CBP (CBP-1), and PGC-1α (PGC-1α-1), but not a corepressor motif from NCoR (NCoR-2), indicating that iloprost functions as a PPARα/δ agonist (Fig. 2A and B). In contrast, iloprost has no effect on coregulator recruitment by PPARγ (Fig. 2C). These results reaffirm that iloprost is a selective PPARα/δ dual agonist and strongly suggest a direct binding of PPARα/δ.

Recognition of Iloprost by PPARα and PPARδ Revealed by Crystal Structures—To determine the molecular basis for the selective binding of iloprost by PPARs, we solved the crystal structures of iloprost complexed with PPARα and PPARδ, respectively (Table 1). The structures reveal that the PPARα LBD-iloprost displays a monomeric fold (Fig. 3A) whereas PPARδ LBD-iloprost forms a dimer complex with the helix 10 from each monomer forming a dimer interface (Fig. 3B). Both PPARα-iloprost and PPARδ-iloprost are folded into three-layer helical sandwiches, with the C-terminal AF-2 helix positioned in the active conformation, in agreement with the agonist nature of the iloprost ligand. The binding mode of iloprost to PPARα/δ was apparent from the highly revealing electron density map shown in Fig. 3, C and D. The iloprost adapts in the center of the ligand-binding pocket and adopts essentially the same configuration for both PPARα and PPARδ. The binding of iloprost to PPARα/δ was stabilized by a combination of hydrogen bonds and hydrophobic interactions in a similar manner (Figs. 3, C and D, and 4). All of the contacts between the iloprost ligand and receptors closely resemble each other for both PPARα and PPARδ.

Functional Correlation of the Iloprost/PPARα/δ Interactions—To validate the roles of pocket residues in iloprost binding and PPARα activation, we mutated several key PPARα residues that contact different groups of iloprost and then tested the transcriptional activity of these mutated PPARα in response to iloprost in cell-based reporter assays using full-length PPARα and a PPAR response reporter. The acidic head group of iloprost forms several hydrogen bonds with the surrounding PPARα residues, including Tyr-314 from helix 5, His-440 from helix 10, and Tyr-464 from helix 12 (Figs. 4A and 5A). These interactions
are also observed between iloprost and PPARδ, thus supporting a critical conserved mechanism for ligand-mediated activation of PPARs (Fig. 4B). Indeed, the Y464L mutation decreases the activation of PPARα by both iloprost and PPARα-selective ligand GW735 in cell-based assays using a PPARα response reporter, highlighting the importance of these hydrogen-bond interactions in ligand binding (Fig. 5E).

In addition to conserved contacts for all PPARα ligands, iloprost also initiates several specific interactions with PPARs using its unique structural groups. The hydrophobic side chain of Met-355 stabilizes ligand binding by making hydrophobic interactions with iloprost backbone (Figs. 4A and 5B). Mutation of this residue decreased PPARα activation by iloprost but had no effects on PPARα activation by PPARα ligand GW735 that...
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has a hydrophilic group at this corresponding position (25), highlighting the differential roles of this residue in recognizing iloprost and other PPARα ligands like GW735 (Fig. 5, B and E). Notably, two unique hydroxy groups from iloprost form hydrogen bonds with Thr-279 and Ser-280 of PPARα, respectively, thereby facilitating the iloprost binding to this nuclear receptor (Fig. 5C). Interestingly, the similar interactions were also observed between iloprost and PPARδ at corresponding positions of Thr-252 and Thr-253, whereas PPARα ligand GW735 and PPARδ ligand GW0472 do not form such interactions (Fig. 5, C and D). To validate further the specific impact of these hydrogen bonds on iloprost binding, we mutated these serine and threonine residues in the ligand-binding pocket surrounding the hydroxy groups of iloprost for both PPARα and PPARδ. Accordingly, all of these mutations significantly decreased the activation of both PPARα and PPARδ by iloprost but not by PPARα ligand GW735 or PPARδ ligand GW0472, respectively (Fig. 5E). Together, these data reveal the important roles of diverse interactions in the specific binding of iloprost by PPARα and PPARδ.

Molecular Determinants of PPAR Subtype Selectivity toward Iloprost Ligand—The inactivity of iloprost to bind PPARγ raises questions as to the molecular basis for the binding selectivity of this ligand toward PPARα/δ, considering high sequence and structural homology among all three PPARs (26). Notably, they all have three-arm and Y-shaped ligand-binding pockets and similar pocket size (27). Structural comparison revealed three key residues from the PPARγ LBD different from PPARα/δ that define a unique characteristic of the PPARα ligand-binding pocket that discriminates the iloprost binding. PPARγ LBD contains two polar residues Tyr-327 from helix 5 (Fig. 6, A and D) and Ser-342 between helix 5 and helix 6 (Fig. 6, B and D) that show conflicts with hydrophobic nature of the iloprost backbone. Mutations of these two residues to the corresponding ones shared by both PPARα and PPARδ enable PPARγ to respond to iloprost treatment (Fig. 6E). Another key pocket residue is Phe-363 located immediate before helix 7 of PPARγ LBD (Fig. 6, C and D). Compared with the smaller iso-leucine side chain in the corresponding positions of PPARα/δ, the bulky phenylalanine is predicted to interfere with the iloprost binding by reducing the pocket size. As expected, iloprost activated the transcriptional activity of PPARγ mutant carrying F363I mutation in contrast to wild-type PPARγ (Fig. 6E). Of note, all of these mutations have no effect on the transcriptional activity of PPARγ in response to ligand rosiglitazone (Fig. 6E). As such, these functional mutations reveal the important roles of the specific hydrophobic interactions with the backbone of iloprost in the selectivity of PPARα/δ for ligand binding among all three PPARs.

DISCUSSION

The functional interaction between iloprost and PPAR receptors is critical for understanding its pharmacologic mechanisms as an important therapeutic agent. Here, we conducted detailed analysis of the binding between iloprost and coactivators by a combination of biochemical binding assay, mutagenesis, and structural analysis. Biochemical studies indicate that iloprost functions as a selective PPARα/δ dual agonist by inducing coactivator binding to these nuclear receptors. The crystal structures of PPARs bound to iloprost further provide an unambiguous evidence for the direct binding of iloprost with PPARα and PPARδ, shedding light on the molecular pathway of this important prostacyclin drug. In addition to conserved binding mechanisms, structural analysis also revealed specific binding of PPARα and PPARδ by iloprost through its unique structural features distinct from other PPAR ligands. Because both iloprost and PPARα/δ are involved in many physiological activities, the structure and functional relationships of iloprost and PPARα/δ signaling provide new opportunities for novel PGL2-based drug development with improved selectivity that can differentiate and enhance individual clinical effect associated with iloprost such as angiogenesis.

The three PPAR subtypes (α, δ, and γ) are characterized as nuclear receptors with not only high sequence homology but also different expression pattern, reflecting distinct but also overlapping roles of the three PPARs played in human physiology (28). Indeed, these PPARs display differential activity profiles in response to various ligands, including eicosanoids (17, 29). Our observations also provide an explanation for why iloprost preferentially activates PPARα and PPARδ rather than the PPARγ subtypes. The molecular basis for the selectivity of PPARα/δ for binding iloprost thus provides critical perspective regarding the differential roles of three PPAR subtypes in prostacyclin signaling and cardiovascular therapeutic effects. Based on the above structural observations, PPARα/δ utilizes both
conserved and also unique epitopes that dictate the discrimination of iloprost binding from other PPAR ligands. The differential ligand binding modes of PPARα/δ versus PPARγ may allow the modulation of distinct ligand binding selectivity and affinity, thereby affecting diverse physiological outcome of different PPAR ligands. As such, the structural mechanism may provide a more rational template for designing novel compounds targeting PPARs with more favorable pharmacologic impact based on existing iloprost drugs.

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