α,β-Unsaturated Ketone Is a Core Moiety of Natural Ligands for Covalent Binding to Peroxisome Proliferator-activated Receptor γ*

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Peroxisome proliferator-activated receptor γ (PPARγ) functions in various biological processes, including macrophage and adipocyte differentiation. Several natural lipid metabolites have been shown to activate PPARγ. Here, we report that some PPARγ ligands, including 15-deoxy-Δ12,14-prostaglandin J2, covalently bind to a cysteine residue in the PPARγ ligand binding pocket through a Michael addition reaction by an α,β-unsaturated ketone. Using rhodamine-maleimide as well as mass spectroscopy, we showed that the binding of these ligands is covalent and irreversible. Consistently, mutation at the cysteine residue abolished abilities of these ligands to activate PPARγ, but not of BRL49653, a non-covalent synthetic agonist, indicating that covalent binding of the α,β-unsaturated ketone in the natural ligands was required for their transcriptional activities. Screening of lipid metabolites containing the α,β-unsaturated ketone revealed that several other oxidized metabolites of hydroxyeicosatetraenoic acid, hydroxyeicosadienoic acid, and prostaglandins can also function as novel covalent ligands for PPARγ. We propose that PPARγ senses oxidation of fatty acids by recognizing such an α,β-unsaturated ketone as a common moiety.

Many structural studies of the PPARγ ligand-binding domain (LBD) with or without synthetic ligands (9–14) have contributed to better understanding of the binding selectivity and activation mechanism of the synthetic PPARγ ligands. In the PPARγ ligand binding pocket, a large hydrophobic region makes contact with ligands, and a hydrogen bond network between PPARγ and ligands stabilizes helix 12, which may promote binding of coactivator proteins (2). However, there is no structural report for the binding mode of the natural PPARγ ligands. In the case of PPARδ, its crystal structure complexed with a fatty acid has been reported (15). The ligand, eicosapentaenoic acid, was attached to its binding pocket in two distinct conformations in the crystal. Meanwhile, it has been reported that a natural PPARγ ligand, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), has the potential to bind covalently to some proteins, such as H-Ras (16), NF-κB (17, 18), IκB kinase (19), and AP-1 (20) via a cysteine residue. In addition, it is reported that the synthetic PPARγ ligands GW9662, T0070907, and L-764406 (21–23) have been shown to react with the cysteine residue of the PPARγ LBD. These lines of evidence led us to investigate the binding mode of 15d-PGJ2 to the PPARγ LBD.

While we built a model structure of 15d-PGJ2–PPARγ complex, we became aware of the possibility that 15d-PGJ2 covalently binds to the PPARγ LBD. Here, we have shown that an α,β-unsaturated ketone is a core moiety of endogenous PPARγ ligands. The ligands containing the α,β-unsaturated ketone can covalently bind to a cysteine residue in PPARγ LBD through a Michael addition, and the covalent binding is required for PPARγ activation by these ligands. According to this core moiety, we have identified oxidized eicosanoids, including 5-oxo-eicosatetraenoic acid (ETE), 15-oxoETE, 15-oxo-eicosadienoic acid (EDE), 15-keto-prostaglandin E1, 15-keto-prostaglandin F2α, 15-keto-prostaglandin F1α, and 15-keto-prostaglandin F2α, as novel endogenous PPARγ ligands. We analyzed the structure–activity relationship of these oxidized prostaglandins in PPARγ activation. We also discuss the biological connection between the ligand synthesis and PPARγ function.

EXPERIMENTAL PROCEDURES

Chemicals—PPARγ ligands and eicosanoids were purchased from Cayman Chemical Co. or Alexis Corporation (Lausen, Switzerland). Rhodamine-maleimide was purchased from Molecular Probes, Inc. Reagents for animal cell culture were obtained from Invitrogen. All other chemicals were purchased from Sigma or WAKO (Osaka, Japan). Plasmids—For bacterial expression of the PPARγ LBD, a PPARγ fragment containing amino acid residues 195–477 was amplified by PCR using oligonucleotides (5'-GA CAT ATG GCG GAG ATC TCC AGT-3') and 5'-GG CCG ATT GTA GCA GTC CTT G-3'; underlines indicate introduced NdeI and BamHI sites, respectively) and subcloned into

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† The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; LBD, ligand-binding domain; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; ETE, eicosatetraenoic acid; 15-keto-PGE1, 15-keto-prostaglandin E1; 15-keto-PGE2α, 15-keto-prostaglandin E2; HODE, hydroxyoctadecadienoic acid; ODE, octadecadienoic acid; ADFP, adipophilin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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Ndel/BamHI-digested pET28a (Invitrogen). After plasmid DNA was recovered from a colony, the sequence was confirmed. To obtain the C285A mutant, pCMX-GAL4-PPARγ1 was amplified by Pyrobest DNA polymerase (TAKARA Bio, Inc.) using oligonucleotides (5'-ATC TTT CAG GCC GCT CAG TTT CGC TCC and 5'-GGA GCG AAA CTG AGC GCC CTG AAA GAT; underlines indicate mutated codon). The template DNA was recovered by digestion with DpnI, and the amplified DNA was directly transformed into Escherichia coli DH5α. After plasmid DNA was recovered from a colony, the sequence was confirmed. The Y475F mutant was obtained by the same procedure using oligonucleotides (5'-CTG GAC ATC TTC AAG GAC TTG TAC and 5'-GTA CAA GTC CTT GAA GAT CTC CTG CAG; underlines indicate mutated codon).

Protein Purification—The human PPARγ LBD was expressed in BL21 (DE3) and purified by nickel affinity chromatography as described previously (14). After removal of a polyhistidine tag by thrombin digestion, the PPARγ LBD was further purified by gel filtration using Superdex200 (Amersham Biosciences). We omitted the reducing agents from all the buffers.

TOF Mass Spectrometry Analysis—After the PPARγ LBD protein was incubated with the ligands, the protein solution was mixed with 33% acetonitrile saturated with sinapinic acid at a 1:1 ratio. Then, 2 μl of the sample was spotted onto the grid and dried completely under vacuum. A MALDI-TOF mass spectrometry spectrum was obtained with a Voyager Elite (PerSeptive Biosystems, Framingham, MA).

Rhodamine-Maleimide Assay—The purified PPARγ LBD (0.1 μM) was mixed with various ligands at the indicated concentrations in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. The mixture was incubated at room temperature for 30 min. After SDS was added to the reaction mixture to a final concentration of 0.5%, rhodamine-maleimide and tris-(2-carboxyethyl)phosphine hydrochloride were added to the mixture to 0.1 and 1 mM final concentrations, respectively. After incubation at room temperature for 30 min, the samples were treated with SDS-PAGE sample buffer containing β-mercaptoethanol and were separated by SDS-PAGE. Fluorescent signals derived from rhodamine were visualized by a FM-BIO II (Hitachi).

Calculation of Binding Constant—We calculated the $K_{on}$ values of irreversible binding of ligands as follows.

$$[\text{NR} + \text{ligand}] \rightarrow [\text{NR-ligand}] \rightarrow [\text{NR-ligand}]_\text{total} = a, [\text{ligand}]_\text{total} = b, [\text{NR-ligand}] = x$$

Here, NR and NR-ligand indicate a nuclear receptor and a ligand-conjugated nuclear receptor, respectively. The reaction speed at certain time ($t$) is expressed as Equation 1.

$$dx/dt = K_{on}(a-x)(b-x) \quad (\text{Eq. 1})$$

If $a$ is not equal to $b$, Equation 1 can be converted to Equation 2.

$$K_{on} = \ln \left[ \frac{(b-a)(a-b)}{(a-x)(b-x)} \right] \quad (\text{Eq. 2})$$

If $a$ is equal to $b$, Equation 1 can be converted to Equation 3.

$$K_{on} = x/a(a-x) \quad (\text{Eq. 3})$$

We obtained the $K_{on}$ value after nonlinear least square fitting of the data using Equation 2 and Equation 3 by the Newton method.

RESULTS

Model Building of the 15d-PGJ2-PPARγ Complex—As the first step toward understanding the binding mechanism of the natural PPARγ ligands, we built a model structure of the 15d-PGJ2-PPARγ complex according to the superimposed structures of the PPARγ-LBD complexed with several synthetic ligands (Protein Data Bank accession codes 1KNU, 1FMC, 1IG5, 4PRG). In the superimposed structures, the polar part of the ligands tended to localize near helix 12 of PPARγ and the non-polar part near helix 3 (Fig. 1A). To fit 15d-PGJ2 to the binding site of BRL49653, we considered four possible orientations of 15d-PGJ2 in the PPARγ ligand binding pocket (Fig. 1B). In the models, the cyclopentene ring (Fig. 1A, $a$ and $b$) or the carboxyl group (Fig. 1B, $c$ and $d$) of 15d-PGJ2 could be assigned as a polar moiety. In the latter case, we noticed that a cysteine residue (Cys-285) of PPARγ located at close proximity to the electrophilic carbon(s) of an α,β-unsaturated ketone in 15d-PGJ2 (Fig. 1B, $c$ and $d$). The PPARγ ligand binding pocket was large enough to accommodate 15d-PGJ2 (Fig. 1C).

Covalent Binding of 15d-PGJ2 to PPARγ-LBD—The model

$K_{off}$ was determined by applying a harmonic potential to the minimized complex and was refined by applying a harmonic potential to the minimized complex and the time step of the molecular dynamics simulation was 1 fs. Finally, the closest structure to the average of the sampled ones was chosen as the complex model.

Reverse Transcription-PCR—THP-1 cells were maintained in RPMI1640 with 10% fetal calf serum at 37 °C. 2 × 10^6 cells were seeded in 6-cm dishes and treated with ligands for 10 h. Cells were collected by centrifugation, and total RNA was purified from cells by Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Quality of isolated RNA was determined by electrophoresis. Single-stranded cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (Promega) with an oligo(dT)15 primer. PCR primers were designed according to the uniSTS data base; detail is available upon request. PCR products were separated by PAGE and stained with SYBR Gold (Molecular Probes). Images of the gels were obtained by the FM-BIO II (Hitachi).
orientations were possible. When the carboxyl group of 15d-PGJ2 was bound to PPAR, 15d-PGJ2 showed the same molecular weight as that calculated from the amino acid composition (MW = 34, 534) (Fig. 2A). In contrast, in the presence of 15d-PGJ2, the PPARγ LBD exhibited a higher molecular weight, which was close to the molecular weight of the PPARγ LBD plus 15d-PGJ2 (calculated MW = 34, 850) (Fig. 2A). This result indicated that 15d-PGJ2 was bound to PPARγ even after the PPARγ was denatured and suggested that 15d-PGJ2 covalently bound to PPARγ.

To confirm that 15d-PGJ2 modifies the cysteine residue in the PPARγ LBD, we took advantage of rhodamine-maleimide to label free cysteine residues in proteins. Fortunately, there is only one cysteine residue in the PPARγ LBD. In these experiments, we denatured the protein by adding 0.5% SDS after ligand-protein complex formation, to facilitate rhodamine-maleimide access to the cysteine residue in the PPARγ LBD (Fig. 2B). The amount of the rhodamine-maleimide-labeled PPARγ LBD decreased by incubating with 15d-PGJ2 in a concentration-dependent manner (Fig. 2C), indicating that 15d-PGJ2 covalently bound to the cysteine residue of PPARγ. Based on the irreversible binding reaction model, we calculated $k_{on}$ value of 15d-PGJ2 as 47,165.3 M$^{-1}$ min$^{-1}$. We next added the known PPARγ ligands to the reaction mixture simultaneously with 15d-PGJ2 (Fig. 2D). Excess amount of the competitors can occupy the ligand binding pocket, and so 15d-PGJ2 cannot reach to the cysteine residue. Then addition of 0.5% SDS removes the non-covalently bound competitors, with the result that the cysteine residue in the denatured PPARγ LBD can be labeled by rhodamine-maleimide. This result indicates that the non-covalent ligands and 15d-PGJ2 share their binding site. Using the $k_{on}$ value of 15d-PGJ2, the $K_v$ values of BRL49653, MCC-555, and 13(S)-HODE were calculated to be 129 nm, 577 nm, and 199 μM, respectively.

Rhodamine-maleimide assay revealed that 15d-PGJ2 specifically modified the cysteine residue of the PPARγ LBD. Concomitantly, 15d-PGJ2 itself must be modified by binding to the PPARγ LBD. Then, we measured the UV spectrum of 15d-PGJ2 in the absence or presence of the PPARγ LBD (Fig. 3A). In the absence of the PPARγ LBD, 15d-PGJ2 showed peak absorbance at 320 nm (Fig. 3A, a, thin line). After incubating with the PPARγ LBD, the resultant peak absorbance of 15d-PGJ2 shifted to 295 nm (Fig. 3A, a, thick line). This blue shift of the peak absorbance was also observed even when 15d-PGJ2 was incubated in a free cysteine solution (Fig. 3A, b). However, the resultant peak absorbance was 310 nm, which was different from the PPARγ-induced peak absorbance but rather similar to that of a 15d-PGJ2 analogue, CAY10410, which lacks the cyclopentenone ring (Fig. 3A, c, thick line). The results suggested that the cysteine residue of the PPARγ LBD targeted an electrophilic carbon within 15d-PGJ2 that is different from that of free cysteine targets. Considering the similarity of the peak absorbance, the free cysteine may target C9 of 15d-PGJ2 (Fig. 3B), whereas the cysteine residue in the PPARγ LBD may target C13 of 15d-PGJ2 (Fig. 3B). The amplitude of the UV spectrum of CAY10410 was reduced by binding to PPARγ (Fig. 3A, c), whose peak absorbance was probably shifted to a shorter wavelength than that we had measured. The result indicated that the cysteine residue in the PPARγ LBD may also target C13 of CAY10410 (Fig. 3B) as well as 15d-PGJ2.

Covalent Binding of Natural Ligands to PPARγ—PPARγ is reportedly activated by several natural and synthetic ligands (5–8). For example, Nagy et al. (7) showed that oxidized fatty acids, e.g. 9- and 13-hydroxyoctadecadienoic acid (HODE) and

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**FIG. 1. Orientation of ligands in PPARγ LBD.** A, structure of the PPARγ LBD with BRL49653 (Protein Data Bank accession code 1FM6, chain D). Overall structure of the PPARγ LBD with BRL49653 showed that the ligand fit its polar part (red) near helix 12 and its non-polar part (gray) near helix 3 (left panel). Close-up view of the ligand binding cavity with BRL49653 (from 1FM6) and YPA (from 1KNU) showed the similarity of the binding mode (right panel). The ligand binding cavity is shown as mesh generated from the data of 1FM6. B, possible orientation of 15d-PGJ2 in the PPARγ ligand binding pocket. Either the cyclopentenone ring (a and b) or the carboxyl group (c and d) of 15d-PGJ2 was positioned at the polar region of the PPARγ ligand binding pocket shown in panel A, right. In each case, another two alternative orientations were possible. When the carboxyl group of 15d-PGJ2 was positioned at the polar region of the PPARγ ligand binding pocket (c and d), β-carbon (blue) of α,β-unsaturated ketone was near the cysteine residue (Cys-285, shown in green) of PPARγ. C, molecular surface of the PPARγ LBD and the ligand binding pocket with 15d-PGJ2 in the same orientation as in panel A. A cutaway rendition of the surface is shown on the left. Close-up view of the ligand binding cavity with 15d-PGJ2 is shown on the right. The position of Cys-285 is indicated as a green tube model.

building described above raised the possibility that 15d-PGJ2 is bound to PPARγ covalently. To test this possibility, we performed a mass spectroscopy experiment. Generally, macromolecules such as proteins are denatured during MALDI-TOF mass spectroscopy analyses, and so small molecules, such as ligands, are detached from the proteins. Actually, with or without the synthetic ligand BRL49653, the PPARγ LBD showed the same molecular weight as that calculated from the amino acid composition (MW = 34, 534) (Fig. 2A). In contrast, in the presence of 15d-PGJ2, the PPARγ LBD exhibited a higher molecular weight, which was close to the molecular weight of the PPARγ LBD plus 15d-PGJ2 (calculated MW = 34, 850) (Fig. 2A). This result indicated that 15d-PGJ2 was bound to PPARγ even after the PPARγ was denatured and suggested that 15d-PGJ2 covalently bound to PPARγ.

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Covalent Binding of Natural Ligands to PPARγ—PPARγ is reportedly activated by several natural and synthetic ligands (5–8). For example, Nagy et al. (7) showed that oxidized fatty acids, e.g. 9- and 13-hydroxyoctadecadienoic acid (HODE) and
9- and 13-oxo-octadecadienoic acid (oxoODE) derived from oxidized low density lipoprotein (oxLDL), activated PPARγ, and mediated the cellular effect of oxLDL in macrophages. Then we assessed whether such known PPARγ ligands possess the ability to bind covalently to PPARγ. We showed that the molecules (e.g., 15d-PGJ2, CAY10410, 9-oxoODE, 13-oxoODE, and T0070907) covalently bound to the cysteine residue in the PPARγ LBD in the rhodamine-maleimide assay (Fig. 4A). CAY10410 also showed weaker blocking activity than 15d-PGJ2. The natural ligands and CAY10410, which covalently bound to the PPARγ LBD, share the common chemical structure of an α,β-unsaturated ketone (Fig. 4B, boxes). Although 9(S)-HODE and 13(S)-HODE have structures quite similar to those of 9-oxoODE and 13-oxoODE, respectively, they lack an α,β-unsaturated ketone and did not covalently bind to the PPARγ LBD, indicating that the α,β-unsaturated ketone was a crucial structure for the covalent binding. It is reported that synthetic ligands GW9662, T0070907, and L-764406 covalently bind to the cysteine residue in the PPARγ LBD (21–23). In this assay, we were able to confirm the cysteine modification by T0070907 (Fig. 4A), which has been shown to react with the cysteine residue of the PPARγ LBD through a nucleophilic aromatic substitution of the chlorine in the structure (21). Because the cysteine residue of the PPARγ LBD targets the β-carbon in the α,β-unsaturated ketone, as shown in Fig. 3, the different mode of action was evident between the 15d-PGJ2-represented natural ligands and these synthetic ligands.

Although the binding mode by 15d-PGJ2, 9-oxoODE and 13-oxoODE was achieved by the same chemical reaction, they showed different IC50 to block the rhodamine-maleimide to label the free cysteine residue in the PPARγ LBD (Fig. 4C). We calculated the k_on values of 15d-PGJ2, 13-oxoODE, and 9-oxoODE as 47,165.3, 8,073.7, and 1,535.3 M−1 min−1, respectively.

![Fig. 2. Covalent binding of 15d-PGJ2 to the PPARγ LBD. A, mass spectrometry analysis of PPARγ LBD with or without ligands. After incubating the PPARγ LBD with either Me2SO, BRL49653, or 15d-PGJ2, the molecular weight of the ligand-PPARγ complex was investigated by MALDI-TOF mass spectrometry. B, schematic representation of the method used to analyze the covalent binding of ligands to the cysteine residue in the PPARγ LBD. After incubating the PPARγ LBD with ligands, proteins were denatured by adding SDS. Free cysteine residues were then detected by rhodamine-maleimide. C, concentration-dependent modification of the cysteine residue in the PPARγ LBD by 15d-PGJ2. D, concentration-dependent competition of 15d-PGJ2 binding to PPARγ with other ligands. Various concentrations of competitor ligands were simultaneously added to the PPARγ LBD with 5 µM 15d-PGJ2, and the cysteine modification by 15d-PGJ2 was investigated by rhodamine-maleimide.]

![Fig. 3. Change of UV spectrum of 15d-PGJ2 by binding to PPARγ. A, UV spectra of 15d-PGJ2 (10 µM) before and after incubating with 58 µM PPARγ indicated the chemical modification in 15d-PGJ2 (a). Blue shift of the peak wavelength of 15d-PGJ2 (10 µM) was also induced by incubating with 2 mM cysteine (b). The resultant peak (thick spectrum) in cysteine-reacted 15d-PGJ2 and CAY10410 are indicated as vertical dashed lines. B, explanation of UV spectral rearrangement by the cysteine residue in PPARγ and the free cysteine. The cysteine residue of the PPARγ LBD reacts with C13 in 15d-PGJ2 and CAY10410, whereas a free cysteine (Cys) reacts with C9 in 15d-PGJ2. C, concentration-dependent modification of the cysteine residue in the PPARγ LBD by 15d-PGJ2. D, concentration-dependent competition of 15d-PGJ2 binding to PPARγ with other ligands. Various concentrations of competitor ligands were simultaneously added to the PPARγ LBD with 5 µM 15d-PGJ2, and the cysteine modification by 15d-PGJ2 was investigated by rhodamine-maleimide.]

Identification of Novel PPARγ Ligands
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BRL49653 was the most potent activator of PPARγ among the ligands we tested (Fig. 5A, left panel). CAY10410 showed transcriptional activity similar to that of 15d-PGJ2 (Fig. 5A, middle panel). On the other hand, when we compared the activities of structurally related molecules, e.g. 13(S)-HODE versus 13-oxoODE and 9(S)-HODE versus 9-oxoODE (Fig. 5A, right panel), the ligands that covalently bind to PPARγ always showed significantly higher activities than the analogous ligands lacking an α,β-unsaturated ketone. This result is consistent with a previous report that oxo metabolites showed greater activities and affinities than HODE did (7). These results suggested that the covalent binding to the receptor helped the ligands to activate PPARγ.

We made three PPARγ mutants with the cysteine exchanged to alanine (C285A), valine (C285V), or serine (C285S). All of the mutants were still activated by BRL49653 at levels equivalent to that of the wild type PPARγ, whereas they totally lost responsiveness to 15d-PGJ2 and 13-oxoODE (Fig. 5B). Thus, these putative natural ligands indeed functioned as ligands, via novel covalent binding to the cysteine residue in the ligand binding pocket, to activate transcription.

Next, we analyzed the effect of an antagonist on activation by both synthetic and natural ligands for PPARγ. Two hours after agonist addition, the PPARγ-specific antagonist GW9662 was applied to the cells expressing GAL4-PPARγ protein and a luciferase gene as a reporter (Fig. 5C). Both 15d-PGJ2- and BRL49653-dependent activities of PPARγ decreased with the addition of GW9662. However, inhibition of 15d-PGJ2-dependent activity of PPARγ by GW9662 was more moderate than that of BRL49653-dependent activity. Rapid inhibition of BRL49653-dependent activity might be due to the displacement of the ligand by the antagonist. On the other hand, resistance of 15d-PGJ2 against the antagonist indicated that 15d-PGJ2 binding to PPARγ in the cells was irreversible. We considered that slower and more moderate inhibition of 15d-PGJ2-dependent activity was not achieved by the displacement of the ligand but by the competitive binding between 15d-PGJ2 and GW9662 to newly synthesized PPARγ.

Screening of Novel PPARγ Ligands—Based on the common structure, α,β-unsaturated ketone in naturally occurring ligands, we looked for other lipid metabolites containing an α,β-unsaturated ketone. Screening the chemical library databases, namiki.db, sumisho_specs.db, and Acd2D_reg.db, we obtained six candidates for PPARγ ligands. Two of them were 9- and 13-oxoODE. Three of them, 5-oxoETE, 15-oxoETE, and 15-oxo-eicosadaenoic acid, were identified as PPARγ ligands (Fig. 6A). Luciferase assay clearly proved that these oxidized eicosanoids, but not their precursors, activated PPARγ (Fig. 6A). Furthermore, these oxidized eicosanoids covalently bound to the cysteine residue in the PPARγ LBD as determined by rhodamine-maleimide assay (Fig. 6B).

In addition to a lipoxygenase-dependent pathway where the above metabolites are involved, we investigated the prostaglandin metabolites containing an α,β-unsaturated ketone in a cyclooxygenase-dependent pathway (Fig. 7, A and B). Using cell-based luciferase assay, we proved that oxidized prostaglandin metabolites 15-keto-PGE1, 15-keto-prostaglandin E2, 15-keto-PGF1, and 15-keto-prostaglandin F2α, activated PPARγ (Fig. 7C, lanes 4, 5, 7, and 8, respectively). Structural relatives, PGE2 and PGF2α, did not activate PPARγ (Fig. 7C, lanes 3 and 6), suggesting that the α,β-unsaturated ketone in the ligands was required for PPARγ activation through the Michael addition to the cysteine residue in the PPARγ. The activation by these oxidized prostaglandins was totally abolished by simultaneous application of PPARγ antagonist T0070907 (Fig. 7D).

Concentration Dependence of PPARγ Activation—Concentration dependence of PPARγ activation by oxidized prostaglandins revealed that 15-keto-PGF1 and 15-keto-PGE2 showed higher EC50 and maximal activation than 15d-PGJ2 did (Fig. 8A). The order of EC50 of oxidized prostaglandins was 15d-PGJ2, 15-keto-PGE2, and 15-keto-PGF1. BRL49653 showed both low EC50 and high maximal activation. This phenomenon indicated that EC50 and maximal activation were independently regulated by each ligand. These oxidized prostaglandins also blocked the maleimide labeling of the PPARγ LBD in a concentration-dependent manner (Fig. 8B), indicating that

Fig. 4. Naturally occurring ligands covalently bind to the PPARγ LBD. A, modification of the cysteine residue in the PPARγ LBD by ligand binding. B, natural ligands that can bind covalently to PPARγ have a common chemical structure, an α,β-unsaturated ketone (indicated as a box). An electrophilic carbon in the α,β-unsaturated ketone is indicated by an arrow. C, dose-dependent modification of PPARγ by ligands. The PPARγ LBD was incubated with various concentrations of 15d-PGJ2, 13-oxoODE or 9-oxoODE, and the covalent binding was detected by using rhodamine-maleimide. Quantifications of the band intensities are plotted.
they bound to the cysteine residue in the PPARγ covalently. The order of the affinities was 15d-PGJ2, 15-keto-PGE1, and 15-keto-PGF1, which was correlated with that of EC50.

Structural Models of the Natural Ligand-PPARγ Complexes—To investigate whether covalent binding of 15d-PGJ2 to the PPARγ LBD is structurally achievable, we built a structural model of the 15d-PGJ2-PPARγ complex, based upon the crystal structure of the PPARγ-retinoid X receptor α heterodimer complexed with BRL49653 and a coactivator peptide (Protein Data Bank accession code 1FM6) (12). The obtained structure was superimposed onto the PPARγ-BRL49653 complex, suggesting some similarities and differences between the BRL49653- and 15d-PGJ2-PPARγ complexes (Fig. 8C). The hydrophobic tail of 15d-PGJ2, which BRL49653 lacked, extended toward the tip of helix 11 (L452) and was stabilized through hydrophobic interactions. This binding mode was different from that observed in the eicosapentaenoic acid-PPARγ complex, where the hydrophobic tail of eicosapentaenoic acid extended toward helix 2 or helix 5, rather than helix 11 (15).

To analyze what causes the difference in the maximal activation, we also built model structures of the PPARγ LBD covalently bound by 15-keto-PGE1 and 15-keto-PGF1. Because these oxidized prostaglandins have very similar structures but very different chemical and physical natures, we took advantage of molecular dynamics simulation to get the model structures instead of homology modeling. Carboxyl groups of these ligands made a hydrogen bond with Tyr-473 (Fig. 8D), which resides in helix 12 and is a key ligand-recognizing residue (12–14). Hydrophobic clusters assisting the ligand binding were also conserved (Fig. 8C and data not shown). In contrast, we observed two differences in hydrogen bonds between the main-chain carbonyl group of Leu-340 and a hydroxyl group of the ligands (Fig. 8D, a) and between the sulfur atom of the cysteine residue and another hydroxyl group (Fig. 8D, b). The former was observed only in the 15-keto-PGF1-PPARγ complex, and the latter was observed both in the 15-keto-PGF1- and 15-keto-PGE1-PPARγ complexes, but not in the 15d-PGJ2-PPARγ complex. These structural differences may produce more stable binding of 15-keto-PGF1 or 15-keto-PGE1 than 15d-PGJ2 and exert the receptor to have higher maximal activity, namely, efficacy.

We observed that all three ligands required the cysteine residue (Fig. 8E, gray bars). According to the structural model described above, we made an additional PPARγ mutant (Y473F) that showed no response to any ligands (Fig. 8E), supporting our model structure.
Regulation of PPARβ/δ Target Genes by Oxidized Prostaglandins—Finally, we determined whether oxidized prostaglandins activate PPARβ/δ target genes in THP-1 cells, which endogenously express PPARβ/δ protein. Adipophilin (ADFP) was upregulated by PPARβ/δ ligands (Fig. 9A, left panel), and the upregulation was blocked by simultaneous application of PPARβ/δ antagonist T0070907 (Fig. 9A, right panel), suggesting that ADFP expression was regulated through PPARβ/δ (28, 29). Among several PPARβ/δ-regulated genes that we have identified by microarray,2 we successfully observed that oxidized prostaglandins 15-ketoPGE₁ and 15-ketoPGF₁ as well as 15d-PGJ₂ and 13-oxoODE activated PPARβ/δ-regulated genes, ADFP, interleukin-1 receptor type I, cyclin-dependent kinase inhibitor 1A, and aldehyde oxidase 1 (Fig. 9B). These results suggested that oxidized prostaglandins acted as PPARβ/δ ligands and regulated the expression of PPARβ/δ target genes in vivo.

**DISCUSSION**

By this study, the currently proposed natural PPARγ ligands were shown for the first time to share a common structure, the α,β-unsaturated ketone, that enables them to bind to PPARγ covalently and to exhibit their activities. Although we do not neces-

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*Fig. 7. Identification of oxidized prostaglandin metabolites as PPARγ ligands. A, synthetic and degradation pathways of lipoygenase- and cyclooxygenase-dependent eicosanoids. Precursors are boxed. Oxidized metabolites are shown in bold. Oxidized prostaglandins investigated in this experiment are underlined. LA, linoleic acid; EDA, eicosadienoic acid; AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid. B, chemical structures of oxidized prostaglandins and their precursors used in this experiment. α,β-Unsaturated ketones are indicated by boxes. C, activities of oxidized prostaglandins in PPARγ-dependent transcription. Indicated ligands were added to the cells expressing GAL4-PPARγ at a concentration of 100 μM. 10 μM BRL49653 were also shown as a positive control (lane 2). Data are represented as mean ± S.D. D, effect of the PPARγ antagonist on oxidized prostaglandin-dependent activation of PPARγ. 15d-PGJ₁ (10 μM), 15-keto-PGE₁ (10 μM), and 15-keto-PGF₁ (50 μM) were added to the cells with or without 1 μM T0070907. Data are represented as mean ± S.D.

1. T. Shiraki and H. Jingami, unpublished data.
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Fig. 8. Structure-activity relationship of PPARγ ligands. A, concentration dependence of oxidized prostaglandin-dependent activation of PPARγ. B, covalent binding of oxidized prostaglandins to the cysteine residue in the PPARγ LBD. Concentrations of each ligand were 0.5, 1, 5, 10, 50, and 100 μM from left to right. No ligand is shown as minus. Stars represent reactive carbons. C, superimposition of the model structure of the 15d-PGJ2-PPARγ complex to the crystal structure of the BRL49653-PPARγ complex. Residues localized within 5 Å of each ligand are shown. The BRL49653- and 15d-PGJ2-PPARγ complexes are shown in pink and blue, respectively. Residues bound to both ligands are colored black, and those bound to either BRL49653 (green) or 15d-PGJ2 (yellow) are shown in red and blue, respectively. D, schematic representation of interactions between 15-ketoPGF1 and PPARγ. Hydrogen bonds are indicated as blue lines. Atoms of oxidized prostaglandins at the positions indicated as a, b, and c are summarized (inset). E, requirement of the cysteine residue in oxidized prostaglandin-dependent activation of PPARγ. 15d-PGJ2 (10 μM), 15-keto-PGE1 (10 μM), and 15-keto-PGF1 (50 μM) were added to the cells expressing either wild type (WT) or C285A or Y473F mutant. Abbreviations are BRL, BRL49653; J2, 15d-PGJ2; F1, 15-keto-PGE1; F2, 15-keto-PGF1. Data are represented as mean ± S.D.

Fig. 9. Oxidized prostaglandin-mediated transcriptional regulation through PPARγ in THP-1 cells. A, reverse transcription-PCR of AD FP gene. AD FP gene was up-regulated by 10 μM PPARγ ligands BRL49653, 15d-PGJ2, and 13-oxoODE (left). The up-regulation was blocked by simultaneous application of PPARγ antagonist T0070907 (right). B, reverse transcription-PCR of PPARγ target genes. Newly identified ligands 15-ketoPGE1 (from left to right, 0.3, 3, and 10 μM) and 15-ketoPGF1 (1.66, 16.6, and 50 μM), as well as 15d-PGJ2 (0.3, 3, and 10 μM) and 13-oxoODE (0.3, 3, and 10 μM), up-regulated PPARγ target genes in a dose-dependent manner. Cdk-like kinase (CLK1) was used as negative control. IL1R1, interleukin-1 receptor type I; AOX1, aldehyde oxidase 1.

First, one can easily imagine that the covalent binding of the ligands to the receptor not only acts as a rigid switch but also enables the receptors to accumulate as active forms, allowing such ligands with low concentrations to function in vivo. Unknown factor(s) may facilitate this covalent binding.

Second, our studies provide clear evidence that the structural moieties within a single molecule are used differently in PPARγ-dependent and -independent functions. In the case of 15d-PGJ2, the electrophilic carbon (C9) within a cyclopentenone ring has been considered to react with the cysteine residue in NF-κB and other proteins (17, 30). On the other hand, we consider that the carbon at position 13 reacts with the sulfur atom of the cysteine residue in PPARγ, according to results from UV absorbance measurements and the activity of CAY10410 lacking the cyclopentenone ring (Figs. 2A, 3A, and 5A). Furthermore, the carboxyl group of 15d-PGJ2 is required for the formation of a hydrogen bond with Tyr-473 in helix 12 of PPARγ. The 15d-PGJ2 biotinylated at the carboxyl group reportedly failed to pull down PPARγ from cells, whereas it still bound to AP-1 and other proteins (20, 30). Thus, the covalent binding of 15d-PGJ2 to the PPARγ LBD is different from that in PPARγ-independent actions.

Third, our finding that several oxidized eicosanoids act as PPARγ ligands fits well with the expected PPARγ functions: PPARγ may function under oxidative conditions and/or inflammation. For example, glucose consumption is related to mitochondrial respiration, which generates free radical. In atherosclerosis, macrophages play important roles in which oxidized low density lipoprotein accumulates as a major source of oxidized lipid (7). Under inflammatory conditions, macrophages express not only cyclooxygenase-2 and 12/15-lipoxygenase, to produce prostaglandins and leukotrienes, respectively (31, 32), but also nitric-oxide synthase to generate nitric oxide, another potent oxidative agent (33). In addition, expressions of prostaglandin transporter and 15-hydroxyprostaglandin dehydrogenase, both of which are involved in prostaglandin metabolism to form 15-keto-prostaglandins (34, 35), were modulated during lipopolysaccharide-induced fever (36). Recently, it has been shown that oxidative stress stimulates inflammatory cells to produce 5-oxoETE from 5(S)-hydroxy-6,8,11,14-eicosatetra-
Further studies will be needed to determine whether they function as an inflammation modulator. They may sense such oxidized fatty acids as signaling molecules and, under certain conditions, such as inflammation. Thus, PPAR-oxidized eicosanoid metabolites is physiologically regulated un-

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