Peroxisome Proliferator-activated Receptor α Interacts with High Affinity and Is Conformationally Responsive to Endogenous Ligands*

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Although the peroxisome proliferator-activated receptor (PPARα) binds and is activated by a variety of synthetic xenobiotics, the identity of the high affinity endogenous ligand(s) is incompletely resolved. Likewise, it is not known how putative endogenous ligands alter PPARα conformation in order to affect transcriptional regulation. Direct fluorescence binding and fluorescence displacement assays showed for the first time that PPARα exhibits high affinity (1–14 nM $K_d$) values for unsaturated long chain fatty acyl-CoAs as well as unsaturated long chain fatty acids commonly found in mammalian cells. Fluorescence resonance energy transfer between PPARα aromatic amino acids and bound corresponding naturally occurring fluorescent ligands (i.e. cis-parinaroyl-CoA, trans-parinaric acid) yielded intermolecular distances of 25–29 Å, confirming close molecular interaction. Interestingly, although PPARα also exhibited high affinity for saturated long chain fatty acyl-CoAs, regardless of chain length (1–13 nM $K_d$ values), saturated long chain fatty acids were not significantly bound. In contrast to the similar affinities of PPARα for fatty acyl-CoAs and unsaturated fatty acids, CoA thioesters of peroxisome proliferator drugs were bound with 5–6-fold higher affinities than their free acid forms. Circular dichroism demonstrated that high affinity ligands (long chain fatty acyl-CoAs, unsaturated fatty acids), but not weak affinity ligands (saturated fatty acids), elicited conformational changes in PPARα structure, a hallmark of ligand-activated nuclear receptors. Finally, these ligand specificities and induced conformational changes correlated functionally with co-activator binding. In summary, since nuclear concentrations of these ligands are in the nanomolar range, long chain fatty acyl-CoAs and unsaturated fatty acids may both represent endogenous PPARα ligands. Furthermore, the finding that saturated fatty acyl-CoAs, rather than saturated fatty acids, are high affinity PPARα ligands provides a mechanism accounting for saturated fatty acid transactivation in cell-based assays.

Peroxisome proliferator-activated receptors (PPARs)1 are crucial nuclear receptors controlling transcription of a variety of genes involved in fatty acid oxidation and cell differentiation (1). Abnormal PPAR activation contributes to lipotoxicity associated with obesity, insulin resistance, type 2 diabetes, and hyperlipidemia (1, 2). PPARs have been identified in several species, with at least three isotypes recognized (α, β/δ, and γ; NR1C1, NR1C2, and NR1C3, respectively). These isotypes are differentially expressed in select tissues, with expression levels depending on cellular processes (reviewed in Ref. 3). The primary amino acid sequence of PPARα is similarly organized as that of other nuclear receptors, with an N-terminal A/B domain containing a ligand-independent transactivation function, the DNA-binding domain (C), a hinge region (D), the ligand-binding domain (LBD) containing a ligand-dependent transactivation function (E), and a C-terminal F domain (4).

Because of their multiple roles in regulating fatty acid metabolism and cell differentiation as well as disease (diabetes, obesity, cancer), much attention has focused on the specificities of the PPAR family for xenobiotics/therapeutic agents that bind and regulate the transcriptional activity of PPARα (5, 6). Although once believed to be orphan receptors, PPARs are now recognized to be ligand-activated members of the steroid/thyroid nuclear hormone receptor superfamily. Each isotype is encoded by a different gene and exhibits unique binding specificities for a broad variety of xenobiotics (reviewed in Refs. 1 and 3). PPARα is now recognized to promiscuously bind with high affinity xenobiotic substances such as hypolipidemic agents, plasticizers, herbicides, and dietary factors (reviewed in Refs. 7–10). The acceptance of such a large variety of structurally diverse, xenobiotic ligands is believed to be due to the large pocket comprising the binding site of the LBD (11). Although the above studies demonstrate high affinity binding of multiple xenobiotics, investigations attempting to identify the endogenous high affinity ligand(s) of PPARα are less clear.

First, although long chain fatty acids (LCFAs) are thought to

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPARαΔAB, peroxisome proliferator-activated receptor α composed of amino acids 101–468 (i.e. missing only the amino-terminal A/B domain); LBD, ligand binding domain; LCFA, long chain fatty acid; LCFA-CoA, long chain fatty acyl-CoA; FRET, fluorescence resonance energy transfer; L-FABP, liver fatty acid-binding protein; SCP-2, sterol carrier protein-2; cis-parinaric acid, (9Z,11E,13E,15Z)-octadecatetraenoic acid; trans-parinaric acid, (9E,11E,13E,15E)-octadecatetraenoic acid; cis-parinaroyl-CoA, (9Z,11E,13E,15Z)-octadecatetraenoic acid; 7-nitrobenz-2-oxa-1,3-diazol-4-y1)-octadecan-1-yl)propionic acid; Beazafibrate, 2-(4-chlorobenzamidoethyl)phenoxy)-2-methylpropionic acid; Medica 16, β,β′-tetrastearilhexadecane dicarboxylic acid; SRC-1, steroid receptor coactivator-1.
be putative endogenous PPARα ligands, most radioligand binding studies suggest that PPARα exhibits only weak affinities for LCFAs. Such radioligand binding assays demonstrate that PPARα binds unsaturated LCFAs (arachidonic, petroselenic, linolenic, linoleic, and oleic acids) with only weak affinities ($K_d$ values in the micromolar range) and saturated LCFA (lauric and palmitic acids) are bound even less well (9, 12–14). These radioligand-based affinities for LCFAs are several orders of magnitude weaker than PPARα exhibits for synthetic xenobiotics. Further, recent confocal fluorescence imaging of nonmetabolizable fluorescent LCFA in living cells shows that nucleoplasmic LCFA concentrations are in the range of 39–68 nM (15, 16). Thus, based on radioligand binding assays, it would appear unlikely that long chain fatty acids are physiologically significant endogenous ligands for PPARα. However, it is known that such radioligand binding assays underestimate the affinities of other fatty acid binding proteins (liver fatty acid binding protein (L-FABP); sterol carrier protein-2 (SCP-2)) by several orders of magnitude (reviewed in Refs. 17–19). Interestingly, more recent data with a direct fluorescent ligand binding assay determined that mouse PPARα binds a naturally occurring fluorescent LCFA, trans-parinaric acid, with high affinity as shown by a $K_d$ of 30 nM (20, 21). Furthermore, displacement of this fluorescent ligand by nonfluorescent LCFA yielded nanomolar $K_i$ values for a variety of naturally occurring, nonfluorescent unsaturated LCFA (arachidonic, linolenic, linoleic, and oleic acids) but not saturated LCFA (stearic and palmitic acids) (20). Whether these high affinities reflect a unique property of the fluorescent ligand, trans-parinaric acid, or whether radioligand binding assays underestimate PPARα affinities remains to be determined. Resolving these issues is important, because the concentration of LCFA in the nucleoplasm is in the nanomolar rather than micromolar range (15).

Second, it is unclear whether only unsaturated but not saturated LCFA represent physiologically significant endogenous PPARα ligands. In transactivation assays wherein cultured cells are supplemented with exogenous LCFA, both saturated (palmitic) and unsaturated (arachidonic, linoleic, linolenic, and oleic acids) fatty acids enhance PPARα transactivation nearly equally well (5, 9, 12, 13, 22–24). In dietary studies wherein rats are fed high fat diets, PPARα-activated gene expression is increased, regardless of whether the dietary lipid composition is mostly polyunsaturated, monounsaturated, or saturated LCFA (25). If LCFA are the exclusive endogenous ligand for PPARα, it is difficult to reconcile these PPARα transactivation and activation data with the ligand binding data indicating that PPARα binds well only to the unsaturated, but not saturated, LCFA (9, 12–14, 20). Taken together, these studies would suggest that, especially in the case of saturated LCFA, a metabolite rather than the saturated LCFA itself might be the active endogenous PPARα ligand (24).

Third, increasing data indicate that a LCFA metabolite such as LCFA-CoA may represent active endogenous high affinity PPARα ligand(s). Early radioligand competition studies show that both unsaturated and saturated LCFA-CoA are weak PPARα ligands. Displacement of a bound radiolabeled substrate by LCFA-CoA (palmitoyl-CoA, oleoyl-CoA, and linoleoyl-CoA) yields micromolar $K_i$ values comparable with those obtained with the corresponding LCFA in the same radioligand binding assay (i.e. $K_i < 5 \mu M$) (26). Since nucleoplasmic levels of LCFA-CoA are very low, in the 3 nM range (16), based on radioligand binding data it would appear unlikely that LCFA-CoA represent physiologically important endogenous ligands for PPARα. In contrast, other data suggest that LCFA-CoA compete with LCFA for binding to PPARα and antagonize PPARα transcriptional activation (26, 27). Unfortunately, the physiological significance of the latter findings is unclear, since the radioligand-binding assay used yielded very weak PPARα affinities for LCFA-CoA (i.e. micromolar $K_i$ values). However, it is known that radioligand binding assays underestimate the affinities of other LCFA-CoA-binding proteins (L-FABP, SCP-2, and acyl-CoA-binding protein) for LCFA-CoAs by several orders of magnitude (reviewed in Refs. 18 and 28–30). Thus, the possibility that the radioligand binding assay significantly underestimates the affinity of PPARα for LCFA-CoAs must be considered.

Fourth, although it is accepted that ligand-induced conformational changes are associated with the transcriptional activation of ligand-activated nuclear receptors (reviewed in Refs. 1 and 31–34), how putative endogenous PPARα ligands (i.e. LCFA or LCFA-CoA) alter the structure of PPARα to affect co-factor recruitment is unknown. Whereas NMR and x-ray crystallography of PPARα demonstrate substantial conformational changes upon binding of xenobiotics (i.e. the dihydrocinnamamate derivative AZ242, 1-tyrosine analogues) (35, 36), there are no direct studies demonstrating that LCFA binding alters PPARα conformation. However, other PPAR isoforms demonstrate such ligand-induced alterations in structure as evidenced by x-ray crystallography of PPARγ and PPARδ, where LCFA (i.e. eicosapentaenoic acid) or xenobiotic (e.g. glutazones) binding alters the structure/conformation of these isoforms (reviewed in Ref. 32). Finally, recent experiments demonstrate that binding of a nonhydrolyzable LCFA-CoA analogue changes PPARα sensitivity to protease digestion and alters its ability to bind to co-activators, suggesting a conformational change (27). Unfortunately, these experiments looked at pure recombinant proteins in the presence of high levels of free ligands, so the effect of ligands on native proteins in the presence of other factors is still unknown. Such ligand-induced conformational changes are a hallmark of ligand-activated nuclear receptors (reviewed in Refs. 1 and 31–34). Thus, if LCFA and/or LCFA-CoAs represent functional ligands for PPARα, it is essential to demonstrate that these endogenous ligands physically alter the structure/conformation of the PPARα isoform.

The objective of the present study was to begin to resolve these issues through use of (i) direct ligand binding assays that do not suffer from the limitations of radioligand binding assays (i.e. fluorescent ligand binding assays, nonfluorescent ligand binding assays based on quenching of tyrosine emission, and displacement of bound fluorescent ligand), (ii) fluorescence resonance energy transfer (FRET) between PPARα aromatic amino acids and bound fluorescent ligand (trans-parinaric acid and cis-parinaroyl-CoA) to calculate the intermolecular distance, (iii) circular dichroism to characterize potential ligand-induced changes in PPARα secondary structure, and (iv) co-immunoprecipitation to determine whether ligands that bind and alter PPARα secondary structure also influence the ability of PPARα to interact with co-activators.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—cis-Parinaric acid, trans-parinaric acid, NBD-chloride, and NBD-stearic acid were purchased from Molecular Probes, Inc. (Eugene, OR). cis-Parinaroyl-CoA was synthesized as previously described (33) and purified by high performance liquid chromatography (37). Coenzyme A, palmitic acid, palmitoleic acid, oleic acid, myristoyl-CoA, palmitoyl-CoA, stearyl-CoA, palmitoleoyl-CoA, oleoyl-CoA, linoleoyl-CoA, arachidonyl-CoA, and bezafibrate were from Sigma, Medica 16, as well as the coenzyme A (cholesterol of Medica 16 and bezafibrate, were chemically synthesized as described (38, 39) and kindly provided along with the glutazones and S-hexadecyl-CoA by Dr. J. Bar-Tana (Hebrew University, Israel). All CoA thioesters, whether freshly synthesized or obtained commercially, were >95% undegraded. Mouse PPARα monoclonal, rabbit PPARα polyclonal, mouse steroid receptor...
coactivator-1 (SRC-1) monoclonal, and rabbit SRC-1 polyclonal antibodies were from Affinity BioReagents (Golden, CO). Anti-rabbit IgG secondary antibodies were from Sigma. Mammalian co-immunoprecipitation kit, chemiluminescent substrate, and film were from Pierce.

Recombinant Mouse PPARα Protein—In the present investigation, PPARαΔAB (amino acids 101–468) was utilized for all ligand binding and structure determinations. This choice, rather than the much smaller LBD, was based on recent studies demonstrating that deletion of additional parts of a nuclear transcription factor can significantly alter ligand binding affinity and specificity.2 The cDNA encoding mouse PPARα with a deletion of the amino-terminal AB domain (i.e., encoding PPARα amino acids 101–468) was cloned into a HisA-tagged bacterial expression vector (pET-PPARαΔAB) and was a generous gift from Dr. N. Noy (Cornell University). The recombinant protein was expressed in the BL21(DE3)pLysS strain of Escherichia coli as described (20) and purified by affinity chromatography with cobalt resin (BD Biosciences Clontech). Purified protein was dialyzed against a buffer containing 10 mM Heps (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 400 mM KCl, and 10% glycerol and stored at −80 °C in 25% glycerol. Protein concentration was determined by Bradford assay. Protein purity was assessed by SDS-PAGE and Western blotting. SDS-PAGE and Coomassie Blue staining detected a single intense band of ∼50 kDa (not shown). Western blotting with rabbit anti-mouse PPARα monoclonal antibody was reacted with goat anti-rabbit IgG alkaline phosphatase conjugate and was processed as described (41). Western blotting with rabbit anti-mouse PPARα monoclonal antibody also resulted in a single band at 50 kDa (not shown). Both SDS-PAGE/Coomassie Blue protein staining and Western blotting confirmed the presence of the PPARαΔAB protein in monomeric form.

Direct Fluorescent Ligand Binding Assays—Direct fluorescent ligand (NBD-stearic acid, NBD-chloride, cis-parinaric acid, trans-parinaric acid, and cis-parinaroyl-CoA) binding measurements were performed as described earlier (29, 33, 37, 42). Briefly, increasing quantities of fluorescent ligand (5–2000 nM) were added from a concentrated stock to 0.1 μM PPARαΔAB in 2 ml of phosphate-buffered saline (pH 7.4). Excitation was carried out at 466 nm for NBD-stearate and at 310 nm for NBD-stearate to 310 nm for NBD-chloride. The dissociation constant (Kd) and number of binding sites (n) obtained by a reciprocal plot of 1/(1–Fmax) obtained above, where a is the maximal fluorescence (Fmax) corresponding to Bmax = nBo, Bmax is the maximal binding site concentration for the given amount of protein, Bo is the protein concentration, b is the number of binding sites (n), and is the Kd.

Direct Fluorescence Binding Assay: Quenching of PPARαΔAB Aromatic Amino Acid Residues by Nonfluorescent Ligands—The direct binding of nonfluorescent ligands to PPARαΔAB was carried out as described (33). Briefly, PPARαΔAB (0.1 μM in 2 ml of phosphate-buffered saline, pH 7.4) was titrated with increasing ligand (5–2000 nM). PPARαΔAB monoclonal antibody was reacted with goat anti-rabbit IgG alkaline phosphatase conjugate and was processed as described (41). Western blotting with rabbit anti-mouse PPARα monoclonal antibody also resulted in a single band at 50 kDa (not shown). Both SDS-PAGE/Coomassie Blue protein staining and Western blotting confirmed the presence of the PPARαΔAB protein in monomeric form.

Displacement Binding Assay: Displacement of Bound Fluorescent NBD-stearic Acid by Nonfluorescent Ligands—PPARαΔAB (0.1 μM in phosphate-buffered saline, pH 7.4) was incubated with NBD-stearic acid (50 nM) for 5 min at 24 °C to obtain maximal fluorescence. NBD-stearic acid was excited at 466 nm, and emission was measured from 490 to 650 nm. The displacement of bound fluorescent ligand was calculated from the decrease in NBD-stearic acid fluorescence intensity with increasing concentrations of nonfluorescent ligand (29, 43). The Kd values for each ligand were calculated according to Equation 3 (20).

\[
EC_{50\text{ligand}} / [\text{NBD-stearate}]_{\text{total}} = K_{\text{d,ligand}} / K_{\text{d,NBD-stearate}} \quad (\text{Eq. 3})
\]

where EC 50 ligand represents the concentration of ligand required for displacing half of the probe from the protein, Kd,ligand is the efficiency of ligand to displace NBD-stearate, and Kd,NBD-stearate is the measured Kd for NBD-stearate obtained as described above. The Ki values for each ligand were calculated by substituting the measured Kd for NBD-stearate and the concentration of ligand required for displacing half of the probe from the protein (EC 50 ligand) in Equation 3 (20).


direct fluorescence binding assay: FRET and Determination of Intermolecular Distance—FRET was used to determine the relative intermolecular distance between PPARαΔAB aromatic amino acid residues and bound trans-parinaric acid or bound cis-parinaroyl-CoA. Since FRET varies as (intermolecular distance)α, the donor (PPARαΔAB aromatic amino acid) and the acceptor (bound trans-parinaric acid or bound cis-parinaroyl-CoA) resides must be in very close proximity for efficient FRET to occur. PPARαΔAB aromatic amino acid excitation was carried out at 280 nm with emission scan from 300 to 450 nm. FRET from PPARαΔAB aromatic amino acids to trans-parinaric acid or cis-parinaroyl-CoA was conducted as described (33, 44, 45) with the following modifications. PPARαΔAB (0.1 μM in 2 ml of phosphate-buffered saline, pH 7.4) was titrated with increasing amounts of ligand (5–2000 nm). The intermolecular distance between PPARαΔAB and bound ligand was calculated according to the Forster equation (Eq. 4) (46), as described earlier (33, 44, 45).

\[
E = R_0^6 / (R_0^6 + R_{\text{trans}}^6) \quad (\text{Eq. 4})
\]

where E is the FRET efficiency, R0 is the critical distance for 50% efficiency, and Rtrans is the actual distance between donor and acceptor. The energy transfer efficiency was first calculated by quenching of donor PPARαΔAB aromatic amino acid fluorescence according to Equation 5.

\[
E = 1 - F_{\text{D,trans}} / F_D \quad (\text{Eq. 5})
\]

where FD,trans and FD are the fluorescence emission intensities of PPARαΔAB aromatic amino acids in the presence or absence of added ligand. Alternately, the energy transfer efficiency E was also calculated by the intrinsic increase of acceptor ligand fluorescence according to Equation 6.

\[
E = (F_{\text{D,trans}} / F_D ) / (1 / e_\text{d} / e_\text{a}) \quad (\text{Eq. 6})
\]

where FD,trans and FD are the fluorescence intensities of the acceptor at 416 nm in the presence and absence of the donor, and eD and eA were the extinction coefficients of the acceptor and donor. The critical distance for 50% energy transfer efficiency (R0) was calculated according to Equation 7 (33, 44–46).

\[
R_0 = 9.79 \times 10^9 / [k^2 \eta^4 Q_{\text{D,trans}}^{1 / 3}] \quad (\text{Eq. 7})
\]

where the orientation factor k2 was assumed to be 0.5, the refractive index = 1.4 for proteins in solution, the quantum yield for PPARαΔAB QD, was calculated to be 0.085, and the overlap integral J was expressed in ml·g−1·cm−1 (44).

Secondary Structure Determination: Effect of Ligand Binding on PPARαΔAB Circular Dichroism (CD)—Circular dichroism spectra of PPARαΔAB (0.8 μM in 125 μM HEPEs, pH 8.0, 12.5 μM dithiothreitol, 5 mM KCl, 0.3% glycerol) were taken in the presence and absence of nonfluorescent fatty acids or fatty acyl-CoA ligands (20 μM) with a J-710 spectropolarimeter (Jasco Inc., Easton, MD) at 23 °C in a 1-mm cell. Spectra were recorded from 250 to 195 nm with a bandwidth of 2 nm, sensitivity of 10 millidegrees, scan rate of 50 nm/min, and a time constant of 1 s. Ten scans were averaged for percentage composition of α-helices, β-strands, turns, and unordered structures with the software package CDPPro (available on the World Wide Web at lamar.colostate.edu/~sreeman/CDPPro), which allows the percentage calculation of various secondary structures by three different methods: SELCON3, CDSPSTR, and CONTIN/ELL (48).
Co-immunoprecipitation: Effects of Ligand Binding on Co-activator Recruitment—It is thought that ligand activation of PPARα stimulates recruitment and interaction with co-activators such as SRC-1 (reviewed in Ref. 49). To test this possibility, co-immunoprecipitation was used to test the ability of monoclonal PPARα or monoclonal SRC-1 antibodies to co-immunoprecipitate both proteins from mouse liver homogenate in the presence or absence of PPARα ligands. Briefly, livers from C57/BL6 male mice were homogenized in M-PER® buffer (Pierce) containing 150 mM sodium chloride and protease inhibitors and centrifuged to pellet insoluble debris. Protein concentration was determined by BCA protein assay (Pierce). Ligands (100 μM) were added to 1 mg of liver homogenate, and co-immunoprecipitation procedures were conducted with the ProFound™ mammalian co-immunoprecipitation kit according to the manufacturer’s instructions. SDS-PAGE, protein transfer, and Western blotting were performed as described (41) with polyclonal primary antibodies made in rabbit and anti-rabbit horseradish peroxidase-conjugated secondary antibodies made in goat, each diluted to the manufacturer’s recommendations. Western blotting was used to detect each protein by visualization with a chemiluminescent substrate and quantified by densitometry, utilizing a single-chip charge-coupled device video camera FluorChemImager and accompanying FluorChem image analysis software from Alpha Innotech (San Leandro, CA). Co-immunoprecipitated protein values were standardized to values obtained for the antibody used to co-immunoprecipitate both proteins, and standardized values were normalized for each ligand sample to the level of interaction observed in the absence of ligands, which was set equal to 1.

RESULTS

Rationale for Choice of Ligands Tested—Although the ligand affinity and specificity of PPARα for a variety of synthetic, nonnatural radioligands has been examined, relatively little is known regarding the identity of putative high affinity endogenous PPARα ligands (13, 20, 21, 26). This is especially the case for LCFA-CoAs and in some cases the corresponding LCFA ligands. Further, it is not known how or to what extent LCFA-CoAs or LCFA ligands alter PPARα conformation upon binding. Three classes of ligands were utilized to begin to examine these issues (Fig. 1). (i) Class A included synthetic (NBD-stearate) and naturally occurring (cis-parinaric acid and trans-parinaric acid) fluorescent LCFA-CoAs as well as the respective fluorescent LCFA-CoAs where applicable (Fig. 1A). (ii) Class B was comprised of naturally occurring, nonfluorescent LCFA-CoAs (palmitic acid, palmitoleic acid, and oleic acid) and their respective LCFA-CoAs (palmitoyl-CoA, stearoyl-CoA, palmitoleoyl-CoA, oleoyl-CoA, and arachidonoyl-CoA) (Fig. 1B). (iii) Class C was composed of known nonfluorescent xenobiotic drugs (bezafibrate, Medica 16, and rosiglitazone) and their respective CoA esters (bezafibroyl-CoA and Medica 16-CoA) (Fig. 1C).
Hydrophobicity of the PPARα Ligand Binding Site: Emission Spectral Shifts of Bound Fluorescent LCFA and LCFA-CoA Ligands—The emission maximum of the NBD fluorophore in NBD-stearic acid (Fig. 1A) is very sensitive to environmental hydrophobicity and thereby provides a useful tool to ascertain whether binding of this ligand to a protein reflects simple adsorption/aggregation or molecular interaction within a hydrophobic binding pocket (43, 50, 51). In the absence of added PPARα protein, the NBD-stearate (Fig. 2A, curve 1) and NBD-Cl (data not shown) fluoresced weakly with an emission maximum near 554 nm. In the presence of PPARα, the emission maximum of NBD-stearate was increased in intensity and blue-shifted to 422 nm (Fig. 2A, curves 2–6). In contrast, the emission of NBD-Cl did not show either increased fluorescence intensity or a blue-shifted emission maximum in the presence of PPARα (Fig. 2B, triangles). Therefore, the acyl chain of NBD-stearic acid was essential for binding to PPARα.

In order to determine whether the hydrophobicity of the PPARα binding site obtained with NBD-stearate reflected that of naturally occurring ligands, the interaction of PPARα with trans-parinaric acid, cis-parinaric acid, and cis-parinaroyl-CoA was examined. Parinaric acids are naturally occurring, polyunsaturated fatty acids with 18 carbons and 4 unsaturated bonds and display close structural similarities to linolenic acid (Fig. 1A). In buffer alone without added PPARα protein, the trans-parinaric acid, cis-parinaric acid, and cis-parinaroyl-CoA fluoresced very poorly, with maximal fluorescence emission at 422 nm (not shown). Upon titration of PPARα with increasing concentrations of trans-parinaric acid (Fig. 2C, circles) and cis-parinaroyl-CoA (Fig. 2D, circles), fluorescence emission intensity increased. Although the parinaric acid emission maximal wavelength is less sensitive to the hydrophobicity of the environment than that of NBD (18, 44, 45), the maximum peak of fluorescence was blue-shifted to 416 nm (data not shown), consistent with a more hydrophobic environment in the PPARα ligand binding site. In contrast, no increase in fluorescence intensity or shift in maximal emission was noted for cis-parinaric acid (Fig. 2C, triangles), suggesting that cis-parinaric acid did not bind to PPARα over the concentration range examined. In summary, the fact that NBD-stearic acid, trans-parinaric acid, and cis-parinaroyl-CoA fluorescence intensities were increased severalfold and blue-shifted nearly 30 and 6 nm, respectively, suggested that both synthetic and naturally occurring fluorescent LCFA and LCFA-CoAs were bound to PPARα whose ligand binding pocket was relatively hydrophobic.

Intermolecular Distance between PPARα and Bound Fluorescent LCFA and LCFA-CoA Ligands: FRET—To obtain further quantitative details about the binding of fluorescent LCFA and fluorescent LCFA-CoA by PPARα, FRET was used to determine the intermolecular distance between these ligands and PPARα aromatic amino acid residues. Since the absorbance spectra of both trans-parinaric acid and cis-parinaroyl-CoA in buffer significantly overlap with the emission spectra of protein aromatic residues when excited at 280 nm, this acceptor/donor pair has been used to provide intermolecular distances for binding of this LCFA and LCFA-CoA to a variety of cytoplasmic LCFA (42, 45) and LCFA-CoA (29) binding proteins. Since this technique requires close molecular proximity, generally in the 5–100 Å range for this donor/acceptor pair, this technique was used to further confirm that the high affinity LCFA and LCFA-CoA binding to PPARα represents close molecular interaction. The average intermolecular distances between the PPARα aromatic amino acid residues and bound trans-parinaric acid (Fig. 3, A and C) or bound cis-parinaroyl-CoA (Fig. 4, A and C) were measured from both the decrease in donor emission at 333 nm and the appearance of sensitized acceptor emission at 416 nm. For each donor and acceptor pair, the spectral overlap integral, the critical distance for 50% efficiency between the donor and the acceptor, the efficiency of energy transfer between the donor and the acceptor, and the relative intermolecular distance between donor and acceptor were calculated as described under “Experimental Procedures.” The overlap inte-
FIG. 3. FRET from PPARαΔAB aromatic residues to bound trans-parinaric acid: Intermolecular distance and FRET binding assay. FRET from PPARαΔAB (0.1 μM) aromatic amino acids to bound trans-parinaric acid was measured by excitation at 280 nm in the presence of increasing trans-parinaric acid (20, 50, 100, 250, 500, 1000, 1500, and 2000 nM) concentration and corrected for background as described under “Experimental Procedures.” FRET was detected as quenching of PPARαΔAB aromatic amino acid fluorescence emission (A and B) and as appearance of sensitized emission from PPARαΔAB-bound trans-parinaric acid (C and D). A, emission spectra of PPARαΔAB with no ligand; spectra 2–9, PPARαΔAB with the addition of increasing trans-parinaric acid. B, plot of the mean ± S.E. (n = 5) change in maximal fluorescence emission (Fo – F) at 333 nm as a function of increasing trans-parinaric acid. Inset, linear plot of the binding curve in B. C, emission spectra of PPARαΔAB bound trans-parinaric acid showing sensitized trans-parinaric acid fluorescence emission near 416 nm. Spectra 1–9, as described for A. D, plot of the average fluorescence emission at 416 nm upon excitation at 280 nm as a function of trans-parinaric acid concentration. Inset, linear plot of the binding curve in D.

FIG. 4. FRET from PPARαΔAB aromatic amino acids to bound cis-parinaroyl-CoA: Intermolecular distance and FRET binding assay. FRET from PPARαΔAB (0.1 μM) aromatic amino acids to bound cis-parinaroyl-CoA acid was measured by excitation at 280 nm in the presence of increasing cis-parinaroyl-CoA (0–2000 nM) concentration and corrected for background as described under “Experimental Procedures.” FRET was detected as quenching of PPARαΔAB aromatic amino acid fluorescence emission (A and B) and as appearance of sensitized emission from PPARαΔAB-bound cis-parinaroyl-CoA (C and D). A, emission spectra of PPARαΔAB with no ligand; spectra 2–9, PPARαΔAB with the addition of 20, 50, 100, 250, 500, 1000, 1500, and 2000 nM cis-parinaroyl-CoA, respectively. B, plot of the average change in maximal fluorescence emission (Fo – F) upon excitation at 280 nm as a function of cis-parinaroyl-CoA concentration. Values represent the mean ± S.E., n = 5. Inset, linear plot of the binding curve in B. C, emission spectra showing an increase in cis-parinaroyl-CoA fluorescence intensity upon excitation of PPARαΔAB; spectra 1–9, as described for A. D, plot of the average fluorescence emission at 416 nm upon excitation at 280 nm as a function of cis-parinaroyl-CoA concentration. Values represent the mean ± S.E., n = 5. Inset, linear plot of the binding curve in D.

Gral of unesterified trans-parinaric acid was slightly less than that of cis-parinaroyl-CoA, and based on these integrals, very similar Ro values (distance for 50% energy transfer between acceptor and donor) were calculated (Table I). Although there was some variation noted for the efficiency of transfer based upon the method of calculation, the relative intermolecular
distances were not significantly different. Based upon transfer efficiency, the bound trans-parinaric acid was within 24.8–27.0 Å of PPARα aromatic amino acids, and similar intermolecular distances were obtained for PPARα and bound cis-parinaroyl-CoA (Table I). Thus, PPARα bound naturally occurring fluorescent LCFA and LCFA-CoA in close molecular proximity to the aromatic amino acids of PPARα. Thus, the finding of very short intermolecular distances (25–28 Å) between PPARα aromatic amino acids and bound naturally occurring fluorescent ligands (trans-parinaric acid and cis-parinaroyl-CoA) were consistent with close molecular interaction rather than nonspecific binding of LCFA-CoA, as well as LCFA, by PPARα.

PPARα Binding Parameters for Fluorescent Fatty Acids and Fatty Acyl-CoA: Direct Fluorescent Ligand Binding Assay—The fluorescence spectral properties of NBD-stearic acid were used in a direct fluorescent ligand binding assay to determine PPARα binding affinity ($K_d$) and number of binding sites for NBD-stearic acid. Binding of NBD-Cl was used as a control for the fluorophore (NBD) without acyl chain. In the absence of added PPARα protein, the NBD-stearate (Fig. 2A, curve 1) and NBD-Cl (Fig. 2B) fluoresced weakly at 525 nm. To obtain binding affinities, a fixed amount of PPARα was titrated with increasing concentrations of NBD-stearic acid (Fig. 2B, circles) or NBD-Cl (Fig. 2B, triangles). Fluorescent intensities of NBD-stearate, measured at 525 nm, corrected for background (ligand without protein) and plotted as a function of total ligand concentration, demonstrated a saturatable binding curve (Fig. 2B, circles). The linear reciprocal plots (Fig. 2B, inset) yielded a $K_d$ of 18.7 ± 3.2 nM and suggested a single binding site ($R^2 > 0.94$). This was confirmed by a Hill plot, which also yielded one NBD-stearic acid binding site ($n = 1.1$) for PPARα. In contrast, NBD-Cl did not show increased fluorescence in the presence of PPARα, and no titration curve could be obtained (Fig. 2B, triangles), suggesting that NBD-Cl does not bind to PPARα.

In order to determine whether the high binding affinity of PPARα for NBD-stearate reflected that of naturally occurring ligands, the direct fluorescence ligand binding assay was repeated with trans-parinaric acid. Fluorescent intensities of trans-parinaric acid (Fig. 2C, circles) measured at 416 nm, corrected for background (ligand without protein), and plotted as a function of total ligand concentration yielded a saturatable binding curve. Furthermore, the reciprocal plot of these binding data were linear with a $R^2 > 0.9$ (Fig. 2C, inset), suggesting a single binding site with a $K_d$ of 35.2 ± 7.0 nM for trans-parinaric acid. The Hill plot confirmed a single binding site for trans-parinaric acid ($n = 1.3$). These data with a naturally occurring fluorescent LCFA demonstrate that PPARα has a single high affinity binding site for trans-parinaric acid, which was in the same range as that displayed by NBD-stearic acid.

Finally, the ability of PPARα to directly bind a naturally-occurring fluorescent LCFA-CoA (i.e. cis-parinaroyl-CoA) was examined. Fluorescent intensities of cis-parinaroyl-CoA (Fig. 2D) measured at 416 nm, corrected for background (ligand without protein), and plotted as a function of total ligand concentration yielded saturatable binding curves. A reciprocal plot of these binding data were linear with a $R^2 > 0.9$ (Fig. 2D, inset), suggesting a single binding site for this ligand with a $K_d$ of 13.3 ± 3.7 nM for cis-parinaroyl-CoA. The Hill plot confirmed a single binding site ($n = 1.1$). Thus, PPARα has a single high affinity binding site for cis-parinaroyl-CoA, a naturally-occurring fluorescent LCFA-CoA.

In summary, these data demonstrate that PPARα has a single binding site, which exhibits high affinity (18–35 nM $K_d$ values) for both synthetic (NBD-stearic acid) and naturally occurring (trans-parinaric acid) fluorescent LCFAs. In addition, PPARα also bound cis-parinaroyl-CoA, a naturally occurring fluorescent LCFA-CoA, with affinity at least as well or better than the fluorescent LCFAs.

**TABLE I**

| Donor Acceptor Interactions | Method of calculation | $J$ | $R$ | $E$ |
|----------------------------|----------------------|-----|-----|-----|
| Binding Parameters for Fluorescent Fatty Acids and Fatty Acyl-CoA: A Fluorescence Resonance Energy Transfer Assay—The finding that FRET from the PPARα aromatic amino acid donors to the bound trans-parinaric acid or cis-parinaroyl-CoA (Fig. 4, A and C) acceptor not only decreased the donor emission at 333 nm but simultaneously increased the sensitized acceptor emission at 416 nm allowed construction of saturation binding curves. As increasing concentrations of trans-parinaric acid were added to PPARα, a decrease in the aromatic amino acid emission (Fig. 3A) and an increase in the trans-parinaric acid fluorescence (Fig. 3C) were noted. Analysis of the binding curves measured at 333 nm for aromatic amino acid quenching (Fig. 3B) and the reciprocal plot (Fig. 3B, inset) provided a $K_d$ value of 67.4 ± 4.3 nM. However, the saturation curve measured at 416 nm for sensitized emission of trans-parinaric acid (Fig. 3D) and its reciprocal plot (Fig. 3D, inset) generated a $K_d$ value of 22.8 ± 4.3 nM. Although these values varied slightly from those obtained by the direct binding assay above, these data further confirmed high affinity binding of trans-parinaric acid. The addition of increasing quantities of cis-parinaroyl-CoA to PPARα also caused a decrease in the intensity of intrinsic PPARα aromatic amino acid emission (Fig. 4A), while concomitantly increasing sensitized cis-parinaroyl-CoA fluorescence (Fig. 4C). Analysis of the saturation curve measured at 333 nm for aromatic amino acid quenching (Fig. 4B) and the reciprocal plot (Fig. 4B, inset) yielded a $K_d$ value of 8.0 ± 1.9 nM. Similarly, the saturation curve measured at 416 nm for sensitized emission of cis-parinaroyl-CoA (Fig. 4D) and its reciprocal plot (Fig. 4D, inset) yielded a $K_d$ value of 13.7 ± 3.4 nM. These values were both very similar to those obtained for PPARα binding of cis-parinaroyl-CoA by direct fluorescence binding methods (Fig. 2) and further support the high affinity binding of the cis-parinaroyl-CoA.

**Binding of Endogenous Fatty Acids and Fatty Acyl-CoAs to PPARα: Displacement of Bound NBD-stearic Acid—Although PPARα bound both synthetic (NBD-stearic acid) and naturally occurring (trans-parinaric acid) fluorescent LCFA-CoAs, the possibility was considered that the high affinities obtained (1–4 nM $K_d$ values) may be attributed at least in part to the nature of these fluorophores. To resolve this issue, the binding efficiency of PPARα for nonfluorescent ligands was examined using a
fluorescent displacement assay not requiring separation of bound from free ligand. In this assay, NBD-stearate was bound to PPARγ/H9251, followed by measurement of NBD-stearic acid displacement upon the addition of increasing concentrations of nonfluorescent endogenous LCFAs (both saturated and unsaturated) and endogenous LCFA-CoAs (both saturated and unsaturated). Displacement was measured as the decrease in fluorescence intensity of NBD-stearate as this ligand was displaced from the binding pocket of PPARγ/H9251 and entered the hydrophilic environment of the buffer. The saturated C-16 palmitic acid did not displace NBD-stearic acid from the PPARγ/H9251-NBD-stearate complex (Fig. 5A), consistent with earlier findings where another saturated fatty acid (C-18 stearic acid) very weakly displaced PPARγ/H9251-bound trans-parinaric acid (20). In contrast, a monounsaturated LCFA (C-16:1 palmitoleic acid) significantly displaced bound NBD-stearate (Fig. 5B). Likewise, other monounsaturated LCFAs (C-18:1 oleic acid) also effectively displaced NBD-stearate (Fig. 5C). In contrast, all types of LCFA-CoAs effectively displaced PPARγ-bound NBD-stearate, including saturated (C-12:0-CoA myristoyl-CoA (Fig.
LCFA-CoAs and Fatty Acids Bind PPARα and Alter Conformation

5D), C-16:0-CoA palmitoyl-CoA (Fig. 5E), and C-18:0-CoA stearoyl-CoA (Fig. 5F), monounsaturated (C-16:1-CoA palmitoleoyl-CoA (Fig. 5G) and C-18:1-CoA oleoyl-CoA (Fig. 5H)), and polyunsaturated (C-18:2-CoA linoleoyl-CoA (Fig. 5I) and C-20:4-CoA arachidonoyl-CoA (Fig. 5J)). However, CoA itself was incapable of displacing the NBD-stearate (Fig. 5K, CoA). Interestingly, LCFA-CoAs very effectively displaced bound NBD-stearate as well as, and in some cases better than, the corresponding LCFA (Fig. 5, A–C). These qualitative observations were confirmed by quantitative analysis of multiple repetitions to obtain mean values (Table II). Ki values for NBD-stearic acid displacement from PPARα were 2–3 nM for unsaturated LCFA-CoAs, whereas the saturated LCFA palmitic acid was not displaced (Table II). In contrast, Ki values for NBD-stearic acid displacement from PPARα by unsaturated LCFA-CoAs as well as saturated LCFA-CoAs were very similar (i.e. 1–4 nM). In summary, the NBD-stearic acid displacement studies showed that PPARα did not bind the endogenous saturated LCFA (C-16:0 palmitic acid) (Fig. 5A), consistent with transparinaric acid displacement data, which showed that PPARα bound C18:0 stearic acid, another naturally occurring saturated LCFA, very weakly (20). In contrast, the NBD-stearic acid displacement assay demonstrated that PPARα exhibited very high affinity (Ki = 2–3 nM) for unsaturated LCFA-CoAs (C-16:1 palmitoleic acid, C-18:1 oleic acid), again consistent with a transparinaric acid displacement data exhibiting PPARα binding C18:1 oleic acid as well as other naturally occurring unsaturated LCFA-CoAs with high affinity (Ki values = 5–17 nM) (20). Finally, the data presented here showed for the first time that PPARα bound all types (saturated, monounsaturated, and polyunsaturated) of naturally occurring LCFA-CoAs with very high affinity as exhibited by Ki values of 1–4 nM (Table II). Thus, whereas PPARα bound both the free acid and the CoA derivative forms of unsaturated LCFA-CoAs with nearly equal high affinity (very low nM Ki values), in the case of the saturated LCFA-CoAs, PPARα only bound the CoA derivatives with high affinity.

**Direct Binding of Endogenous Fatty Acids and Fatty Acyl-CoAs to PPARα: Quenching of PPARα Intrinsic Aromatic Amino Acid Fluorescence**—To further confirm the ligand specificity of PPARα and high affinity obtained by the NBD-stearic acid displacement assay, we took advantage of the intrinsic aromatic amino acid fluorescence of PPARα to develop an independent ligand binding assay not dependent upon an exogenous fluorescent ligand. In this assay, quenching of the intrinsic aromatic amino acid fluorescence of PPARα was examined in response to increasing concentration of a series of LCFA-CoAs, and oleic acid was used as a control.

The results of the direct aromatic amino acid fluorescence quenching assay closely confirmed those obtained with the NBD-stearic acid displacement assay. In both cases, PPARα bound unsaturated (but not saturated) LCFA-CoAs with high affinity. In contrast, PPARα exhibited high affinity binding for both unsaturated and saturated LCFA-CoAs nearly equally.

**Comparison of PPARα Binding of Synthetic Peroxisome Proliferators and Their CoA Thioesters—Several fibrates (Fig. 1C) belonging to the hypolipidemic class of drugs are known to be PPARα activators, whereas glitazones (Fig. 1C), which belong to the class of thiazolidinediones, are known to be PPARγ activators. Although bezafibrate has been found to bind to PPARα-LBD in the low nanomolar range (21), relatively little is known regarding the affinity of PPARα for these drugs and their CoA derivatives as compared with endogenous ligands (i.e. LCFA-CoAs and LCFA-CoA). Quenching of PPARα intrinsic aromatic amino acid fluorescence with increasing concentrations of bezafibrate resulted in approximately a 20% decrease in fluorescence emission (Fig. 7A), whereas binding of MEDICA 16 (another PPARα-inducing drug) resulted in only an 8% decrease in fluorescence (Fig. 7E). Transformation of these data showed saturable binding (Fig. 7, B and F), and reciprocal plots of the binding data best fit straight lines with R² > 0.9 (Fig. 7, B (inset) and F (inset), suggesting a single binding site for these peroxisome proliferator drugs. Despite the low degree of quenching, analysis of multiple binding curves yielded Ki values ranging from 13 nM for bezafibrate to 57.5 nM for MEDICA 16 (Table IV). In contrast, the CoA derivatives elicited much greater quenching, with bezafibroyl-CoA inducing a 45% decrease in PPARα intrinsic aromatic amino acid fluorescence (Fig. 7C) and MEDICA 16 CoA inducing an almost 40% decrease (Fig. 7G). Furthermore, transformation of these data (Fig. 7, D and H) showed even steeper saturation curves, and analysis of multiple replicates yielded Ki values of 3–8 nM for the CoA thioesters of these peroxisome proliferator drugs (Table IV). Clearly, the CoA derivatives of these drugs, especially MEDICA 16 CoA, exhibited significantly higher binding than the free acid form of the peroxisomal proliferating drug. The high affinity binding of bezafibroyl-CoA and MEDICA 16 CoA binding determined by PPARα intrinsic aromatic amino acid fluorescence quenching was confirmed by displacement of PPARα-bound NBD-stearate, which yielded Ki values of 2.1 ± 0.7 and 3.0 ± 0.6 nM, respectively (data not shown). These high

**Table II**

| Ligand             | Ki ± S.E. | nM |
|--------------------|-----------|----|
| Fatty acids        |           |    |
| Palmitic acid (16:0) | ND        |    |
| Palmitoleic acid (16:1) | 3.2 ± 0.9 |    |
| Oleic acid (18:1)  | 2.0 ± 0.6 |    |
| Fatty acyl-CoAs    |           |    |
| Myristoyl-CoA (14:0) | 1.9 ± 0.5 |    |
| Palmitoyl-CoA (16:0) | 1.1 ± 0.3 |    |
| Stearoyl-CoA (18:0) | 4.2 ± 1.1 |    |
| Palmitoleoyl-CoA (16:1) | 2.4 ± 0.4 |    |
| Oleoyl-CoA (18:1)  | 1.4 ± 0.3 |    |
| Linoleoyl-CoA (18:2) | 1.0 ± 0.3 |    |
| Arachidonoyl-CoA (20:4) | 2.0 ± 0.5 |    |
| CoA                | ND        |    |

**Comparison of PPARα Binding of Synthetic Peroxisome Proliferators and Their CoA Thioesters—**Several fibrates (Fig. 1C) belonging to the hypolipidemic class of drugs are known to be PPARα activators, whereas glitazones (Fig. 1C), which belong to the class of thiazolidinediones, are known to be PPARγ activators. Although bezafibrate has been found to bind to PPARα-LBD in the low nanomolar range (21), relatively little is known regarding the affinity of PPARα for these drugs and their CoA derivatives as compared with endogenous ligands (i.e. LCFA-CoAs and LCFA-CoA). Quenching of PPARα intrinsic aromatic amino acid fluorescence with increasing concentrations of bezafibrate resulted in approximately a 20% decrease in fluorescence emission (Fig. 7A), whereas binding of MEDICA 16 (another PPARα-inducing drug) resulted in only an 8% decrease in fluorescence (Fig. 7E). Transformation of these data showed saturable binding (Fig. 7, B and F), and reciprocal plots of the binding data best fit straight lines with R² > 0.9 (Fig. 7, B (inset) and F (inset), suggesting a single binding site for these peroxisome proliferator drugs. Despite the low degree of quenching, analysis of multiple binding curves yielded Ki values ranging from 13 nM for bezafibrate to 57.5 nM for MEDICA 16 (Table IV). In contrast, the CoA derivatives elicited much greater quenching, with bezafibroyl-CoA inducing a 45% decrease in PPARα intrinsic aromatic amino acid fluorescence (Fig. 7C) and MEDICA 16 CoA inducing an almost 40% decrease (Fig. 7G). Furthermore, transformation of these data (Fig. 7, D and H) showed even steeper saturation curves, and analysis of multiple replicates yielded Ki values of 3–8 nM for the CoA thioesters of these peroxisome proliferator drugs (Table IV). Clearly, the CoA derivatives of these drugs, especially MEDICA 16 CoA, exhibited significantly higher binding than the free acid form of the peroxisomal proliferating drug. The high affinity binding of bezafibroyl-CoA and MEDICA 16 CoA binding determined by PPARα intrinsic aromatic amino acid fluorescence quenching was confirmed by displacement of PPARα-bound NBD-stearate, which yielded Ki values of 2.1 ± 0.7 and 3.0 ± 0.6 nM, respectively (data not shown). These high
affinities of PPARα for peroxisomal proliferating drugs were specific, since other peroxisomal proliferators known to induce PPARγ but not PPARα (pioglitazone, troglitazone, and rosiglitazone) did not bind (Table IV).

Effect of Endogenous Fatty Acids and Fatty Acyl-CoAs on PPARα Secondary Structure: Circular Dichroism—Ligand-induced conformational change is a hallmark of ligand-regulated nuclear transcription factors, including PPARs (35, 52). The observation that binding of nonfluorescent ligands significantly quenched the emission of PPARα/β aromatic amino acid residues (Figs. 6 and 7) suggested that ligand binding altered the conformation of PPARα. To further examine whether the binding of naturally occurring LCFAs and LCFA-CoAs alters PPARα structure, the effect of these ligands on CD spectra of PPARα was examined. CD is a sensitive method to measure conformational changes induced by ligand binding to nuclear receptors (33).

The far UV spectrum of PPARα exhibited two minima at 207 and 221 nm and one maximum at 194 nm (filled circles), suggesting the presence of substantial α-helical content in the PPARα polypeptide chain. This was confirmed by quantitative analysis of the CD spectra as described under “Experimental Procedures,” which indicated that PPARα was composed of 37.2% α-helix, 16.1% β-sheets, 19.9% β-turns, and 27.2% unordered structures (Table V). The addition of a 25-fold molar excess of saturated fatty acid (C-16:0 palmitic acid) did not elicit any shifts in the PPARα CD spectra (Fig. 8A, open squares), consistent with the very weak affinity of PPARα for

**Table III**

| Ligand                  | Kd  ± S.E. | nM  |
|-------------------------|-----------|-----|
| Fatty acids             |           |     |
| Palmitic acid (16:0)    | Weak      |     |
| Oleic acid (18:1)      | 4.0 ± 0.4 |     |
| Fatty acyl-CoAs         |           |     |
| Myristoyl-CoA (14:0)   | 5.6 ± 0.5 |     |
| Palmitoyl-CoA (16:0)   | 8.4 ± 0.7 |     |
| Stearoyl-CoA (18:0)    | 14.0 ± 0.9|     |
| Palmitoleoyl-CoA (16:1)| 13.5 ± 0.9|     |
| Oleoyl-CoA (18:1)      | 10.7 ± 1.2|     |
| Linoleoyl-CoA (18:2)   | 4.3 ± 0.3 |     |
| Arachidonoyl-CoA (20:4)| 3.5 ± 0.4 |     |

The affinities of PPARα for peroxisomal proliferating drugs were specific, since other peroxisomal proliferators known to induce PPARγ but not PPARα (pioglitazone, troglitazone, and rosiglitazone) did not bind (Table IV).
this saturated fatty acid (see above). In contrast, in the presence of a monounsaturated (C-16:1 palmitoleic acid; Fig. 8B, open squares) or polyunsaturated (C-20:4 arachidonic acid; Fig. 8B, open triangles) fatty acid, the CD spectra of PPAR\(\alpha\)/H9251 were significantly altered. The unsaturated LCFA increased PPAR\(\alpha\)/H9251 molar ellipticity values at 207 and 221 nm and concomitantly decreased PPAR\(\alpha\)/H9251 molar ellipticity values at 194 nm. These CD changes elicited by unsaturated (but not saturated) LCFA-CoAs were consistent with ligand-induced reductions in PPAR\(\alpha\)/H9251/\(\beta\)-helical content. Quantitative analysis of multiple CD spectra confirmed that the monounsaturated (C-16:1 palmitoleic acid) and more so the polyunsaturated (C-20:4 arachidonic acid) LCFA-CoAs significantly altered PPAR\(\alpha\)/H9251 structure as shown by a 12.4 and 18.5% decrease in total \(\alpha\)-helices (H(r) and H(d)), respectively, and a 13.9 and 21.1% increase in total \(\beta\)-sheets (S(r) and S(d)), respectively (Table V).

Although the secondary structure of PPAR\(\alpha\) was highly sensitive to unsaturated, but not saturated, LCFA, saturated LCFA-CoAs (C-16:0-CoA palmitoyl-CoA and C18:0-CoA stearoyl-CoA) significantly reduced the relative proportions of PPAR\(\alpha\) \(\alpha\)-helix structure by 19.6 and 18.0%, respectively, and increasing the proportion of \(\beta\)-sheets by 20.3 and 16.1%, respectively, with smaller increases in \(\beta\)-turns and unordered structures (Table V). The addition of unsaturated LCFA-CoAs (C-16:1-CoA palmitoleoyl-CoA and C-20:4-CoA arachidonoyl-CoA) resulted in molar ellipticity changes almost exactly the same as those for the saturated LCFA-CoAs (Fig. 8D). Binding of palmitoleoyl-CoA and arachidonoyl-CoA decreased the PPAR\(\alpha\)/H9251 content of \(\alpha\)-helices by 18.8 and 18.3%, respectively, while concomitantly increasing \(\beta\)-sheets by 18.7 and 17.9%, respectively, and eliciting smaller changes in \(\beta\)-turns and unordered structures (Table V). Thus, not only the saturated acyl-CoAs, but also the polyunsaturated acyl-CoAs, significantly altered the secondary structure of PPAR\(\alpha\). Taken together, the data illustrate that unsaturated LCFA (but not saturated LCFA), as well as CoA thioesters of both saturated and unsaturated LCFA, elicited significant changes in PPAR\(\alpha\) structure evidenced by decreased proportion of \(\alpha\)-helices and increased proportion of \(\beta\)-sheets and other structures.

**Effect of Ligand Binding on Co-activator Recruitment: Co-immunoprecipitation**—Although the unsaturated fatty acids...
and all fatty acyl-CoAs tested elicited a conformational change on PPARs (i.e. decreased α-helices and increased β-sheets), the effect of the acyl-CoA binding was in most cases greater than that of fatty acid binding. In order to determine how these changes might affect co-activator recruitment to PPARs, co-immunoprecipitation was performed in the presence and absence of selected ligands tested in the above assays. These results show that in the absence of any extraneous ligands or in the presence of saturated fatty acids (which do not bind to PPARs), the PPARs and SRC-1 were co-immunoprecipitated by the respective monoclonal antibodies from liver homogenate (Fig. 9). The addition of bezafibrate resulted in co-immunoprecipitation of both proteins at levels equal to those for no added ligand. However, the addition of the unsaturated fatty acid oleic acid resulted in a significant (p < 0.05) increase in PPARs co-immunoprecipitation with SRC-1 (Fig. 9A), although this increase was not noted for SRC-1 co-immunoprecipitation with PPARs. Since LCFA-CoAs are rapidly hydrolyzed to fatty acids, S-hexadecyl-CoA (nonhydrolyzable palmitoyl-CoA derivative) was utilized as a control. With either monoclonal antibody, a significant (p < 0.05) increase in co-immunoprecipitation occurred in the presence of S-hexadecyl-CoA (Fig. 9, A and B). Although the contribution of the acyl group for each hydrolyzable CoA thioester was probably not 100% due to partial hydrolysis, increased co-immunoprecipitation was noted upon the addition of palmitoyl-CoA (p < 0.06), oleoyl-CoA (p < 0.01), and bezafibroyl-CoA (p < 0.05) for the PPARs protein (Fig. 9A) and bezafibroyl-CoA (p < 0.05) for the SRC-1 protein (Fig. 9B). Thus, these data show that binding of high affinity ligands, especially fatty acyl-CoAs, not only altered PPARs conformation, but in addition enhanced the recruitment of the co-activator SRC-1.

**DISCUSSION**

Although PPARs exhibits high affinity (i.e. nanomolar Kd values) for many xenobiotic peroxisome proliferators and is activated by these agents, identification of endogenous ligands, which are bound with high affinity and conformationally alter PPARs, is less clear. As indicated in the Introduction, a variety of functional (transactivation, activation, nutritional) and ligand binding studies suggest that long chain fatty acids (LCFAs) and possibly LCFA-CoAs may be endogenous PPARs ligands. However, several issues complicate this interpretation. (i) The majority of radioligand binding assays yield very weak affinities (i.e. nanomolar Kd values) of PPARs for LCFAs (9, 12–14) and LCFA-CoAs (26). (ii) It is not clear whether the radioligand binding assays of LCFA and LCFA-CoA binding to PPARs represent close molecular interaction. Both LCFA and LCFA-CoAs are relatively hydrophobic lipids with low critical micellar concentrations, especially LCFA. Further, LCFA often appear as “fortuitous” ligands not specifically associated with proteins (53, 54). (iii) The concentration of LCFA and LCFA-CoAs in the nucleoplasm are very low, in the 39–68 nM (15, 16) and 3 nM (16) range, respectively. (iv) Although both unsaturated and saturated LCFA induce PPARs transactivation (5, 9, 12, 13, 22–24) and increase expression of PPARs activated proteins in dietary studies (25), the ligand binding data suggest that PPARs binds unsaturated LCFA, but not (or much less so) the saturated LCFA (9, 12–14, 20). (v) There are little data demonstrating how LCFA-CoAs or LCFA alter the structure/conformation of PPARs. (vi) Although pure recombinant proteins have been utilized to study co-factor interactions, there are no data of how ligand-induced conformational changes in PPARs affect co-activator recruitment in a physiological environment. The data presented herein contribute the following significant new insights to our understanding of these issues.

First, the current work presented evidence that endogenous LCFA-CoAs and unsaturated (but not saturated) LCFA directly bind to PPARs with very high affinity. Several different fluorescent approaches were used to determine the binding efficiency of PPARs for LCFA-CoAs and LCFA of variable chain length and saturation: direct binding of naturally occurring LCFA-CoAs and Fatty Acids Bind PPARs and Alter Conformation

**TABLE IV**

| Ligand       | Kd ± S.E. |
|--------------|-----------|
| Bezafibrate  | 13.1 ± 1.4|
| Bezafibroyl-CoA | 2.7 ± 0.2|
| Medica 16    | 575.5 ± 4.9|
| Medica 16-CoA | 8.4 ± 0.8|
| Pioglitazone | ND        |
| Troglitazone | ND        |
| Rosiglitazone| ND        |

**FIG. 8. Effect of fatty acid and fatty acyl-CoA binding on PPARαΔAB conformation: CD spectra.** Far-UV CD spectra of 0.8 μM PPARαΔAB in the absence or presence of 20 μM added ligand were obtained as described under “Experimental Procedures.” A, CD spectrum of PPARαΔAB in the absence (filled circles) and presence of added ligand: palmitic acid (empty squares); B, in the presence of palmitoleic acid (empty squares) or arachidonic acid (empty triangles); C, in the presence of palmitoyl-CoA (empty squares) or stearoyl-CoA (empty triangles); D, in the presence of palmitoleoyl-CoA (empty squares) or arachidonoyl-CoA (empty triangles). Each spectrum represents an average of 10 scans for a given representative spectrum from three replicates.
LCFA-CoAs and Fatty Acids Bind PPARα and Alter Conformation

TABLE V

Effect of fatty acids and fatty acyl-CoAs on the relative proportion of PPARαΔAB secondary structure determined by CD

|            | H(r) ± S.E. | H(d) ± S.E. | S(r) ± S.E. | S(d) ± S.E. | Turns ± S.E. | Unrd ± S.E. |
|------------|-------------|-------------|-------------|-------------|--------------|-------------|
| PPARαΔAB   | 22.1 ± 0.4  | 15.1 ± 0.1  | 8.8 ± 0.2   | 7.3 ± 0.1   | 19.9 ± 0.4   | 27.2 ± 0.4  |
| Palmitic   | 21.2 ± 0.3  | 15.0 ± 0.1  | 9.2 ± 0.3   | 7.3 ± 0.1   | 20.4 ± 0.4   | 27.0 ± 0.3  |
| Palmitoleic| 19.0 ± 0.4* | 13.6 ± 0.1* | 10.9 ± 0.6* | 7.8 ± 0.2*  | 20.4 ± 0.5   | 27.2 ± 1.1  |
| Arachidonic| 17.6 ± 0.6* | 12.7 ± 0.3* | 12.0 ± 0.7* | 8.4 ± 0.3*  | 21.0 ± 0.7   | 28.5 ± 0.8  |
| Stearoyl-CoA| 17.1 ± 0.5* | 12.8 ± 0.2* | 11.7 ± 0.7* | 8.5 ± 0.2*  | 21.3 ± 0.5   | 28.8 ± 0.6b |
| Palmitoleoyl-CoA| 17.2 ± 0.3* | 13.3 ± 0.1* | 11.2 ± 0.5* | 8.0 ± 0.2*  | 21.3 ± 0.4*  | 28.8 ± 0.6b |
| Arachidonoyl-CoA| 17.1 ± 0.3* | 13.1 ± 0.1* | 11.7 ± 0.8b | 8.1 ± 0.3b  | 21.4 ± 0.5b  | 28.5 ± 0.7  |

*a p < 0.001.
b p < 0.05.

Fig. 9. Effect of ligand binding on co-activator recruitment: Co-immunoprecipitation. The ability of monoclonal PPARα or monoclonal SRC-1 antibodies to precipitate both native proteins from mouse liver homogenate in the presence or absence of PPARα ligands was tested. Co-immunoprecipitated protein values were standardized to protein values obtained for the antibody used to co-immunoprecipitate both proteins, and standardized values were normalized for each ligand sample to the level of interaction observed in the absence of ligand, which was set equal to 1. A, amount of PPARα co-immunoprecipitated from SRC-1 antibody; B, amount of SRC-1 co-immunoprecipitated from PPARα antibody. Values represent standardized mean protein quantities ± S.E., n = 3–4.

The observation that PPARα exhibits high affinity for all types of LCFA-CoAs tested, as well as unsaturated (but not saturated) LCFA-CoAs, may be taken in context of the known structure of the ligand-binding pocket of this protein. Although the complete PPARα protein has not been crystallized, the LBD region of each PPAR subtype has been crystalized, and super-positioning of these structures suggests that the overall structure is very similar, with specific amino acid changes determining ligand specificity between the subtypes (11). The general structure of the PPAR-LBD consists of 13 α-helices and four small β-strands, with the binding pocket forming a “Y”-shaped cavity of ~1400 Å³ (55). This volume appears sufficient to accommodate LCFA-CoAs as well as LCFA-CoAs, which have typical volumes of <430 Å³ (56) and <700 Å³² respectively. Second, the current work presented evidence that LCFA-CoAs and unsaturated (but not saturated) LCFA-CoAs binding to PPARα represented close molecular interaction. The ligand-based assays, it is known that such radioligand binding assays underestimate the affinities of other LCFA binding proteins (L-FABP and SCP-2) (reviewed in Refs. 17–19) and LCFA-CoA binding proteins (acyl-CoA-binding protein, SCP-2, and L-FABP) (reviewed in Refs. 18 and 28–30). Together, these data provide significant evidence indicating that PPARα exhibits high affinity (low nanomolar Kᵰ values) for unsaturated LCFA-CoAs, saturated LCFA-CoAs, and unsaturated (but not saturated) LCFA-CoAs. These findings are consistent with endogenous LCFA-CoAs as well as unsaturated (but not saturated) LCFA-CoAs being physiologically significant ligands for PPARα.

ring and synthetic fluorescent ligands, displacement of bound fluorescent ligand (NBD-stearate) by endogenous ligands (LCFA-CoAs and LCFA), direct quenching of PPARα aromatic amino acid fluorescence by endogenous ligands, and FRET from PPARα aromatic residues to bound naturally occurring cis-parinaroyl-CoA or trans-parinaric acid. The major advantage of these assays is that, unlike radioligand binding assays, they do not require separation of bound from free ligand and do not require an additional matrix (e.g. charcoal, lipidx, etc.) that competes for bound ligand. In general, the fluorescence assays yielded very high binding efficiency of PPARα for naturally occurring saturated and unsaturated LCFA-CoAs with Kᵰ values of 1–14 nM. Whereas the same assays yielded very high binding efficiency of PPARα for naturally occurring unsaturated LCFA-CoAs (Kᵰ values of 1–4 nM), saturated LCFA-CoAs were not or only very weakly bound. These Kᵰ values for all LCFA-CoA thioesters as well as the unsaturated (but not saturated) LCFA-CoAs were in the same range as PPARα exhibited for peroxisome proliferators (bezafibrate) and their CoA thioesters (Bezafibroyl-CoA and Medica 16-CoA). Finally, in support of these assays showing that PPARα has high affinity for unsaturated, but not saturated LCFA-CoAs, recent data with a fluorescent ligand (trans-parinaric acid) displacement assay also determined that PPARα binds naturally occurring unsaturated (but not saturated) LCFA-CoAs with high affinity as shown by Kᵰ values of 5–17 nM (20). Although these high affinity Kᵰ values for LCFA-CoAs and unsaturated LCFA-CoAs are in contrast to the 2–3-order of magnitude weaker Kᵰ values obtained by radioligand-based assays, it is known that such radioligand binding assays underestimate the affinities of other LCFA binding proteins (L-FABP and SCP-2) (reviewed in Refs. 17–19) and LCFA-CoA binding proteins (acyl-CoA-binding protein, SCP-2, and L-FABP) (reviewed in Refs. 18 and 28–30). Together, these data provide significant evidence indicating that PPARα exhibits high affinity (low nanomolar Kᵰ values) for unsaturated LCFA-CoAs, saturated LCFA-CoAs, and unsaturated (but not saturated) LCFA-CoAs. These findings are consistent with endogenous LCFA-CoAs as well as unsaturated (but not saturated) LCFA-CoAs being physiologically significant ligands for PPARα.
binding pockets of many transcription factors are highly hydrophobic, and it has been proposed that the ligand-binding pocket of PPARα is more hydrophobic than the binding pockets of other LCFA-binding proteins (20). In the work presented herein, the hydrophobicity of the PPARα ligand-binding pocket was shown by the increase in fluorescence intensity, as well as the blue shift of NBD-stearate emission maxima, as binding occurs. This shift of about 30 nm for NBD-stearate fluorescence emission was similar to that previously reported for NBD-stearate bound to another nuclear transcription factor (hepatocyte nuclear factor-4α), which displays a blue shift of 32 nm (33). Similar studies involving cytoplasmic binding proteins demonstrate smaller blue shifts, with an NBD-stearate-bound SCP-2 shift of 25.5 nm and an NBD-stearate-bound L-FABP shift of only 12.7 nm (50). Comparison of these shifts suggests that the ligand-binding pocket of PPARα is similar in hydrophobicity to that of other nuclear transcription factors (e.g. hepatocyte nuclear factor-4α) and more hydrophobic than that of many LCFA-binding proteins (SCP-2 and L-FABP). PPARα binding to trans-parinaric acid and cis-parinaroyl-CoA resulted in a similar, although smaller, blue shift in fluorescence emission maxima, from 422 to 416 nm. Smaller blue shifts have previously been reported for several cytoplasmic binding proteins, including cis- or trans-parinaroyl-CoA-bound acyl-CoA-binding protein (29), cis- or trans-parinaric acid-bound L-FABP (18), and cis- or trans-parinaric acid-bound SCP-2 (30).

Previous work focusing on the effect of the dielectric constant of the environment of NBD-stearate on maximum emission wavelength allows for the correlation of this blue shift to the dielectric properties of the microenvironment of the ligand-binding site (50). This extrapolation based upon the maximal emission wavelength of NBD-stearate bound to PPARα yielded a relative dielectric constant less than 2, indicating that the bound substrate was localized within a highly hydrophobic microenvironment. This value was essentially the same as that previously acquired for NBD-stearate bound to hepatocyte nuclear factor-4α protein (33) and smaller than those obtained for the cytoplasmic binding proteins mentioned above (50). Finally, the intermolecular distance between PPARα aromatic residues and bound trans-parinaric acid and bound cis-parinaroyl-CoA was determined to be ~25 Å by FRET techniques. Together, these data were consistent with LCFA and LCFA-CoA binding representing close molecular interaction with PPARα rather than nonspecific binding.

Third, the high affinities (i.e. low nanomolar $K_d$ values) exhibited by PPARα for naturally occurring LCFA-CoAs and LCFAs were in the range of concentrations of these ligands in the nucleoplasm. Resolving these issues was important, because the concentration of LCFA-CoA in the nucleoplasm is thought to be in the nanomolar rather than micromolar range. Recent confocal fluorescence imaging of nonmetabolizable fluorescent LCFA-CoAs in living cells shows that nucleoplasmic LCFA-CoAs represents 3–12% of cellular LCFA-CoAs, depending on the fatty acid, such that nucleoplasmic LCFA-CoA concentrations are in the range of 39–68 nM (15, 16). Likewise, LCFA-CoA levels in the nucleoplasm are even lower, in the 3 nM range (16). Although expression of cytoplasmic LCFA- and LCFA-CoA-binding proteins such as the liver fatty acid-binding protein can significantly increase the distribution of both LCFA-CoA and LCFA-CoAs to the nucleoplasm, the nucleoplasmic LCFA and LCFA-CoA levels are still in the nanomolar range, 120–500 nM and 8 nM, respectively (15).

Fourth, the finding that PPARα exhibits very high affinity (1–14 nM $K_d$ values) for both saturated and unsaturated LCFA-CoAs may help to explain the similar effects of dietary saturated and unsaturated LCFA-CoAs on PPARα activation. In trans-activation assays wherein cultured cells are supplemented with exogenous LCFA-CoAs, both saturated (palmitic) and unsaturated (arachidonic, linoleic, and linolenic, oleic) fatty acids enhance PPARα transactivation nearly equally well (5, 9, 12, 13, 22–24). Likewise, in dietary studies, wherein rats were fed high fat diets, PPARα-activated gene expression was increased, regardless of whether the dietary lipid composition was mostly polyunsaturated, monounsaturated, or saturated (25). Since PPARα exhibits high affinity (low nM $K_d$ values) for unsaturated LCFA-CoAs as well as their CoA derivatives, either or both of these ligands could contribute to PPARα activation. In contrast, since PPARα exhibits high affinity (low nanomolar $K_a$ values) for saturated LCFA-CoAs, but not for saturated LCFA-CoAs, the LCFA-CoAs formed by intracellular fatty acyl-CoA synthetases from dietary saturated LCFAs may represent the high affinity endogenous ligands, accounting for PPARα transactivation and activation by exogenous saturated LCFA-CoAs.

Thus, the ligand binding data obtained herein may reconcile the PPARα transactivation and PPARα activation data obtained using similar effects with saturated and unsaturated LCFA-CoAs. Taken together, these studies would suggest that, especially in the case of saturated LCFAs, a metabolite (e.g. LCFA-CoA) rather than the saturated LCFA itself might be the active endogenous PPARα ligand.

Fifth, PPARα binding of both saturated and unsaturated LCFA-CoAs as well as unsaturated (but not saturated) LCFA-CoAs significantly altered PPARα structure. Such ligand-induced conformational alterations are a requisite for ligand-regulated nuclear receptors (35, 52). Two studies suggested that binding of saturated LCFA-CoAs, unsaturated LCFA-CoAs, and unsaturated (but not saturated) LCFA-CoAs to PPARα elicited conformational change therein. (i) Binding of these ligands significantly quenched the PPARα aromatics amino acid fluorescence emission, consistent with a reorientation of one or more of these residues from the more hydrophobic interior of the protein to the more aqueous exposed protein surface. (ii) This ligand-induced structural change in PPARα was also confirmed by circular dichroism, which resulted in large molar ellipticity changes in the presence of compounds demonstrated to bind with high affinity (low nanomolar $K_d$ values) to PPARα and no change in the presence of compounds that do not bind or only weakly bind (e.g. saturated LCFA) to PPARα. Binding of unsaturated (but not saturated) LCFA-CoAs, led to an overall decrease in PPARα content of α-helices and an increase in β-sheets. Similar results were obtained for both saturated and unsaturated LCFA-CoAs with even larger conformational changes. Thus, both types of ligands elicited the requisite conformational change in PPARα required for ligand-activated nuclear receptors, providing further support that these compounds may be the endogenous ligands.

Sixth, co-immunoprecipitation of native PPARα and SRC-1 proteins from liver homogenate in the presence of exogenous ligands demonstrated that these ligand-induced conformational changes correlated to co-activator recruitment. In the presence of high affinity PPARα ligands (unsaturated fatty acid, fatty acyl-CoA, bezafibrate-CoA, and nonhydrolyzable S-hexadecyl-CoA), co-immunoprecipitation of PPARα and SRC-1 was increased, whereas in the presence of ligands that bound with lower affinity (saturated fatty acid, bezafibrate), co-immunoprecipitation was similar to that in the absence of ligand. These data suggest that PPARα ligands, which induce strong conformational changes (i.e. decreased α-helices and increased β-sheets), are required for maximal PPARα transactivation or activation. Previous experiments suggest that LCFA-CoA binding does not affect recruitment of co-activators such as PBP (49), whereas antagonizing the recruitment of other co-activa-
tors such as SRC-1 (26, 27, 49). Whereas the basis for the discrepancy between the latter findings and the results presented herein are not known, several factors may contribute. (i) In the present investigation, co-activator recruitment was determined from liver homogenates containing full-length native PPARα and SRC-1 proteins rather than the truncated recombinant PPARα ligand binding domain and co-activator proteins in buffer (26, 27, 49). (ii) In the present investigation, more physiologically relevant concentrations of ligands were added, in contrast to the much higher levels of free ligand utilized in the earlier studies (26, 27, 49). (iii) The present investigation co-immunoprecipitated PPARα and SRC-1 from liver homogenate, which contains not only SRC-1, but also many other co-factors that may contribute to co-activator binding/stability. In contrast, the earlier in vitro assays with purified PPARα ligand binding domain and SRC-1 did not contain such components (26, 27, 49). (iv) In contrast to the data in Fig. 9, some earlier studies presented only single values (49), which precluded evaluation of significance. Finally, the data presented herein, but not those of the earlier in vitro studies with purified and truncated PPARα (26, 27, 49), further explain how the addition of saturated LCFA-CoAs can increase transactivation and activation of PPARα in cultured cells (5, 9, 12, 13, 22–24) and in vivo (25), thereby supporting the idea that both LCFA-CoAs and unsaturated LCFA-CoAs function as endogenous ligands for PPARα.

Finally, studies involving gene targeting in mice provide physiologically relevant evidence supporting the possibility that both unsaturated fatty acids and fatty acyl-CoAs are endogenous ligands of PPARα. Although early studies with bound xenobiotics and LCFA-CoAs suggest that the carboxyl group was necessary for ligand binding, there has been some speculation as to whether the activation of ligands to LCFA-CoA thioesters was a prerequisite for ligand binding. For many years, there has been an on-going debate as to the role of the carboxylic function of xenobiotic carboxylic compounds regarding peroxisomal proliferation, and there is much evidence to suggest that acylation of this carboxylic group occurs and may even be required for peroxisomal proliferation. The administration of peroxisome proliferators to mice has been found to cause an increase in acyl-CoA oxidase and bifunctional protein, the first two enzymes of the β-oxidation pathway (57), as well as an increase in the level of CoA thioesters of the peroxisome proliferators (58, 59). Furthermore, the addition of 2-bromopalmityl-CoA, which functions as a general inhibitor of fatty acid thioesterification, was found to inhibit the effects of bezafibrate on peroxisomal proliferation (60). More recent experiments utilizing knock-out mice to study the expression of genes regulated by PPARα have implicated LCFA-CoAs and other putative substrates for acyl-CoA oxidase as the biological ligands of PPARα. For animals deficient for the acyl-CoA oxidase gene, there is an increase in very long chain fatty acid levels in the serum, and since the very long chain LCFA-CoAs are incapable of entering the β-oxidation pathway, they accumulate and hyperactivate PPARα (61). Conversely, animals deficient for the adrenoleukodystrophy protein gene, which prevents very long chain fatty acids and/or their CoAs from being transported to the peroxisomes, display elevated levels of very long chain fatty acids and normal levels of their CoAs and normal PPARα activity (62). Although 1-peroxisomal bifunctional enzyme and 2-peroxisomal bifunctional enzyme double knock-out mice do not display peroxisomal proliferation, up-regulation of PPARα target genes occurs, further suggesting that substrates of peroxisomal β-oxidation are PPARα ligands (40). This suggests that very long chain acyl-CoAs (>20C) and very long chain enoyl-CoAs may be functioning as PPARα ligands to activate PPARα in acyl-CoA oxidase and peroxisomal bifunctional enzyme knock-out mice, respectively. Although beyond the scope of the current study, the possibility that very long chain acyl-CoAs and very long chain enoyl-CoAs may also serve as endogenous PPARα ligands warrants further study. Overall, these data show that gene alteration resulting in high levels of acyl-CoAs cause PPARα activation, whereas gene alterations resulting in reduced CoA levels result in PPARα inactivation.

Finally, the studies presented herein also bear on the role of acyl-CoAs interacting with other PPAR isoforms. Early radio-ligand binding assays of human PPARγ and LCFA-CoAs (palmitoyl-CoA, oleoyl-CoA, linoleoyl-CoA) yielded micromolar Kᵣ values comparable with those obtained with the corresponding LCFA-CoAs; however, this affinity was 4–17-fold lower than for PPARα (26). More recently, PPARα and PPARδ have displayed similar sensitivity to chymotrypsin digestion in the presence of S-hexadecyl-CoA, whereas only a minor effect was noted for PPARγ (49). GST pull-down experiments have shown that the addition of S-hexadecyl-CoA has similar effects on PPARα and PPARδ, and opposing effects in PPARγ (49). This, as well as the overall structure similarity, suggests that each PPAR subtype may interact with acyl-CoAs at some level, but the effects in vivo may differ. Although beyond the scope of the present investigation, it would be interesting in future studies to directly examine the affinities of these other PPAR isoforms for acyl-CoAs using the approaches presented herein.

In conclusion, several fluorescence spectroscopic assays as well as circular dichroism techniques clearly demonstrate that PPARα binds LCFA-CoAs and unsaturated (but not saturated) LCFA-CoAs with high affinity (nanomolar Kᵣ values). PPARα bound these ligands with affinities similar to those for certain peroxisome proliferators and, even more so, their CoA derivatives. Only high affinity ligands induced a conformational change in PPARα structure upon binding. The high affinities for these ligands (i.e. very low nanomolar Kᵣ values) were in the same range as concentrations of LCFA-CoAs and LCFA-CoAs in the nucleoplasm of living cells. Thus, the LCFA-CoAs and unsaturated (but not saturated) LCFA-CoAs fulfill three of the most important criteria for ligand-activated nuclear receptors such as PPARα (i.e. high affinity (i.e. nanomolar Kᵣ), ligand-induced conformation change, and affinities in the range of nuclear concentrations of these ligands (i.e. low nanomolar). These findings suggest that the both saturated and unsaturated LCFA-CoAs as well as unsaturated (but not saturated) LCFA-CoAs may represent specific, high affinity, endogenous, functional ligands for PPARα.

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LCFA-CoAs and Fatty Acids Bind PPARα and Alter Conformation
