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## Very-Long-Chain and Branched-Chain Fatty Acyl-CoAs are High Affinity Ligands for the Peroxisome Proliferator-Activated Receptor $\alpha$ (PPAR $\alpha$ )<sup>†</sup>

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### Abstract

Very long chain fatty acids (VLCFA) and branched-chain fatty acids (BCFA) are potent inducers of the peroxisome proliferator-activated receptor PPAR $\alpha$ , a nuclear receptor that enhances transcription of peroxisomal enzymes mediating  $\beta$ -oxidation of these potentially toxic fatty acids. However, it is not known whether the respective free fatty acids or their activated metabolites, i.e. CoA thioesters: (i) are the endogenous high-affinity PPAR $\alpha$  ligands, (ii) alter PPAR $\alpha$  conformation, and (iii) alter recruitment of coregulatory proteins to PPAR $\alpha$ . As shown by quenching of PPAR $\alpha$  intrinsic amino acid fluorescence, PPAR $\alpha$  exhibited high affinity (3–29 nM  $K_d$ s) for the CoA thioesters of the common (C20–C24) VLCFA. In contrast, with the exception of arachidonic acid ( $K_d$ =20 nM), PPAR $\alpha$  only weakly bound the VLCFA. PPAR $\alpha$  also exhibited higher affinity for the CoA thioesters of BCFA (phytanoyl-CoA, pristanoyl-CoA;  $K_d$ s near 11 nM) than for the respective free branched-chain fatty acids. As shown by circular dichroism, the high affinity VLCFA-CoA and BCFA-CoA strongly altered PPAR $\alpha$  conformation. Likewise, the high affinity VLCFA-CoA and BCFA-CoA altered co-factor recruitment to PPAR $\alpha$  as shown by co-immunoprecipitation from liver homogenates. In contrast, nearly all the respective free fatty acids elicited only weak conformational changes in PPAR $\alpha$  and did not alter co-factor recruitment to PPAR $\alpha$ . In summary, the CoA thioesters of very-long-chain and branched-chain fatty acids are much more potent PPAR $\alpha$  ligands than the free acids, resulting in altered PPAR $\alpha$  conformation and cofactor recruitment. Since these are hallmarks of ligands-activated nuclear receptors, this suggests that the CoA thioesters are the active forms of these PPAR $\alpha$  ligands.

### Keywords

PPAR $\alpha$ ;  $\beta$ -oxidation; fatty acyl-CoA; phytanic acid; fluorescent binding assays; circular dichroism; nuclear transcription factors

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Peroxisome proliferator-activated receptors (PPAR) are ligand-activated members of the steroid/thyroid nuclear hormone receptor superfamily. PPAR have been identified in several species, with at least three isotypes recognized ( $\alpha$ ,  $\beta/\delta$ ,  $\gamma$ ). Each isotype, encoded by a different gene, is differentially expressed in select tissues and expression levels depend on cellular

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processes (rev. in (1). Although there is some overlap in ligand specificity, unique isotype binding occurs by a variety of xenobiotics depends upon specific amino acid substitutions within the PPAR ligand-binding pocket (1,2). Independent of ligand binding, a basal level of transcription occurs by PPAR forming a heterodimer with the retinoid X receptor (RXR) which binds to a specific DNA response element (PPRE) within the target genes (3,4). Ligand binding to one and, even more so, both receptors greatly potentiates transcription (rev. in (4). PPRE exist within many of the genes involved in fatty acid oxidation and cell differentiation, suggesting that PPAR are responsible for regulating transcription of these genes (5).

An especially important role of PPAR $\alpha$  is inducing transcription of multiple peroxisomal enzymes required for  $\beta$ -oxidation of very-long-chain fatty acids (VLCFA) and branched-chain fatty acids (BCFA). VLCFA and BCFA accumulation are associated with human neurological disorders including Zellweger syndrome, X-linked adrenoleukodystrophy, Refsum disease, and MFP-2 deficiency (6-8). These genetic disorders arise from defects in enzymes of the peroxisomal  $\beta$ -oxidation pathway which result in accumulation of VLCFA, BCFA, and their thioesters (6-8). Both VLCFA and isoprenoid-derived BCFA preferentially undergo peroxisomal  $\beta$ -oxidation following acylation of the respective fatty acids to CoA thioesters. The classical peroxisomal  $\beta$ -oxidation pathway catalyzed by palmitoyl-CoA oxidase (AOX), L-bifunctional protein (L-PBE), and 3-ketoacyl-CoA thiolase (PTL) is responsible for the oxidation of straight-chain fatty acids (9). All three enzymes of the classical peroxisomal  $\beta$ -oxidation system are transcriptionally activated by PPAR $\alpha$  ligands (rev. in (10). In contrast, although the branched-chain phytanic acid is considered a  $\beta$ -oxidation pathway precursor, a different set of peroxisomal enzymes are utilized for oxidation of BCFA. For example, phytanic acid must first undergo  $\alpha$ -oxidation to produce pristanoyl-CoA before entering the  $\beta$ -oxidation cycle (11). BCFA-CoAs are then utilized by the branched-chain acyl-CoA oxidase, D-bifunctional protein (D-PBE), and sterol carrier protein-x (SCP-x). Both D-PBE (9) and SCP-x (12,13) are inducible upon administration of branched-chain PPAR $\alpha$  ligands.

While there is evidence indicating that VLCFA and BCFA are peroxisome proliferators and may be PPAR $\alpha$  ligands, it is not completely clear whether the unesterified VLCFA and BCFA directly and/or metabolites thereof activate PPAR $\alpha$ . For example, early transactivation assays show: (i) PPAR $\alpha$  is activated by polyunsaturated (20:4, 20:5, 22:6), but not monounsaturated (24:1, 22:1) VLCFA (3), (ii) n-3 polyunsaturated VLCFA elicited similar PPAR $\alpha$  activation levels as n-6 polyunsaturated VLCFA (3). These findings of VLCFA being potential PPAR $\alpha$  ligands are supported by gel-shift assays (14) and co-activator-dependent receptor ligand assays (15). Likewise, transactivation studies with branched-chain fatty acids also show PPAR $\alpha$  activation (11). It must be noted that most of these assays utilize very high concentrations of VLCFA (i.e. 30-100  $\mu$ M) and BCFA (20  $\mu$ M)--several orders of magnitude higher than concentrations of unesterified fatty acids detected in nuclei of living cells (16, 17). These data suggested that affinities of PPAR $\alpha$  for these ligands are very weak, i.e.  $K_d$ s in the  $\mu$ M range, and the free fatty acids may not represent the endogenous ligands. The finding that, since unesterified fatty acids are potent detergents, they are rapidly activated/metabolized to their CoA thioesters in cells and tissues (rev. (18-20), suggests that metabolites such as the CoA thioesters may represent the endogenous PPAR $\alpha$  ligands. This possibility is supported by recent findings in mice. For instance, peroxisome proliferators increase the level of CoA thioesters of the peroxisome proliferators in mice (21,22). Inhibitors of fatty acid thioesterification such as 2-bromopalmitic acid inhibit the effects of bezafibrate on peroxisomal proliferation (23). Mice with a disruption of the AOX gene exhibit peroxisomal proliferation, elevated serum levels of VLCFA, as well as elevated VLCFA-CoAs, hyperactivation of PPAR $\alpha$ , and up-regulation of down-stream genes which contain PPRE (23). On the other hand, ablation of the adrenoleukodystrophy gene inhibits VLCFA transport into peroxisomes and results in elevated serum VLCFA, unaltered liver VLCFA-CoA, and normal PPAR $\alpha$  activity

(10). Taken together with other studies (24,25) these data in both normal and gene-targeted mice suggest that intermediates of peroxisomal  $\beta$ -oxidation are PPAR $\alpha$  ligands.

In summary, while many of the above experiments are suggestive that the CoA thioesters of VLCFA and BCFA represent functional ligands for PPAR $\alpha$ , it remains to be shown whether the VLCFA, BCFA, or their CoA thioesters exhibit characteristic hallmarks of endogenous ligands that bind to PPAR $\alpha$ : (i) high ( $K_{ds}$  in nM range) affinity, (ii) physically alter the structure/conformation of the PPAR $\alpha$  protein, and (iii) affect co-factor recruitment PPAR $\alpha$  (26). The objective of the present study was to begin to resolve these issues through use of direct ligand binding assays (i.e. non-fluorescent ligand binding assays based on quenching of tyrosine emission), circular dichroism to characterize potential ligand-induced changes in PPAR $\alpha$  secondary structure, and co-immunoprecipitation to study co-factor recruitment.

## EXPERIMENTAL PROCEDURES

### Chemicals

Arachidic acid (C20:0), arachidonic acid (C20:4, n-6), eicosapentaenoic acid (C20:5, n-3), behenic acid (C22:0), docosapentaenoic acid (C22:5, n-3), docosahexaenoic acid (C22:6, n-3), lignoceric acid (C24:0), nervonic acid (C24:1, n-6), phytanic acid (3,7,11,15-tetramethyl-hexadecanoic acid), 2-fluoropalmitic acid, 1-Iodohexadecane, and coenzyme A were from Sigma (St. Louis, MO). Pristanic acid (3,7,11,15-tetramethyl-pentadecanoic acid) was from Larodan Fine Chemicals (Malmö, Sweden). A-bromopalmitate was from City Chemical LLD (West Haven, CT). Purity of fatty acids was examined by HPLC (27) and determined to be greater than 95%. All fatty acyl-CoAs were synthesized as previously described (28), purified by HPLC (27), and found to be >98% unhydrolyzed. Mouse anti-PPAR $\alpha$  monoclonal antibodies, as well as rabbit anti-PPAR $\alpha$ , anti-steroid receptor coactivator-1 (SRC-1), and anti-cyclic AMP-responsive enhancer binding protein binding protein (CBP) polyclonal antibodies, were from Affinity BioReagents (Golden, CO). Anti-rabbit IgG secondary antibodies-HRP conjugated were from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein assay, mammalian co-immunoprecipitation kit, chemiluminescent substrate, and film were from Pierce Chemical Co. (Rockford, IL).

### Recombinant mouse PPAR $\alpha$ protein

The cDNA encoding mouse PPAR $\alpha$  with a deletion of the amino-terminal A/B domain (i.e. encoding PPAR $\alpha$  amino acids 101-468) was cloned into a (His)<sub>6</sub>-tagged bacterial expression vector (pET-PPAR $\alpha$  $\Delta$ AB) and was a generous gift from Dr. Noa Noy (Cornell University, USA) (29). The recombinant protein was expressed, purified, and analyzed by SDS-PAGE and Western blotting as previously described (30). Protein concentration was determined by Bradford assay. Since Bradford assays underestimate the mass of proteins such as liver fatty acid binding protein, uncorrected Bradford assays can result in considerable disagreement in the number of binding sites obtained by solution binding assays (e.g. radioligand, fluorescence) as compared to crystallography (18,31,32). Since both X-ray crystallography (33,34) and fluorescence binding assays (29,30) are in complete agreement that PPAR $\alpha$  has only a single binding site, this indicates that the Bradford assay is not underestimating PPAR $\alpha$  protein mass.

### Direct fluorescence binding assay: Quenching of PPAR $\alpha$ $\Delta$ AB aromatic amino acid residues by non-fluorescent ligands

The direct binding of non-fluorescent ligands to PPAR $\alpha$  $\Delta$ AB was carried out as described (30,35). Briefly, PPAR $\alpha$  $\Delta$ AB (0.1 $\mu$ M in 2ml of PBS, pH 7.4) was titrated with increasing ligand (0-2000nM). Both fatty acids and fatty acyl-CoAs are molecules with limited solubility in aqueous buffer as evidenced by critical micelle concentrations in the typical range of 1 $\mu$ M (36) and 60-200 $\mu$ M (20), respectively. For the majority of the binding assays, the ligand

concentration examined was 0-300nM, well below the critical micelle concentration. Only in the rare exception where fatty acid binding was not seen at 300nM was the examined concentration increased above 500nM. PPAR $\alpha$ ΔAB aromatic amino acid excitation was carried out at 280nm and emission was measured from 300-400nm. Fluorescence emission spectra were obtained at 24°C with a PC1 photon counting spectrofluorometer (ISS Inc., Champaign, IL), corrected for background (buffer and solvent effects), and maximal intensities measured. The dissociation constant ( $K_d$ ) and the number of binding sites ( $n$ ) were calculated as previously described (30).

### Secondary Structure Determination: Effect of Ligand Binding on PPAR $\alpha$ ΔAB Circular Dichroism (CD)

Circular dichroic spectra of PPAR $\alpha$ ΔAB (0.8 $\mu$ M in 125 $\mu$ M HEPES, pH 8.0, 12.5 $\mu$ M DTT, 5mM KCl, 0.3% glycerol) were taken in the presence and absence of ligands (20 $\mu$ M) with a J-710 spectropolarimeter (JASCO Inc., Easton, MD) at 23°C in a 1-mm cuvette as described (30,35). Spectra were recorded from 280 to 185nm with a bandwidth of 2nm, sensitivity of 10 millidegrees, scan rate of 50nm/min, and a time constant of 1s. Ten scans were averaged for percent composition of  $\alpha$ -helices,  $\beta$ -strands, turns, and unordered structures with the software package CDPro (downloaded from the website: <http://lamar.colostate.edu/~sreeram/CDPro>), which allows the percent calculation of various secondary structures by three different methods; SELCON3, CDSSTR, and CONTIN/LL (37).

### Co-immunoprecipitation: Effects of ligand binding on co-factor recruitment

Livers from C57BL/6 male mice were homogenized in M-PER® buffer (Pierce Chemical Co., Rockford, IL) containing 150mM sodium chloride and protease inhibitors, and centrifuged to pellet insoluble debris. Protein concentration was determined by BCA protein assay. Ligands (100 $\mu$ M) were added to 2mg of liver homogenate, and co-immunoprecipitation procedures with a PPAR $\alpha$  monoclonal antibody SDS-PAGE were conducted with the ProFound™ mammalian co-immunoprecipitation kit according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Protein transfer and western blotting were performed using standard techniques (30). Proteins were 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 PPAR $\alpha$  values, 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. Statistical analysis was performed using the Student's *t* test.

## RESULTS

### Direct Binding of Very-Long-Chain Fatty Acids to PPAR $\alpha$ : Quenching of PPAR $\alpha$ Intrinsic Aromatic Amino Acid Fluorescence

Since early radioligand fatty acid and fatty acyl-CoA binding assays are known to underestimate the affinities of binding proteins by several orders of magnitude (rev. in (18,19)), an assay not dependent upon an exogenous radiolabeled or fluorescent ligand or upon physical separation of bound from free ligand was utilized. This assay, recently developed by our laboratory (30, 35), takes advantage of PPAR $\alpha$ 's intrinsic aromatic amino acid fluorescence which is quenched upon ligand binding. In the case of saturated VLCFA, saturation binding curves were obtained for the 20-carbon arachidic acid (Fig. 1A), but not the longer chain 22-carbon behenic acid (Fig. 2A) and 24-carbon lignoceric acid (Fig. 3A). Analysis of multiple replicates showed that PPAR $\alpha$  bound arachidic acid with a  $K_d$  of 76 $\pm$ 12nM at a single binding site (Table 1). This was confirmed by Hill plot (Fig. 1A) which also yielded one arachidic binding site ( $n = 1.0$ ) for PPAR $\alpha$ . Thus, saturated VLCFA were poorly bound by PPAR $\alpha$ .

In general, inclusion of unsaturation in the VLCFA enhanced the abilities of 20-carbon, and to a much lesser extent 22- and 24-carbon, VLCFA to be bound by PPAR $\alpha$ . For example, nervonic acid (24-carbon fatty acid containing a single double bond) exhibited a modest saturation curve (Fig. 3B) and very weak  $K_d$  of 769nM (Table 1). VLCFA with larger numbers of double bonds were bound better, but in a pattern highly dependent on chain-length and position of the double bonds. For instance, the n-6 polyunsaturated 20-carbon fatty acid arachidonic acid (C20:4) (Fig. 1B) exhibited a steeper saturation curve than the n-3 polyunsaturated 22-carbon fatty acid docosahexaenoic acid (C22:6) (Fig. 2C), while the n-3 eicosapentaenoic acid (C20:5) did not exhibit significant binding except at very high concentrations (Fig. 1C) and the n-3 22-carbon docosapentaenoic acid (C22:5) was not bound (Fig. 2B). This was reflected in much higher affinity exhibited by PPAR $\alpha$  for the n-6 arachidonic acid ( $K_d=20\pm 5$ nM) than for the n-3 docosahexaenoic acid ( $K_d=145\pm 48$ nM), eicosapentaenoic acid, and docosapentaenoic acid whose  $K_d$ s were so weak as not to be measurable (Table 1). In summary, with the exception of the n-6 polyunsaturated arachidonic acid, affinities for the other saturated and n-3 VLCFA were relatively weak (arachidic acid, docosahexaenoic acid) or not detectable (eicosapentaenoic acid, docosapentaenoic acid, lignoceric acid), regardless of the presence of double bonds. These data were not consistent with most of the VLCFA being physiologically significant PPAR $\alpha$  ligands.

### Direct Binding of Very-Long-Chain Fatty Acyl-CoAs to PPAR $\alpha$

To test PPAR $\alpha$ 's specificity and affinity for the CoA thioesters of the above VLCFA, the effect of the respective VLCFA-CoAs on quenching of PPAR $\alpha$  intrinsic aromatic amino acid fluorescence was examined. The results showed for the first time, contrary to the free VLCFA, the CoA thioesters of all the VLCFA bound with high affinity to PPAR $\alpha$  and at a single site on PPAR $\alpha$ . For example, each of the saturated VLCFA-CoAs exhibited saturation binding curves that were significantly steeper than the respective VLCFA: the 20-carbon arachidonyl-CoA (Fig. 1D) > arachidonic acid (Fig. 1A); the 22-carbon behenoyl-CoA (Fig. 2D) >>> behenic acid (Fig. 2A); the 24-carbon lignoceroyl-CoA (Fig. 3C) >>> lignoceric acid (Fig. 3A). Analysis of multiple binding curves yielded  $K_d$ s in the following order: arachidonoyl-CoA ( $K_d=16\pm 1$ nM) > behenoyl-CoA ( $K_d=6\pm 1$ nM) > lignoceroyl-CoA ( $K_d=3\pm 1$ nM) (Table 1). Likewise, the CoA thioester of nervonic acid was bound with high affinity ( $K_d=9\pm 1$ nM)—85-fold better than the free nervonic acid (Table 1). In contrast to the free VLCFA, the CoA thioesters of both n-6 VLCFAs (arachidonoyl-CoA,  $K_d=16\pm 1$ nM) and n-3 VLCFA (eicosapentaenoyl-CoA,  $K_d=29\pm 4$ nM; docosapentaenoyl-CoA,  $K_d=10\pm 1$ nM; docosahexaenoyl-CoA,  $K_d=11\pm 2$ nM) were bound with very high and essentially the same affinities by PPAR $\alpha$  (Table 1). Thus, PPAR $\alpha$  exhibited very high affinities for all the CoA thioesters of VLCFA. In contrast, PPAR $\alpha$  exhibited very weak or no measurable affinity for the free VLCFA. Since polyunsaturated VLCFA (both n-6 and n-3) are known to activate PPAR $\alpha$  in cells and animals (3), these data suggest that the respective VLCFA-CoAs, rather than the free acids, may be the active endogenous ligands for PPAR $\alpha$ .

### Direct Binding of Branched-Chain Fatty Acids and their CoA Thioesters to PPAR $\alpha$

Although phytanic acid is also a 20-carbon fatty acid, it differs markedly from the  $\geq 20$ -carbon chain VLCFA. Unlike the straight-chain VLCFA, phytanic acid's chain is only 16 carbons long and has 4 methyl side chains. Further, branched-chain fatty acids such as phytanic acid and its  $\alpha$ -oxidation product pristanic acid are the most potent known naturally-occurring PPAR $\alpha$  transcriptional activators—much more potent than even the unsaturated VLCFA (rev. in (38-40)). However, it is not known whether: (i) phytanic acid or its  $\alpha$ -oxidation product (i.e. pristanic acid) is the endogenous PPAR $\alpha$  ligand, and (ii) the respective CoA thioesters of the branched-chain fatty acids are the endogenous PPAR $\alpha$  ligands. These questions were resolved by the direct fluorescent binding assay described above. PPAR $\alpha$  exhibited saturation binding curves for both phytanic acid (Fig. 4A) and pristanic acid (Fig. 4B). Double reciprocal plots



were linear (inserts, Figs. 4A,B), indicating PPAR $\alpha$  had a single binding site for each of these branched-chain fatty acids. Analysis of multiple binding curves indicated that PPAR $\alpha$  bound branched-chain fatty acids with high affinities in the order: pristanic acid ( $K_d=19\pm 2$  nM) > phytanic acid ( $K_d=34\pm 4$ nM) (Table 1). Although pristanic acid was a 2-fold higher affinity ligand, nevertheless the parent phytanic acid itself was a high affinity PPAR $\alpha$  ligand. Thus, phytanic acid did not need to be metabolized to pristanic acid in order to be a high affinity ligand for PPAR $\alpha$ . The CoA thioesters of branched-chain fatty acids were also bound with high affinity by PPAR $\alpha$  in the order: pristanoyl-CoA ( $K_d=12\pm 1$ nM) > phytanoyl-CoA ( $K_d=11\pm 1$ nM) (Table 1). While the affinities of PPAR $\alpha$  for the respective CoA thioesters of branched-chain fatty acids were higher than for the free acids, the difference was only 2-3 fold and PPAR $\alpha$  exhibited high affinities (nM  $K_d$ s) for both the free branched-chain fatty acids and their CoA thioesters. Thus, unlike the VLCFA, the branched-chain fatty acids as well as their CoA thioesters were high affinity ligands for PPAR $\alpha$ .

### Direct Binding of Non-metabolizable Fatty Acids and Non-hydrolyzable Fatty Acyl-CoAs to PPAR $\alpha$

Since all the above fatty acids (saturated, unsaturated, and branched-chain) can be metabolized to their CoA thioesters and further transesterified to more complex lipids (41), the ability of PPAR $\alpha$  to bind non-metabolizable saturated fatty acids, 2-bromopalmitic acid and 2-fluoropalmitic acid, was examined. PPAR $\alpha$  exhibited a shallow saturation binding curve for 2-bromopalmitic acid (Fig. 4C) at a single site as indicated by the double reciprocal plot (Fig. 4C, insert). Similar results were obtained with 2-fluoropalmitic acid (binding curves not shown). Multiple replicates showed that PPAR $\alpha$ 's affinity for both 2-bromopalmitic acid and 2-fluoropalmitic acid was weak,  $K_d=808\pm 195$ nM and  $K_d=219\pm 33$ nM, respectively (Table 1).

Conversely, PPAR $\alpha$  is known to enhance the hydrolysis of fatty acyl-CoA thioesters (42) and therefore the possibility that the above findings of high affinity LCFA-CoA binding to PPAR $\alpha$  might reflect in part the binding of the breakdown products of the fatty acyl-CoAs, i.e. the hydrolytically released free fatty acids, was considered. Although most of the straight-chain VLCFA do not bind to PPAR $\alpha$  with high affinity, a few exceptions (arachidonic acid, phytanic acid, and pristanic acid) were bound by PPAR $\alpha$  with high affinity. Therefore, the affinity of PPAR $\alpha$  for a non-hydrolyzable fatty acyl-CoA analogue, *S*-hexadecyl-CoA (42), was examined. A sharp, saturable binding curve was observed upon titration of PPAR $\alpha$  with increasing amounts of *S*-hexadecyl-CoA (Fig. 4F). Analysis of multiple binding curves showed that PPAR $\alpha$  exhibited very high affinity for *S*-hexadecyl-CoA as indicated by a  $K_d=10\pm 1$ nM (Table 1). Thus, the data obtained with the non-metabolizable fatty acids and non-hydrolyzable CoA analogue further substantiated the role of CoA thioesters as the active form of PPAR $\alpha$  endogenous ligands.

### Effect of Very-Long-Chain Fatty Acids on PPAR $\alpha$ Secondary Structure: Circular Dichroism

Ligand-induced conformational change is a hallmark of ligand-regulated nuclear transcription factors, including PPAR (34,35,41,43). The observation that binding of non-fluorescent ligands significantly quenched the emission of PPAR $\alpha$  $\Delta$ AB aromatic amino acid residues (Figs. 1-4) suggests that ligand binding altered the conformation of PPAR $\alpha$ . To further examine whether the binding of naturally-occurring VLCFA alter PPAR $\alpha$  structure, the effect of these ligands on the circular dichroic (CD) spectra of PPAR $\alpha$  was examined. The far UV spectrum of PPAR $\alpha$  exhibited two minima at 207 and 221nm and one maximum at 193nm (Fig. 5, *filled circles*; Fig. 6-7, *open circles*), suggesting the presence of substantial  $\alpha$ -helical content in the PPAR $\alpha$  polypeptide chain. This was confirmed by quantitative analysis of the CD spectra as described in "Experimental Procedures", which indicated that PPAR $\alpha$  was comprised of 40.2%  $\alpha$ -helix, 14.5%  $\beta$ -sheets, 18.7%  $\beta$ -turns, and 26.6% unordered structures (Table 2). The addition of increasing concentrations of a high affinity ligand (arachidonic acid) caused conformational

changes that increased in intensity until starting to plateau off at a ratio of PPAR $\alpha$ :C20:4 of 1:5 (Fig. 5). The relative proportion of regular (Fig. 5, *open bars*) and distorted (Fig. 5, *diagonal bars*)  $\alpha$ -helices decreased, concomitant with regular  $\beta$ -sheets increasing (Fig. 5, *hatched bars*) as the ratio of PPAR $\alpha$ :C20:4 increased. Based upon these data and the fact that PPAR $\alpha$  had varying affinities for the examined ligands, a PPAR $\alpha$ /ligand ratio was chosen for the following CD experiments to allow comparison of maximal ligand-induced conformational changes, i.e. at saturated PPAR $\alpha$  ligand binding site.

Addition of saturated VLCFA only minimally affected the structure of PPAR $\alpha$ ; as seen by the 20-carbon arachidic acid (Fig. 6A, *filled circles*), but not the 22-carbon behenic acid (Fig. 6D, *filled circles*) and 24-carbon lignoceric acid (Fig. 6G, *filled circles*). Only in the case of arachidic acid were PPAR $\alpha$ 's molar ellipticity values at 207 and 221nm increased while concomitantly the values at 193nm decreased, indicating a reduction in PPAR $\alpha$   $\alpha$ -helical content and an increase in  $\beta$ -sheets. Quantitative analysis of multiple CD spectra confirmed that the 20-carbon, but not the 22- or 24-carbon, saturated VLCFA significantly altered PPAR $\alpha$  structure as shown by a 6.5% decrease in total  $\alpha$ -helices (Hr and Hd) concomitant with 3.6% increases in total  $\beta$ -sheets (Sr and Sd), 1.4% increase in  $\beta$ -turns, and 1.3% increase in unordered structures (Table 2). Thus, with the exception of the 20-carbon arachidic acid the saturated VLCFA did not significantly alter PPAR $\alpha$  secondary structure. Thus, these findings were entirely consistent with the relative affinities of PPAR $\alpha$  for the respective saturated VLCFA: 20-carbon arachidic (modest binding/CD change) >>> 22-behenic acid (no binding/no CD change), 24-carbon lignoceric acid (no binding/no CD change).

Unsaturated 20-carbon VLCFA altered the PPAR $\alpha$  secondary structure much more than the longer chain unsaturated VLCFA. For instance, the n-6 polyunsaturated 20-carbon fatty acid arachidonic acid (C20:4) (Fig. 6B, *filled circles*) significantly altered the shape of the circular dichroic spectrum of PPAR $\alpha$ , increasing the molar ellipticity values at 207 and 221nm while concomitantly decreasing the values at 193nm; indicating less  $\alpha$ -helical content and more  $\beta$ -sheets. Quantitative analysis of multiple CD spectra confirmed that the 20-carbon n-6 arachidonic acid (20:4) significantly altered PPAR $\alpha$  structure as shown by a 9.9% decrease in total  $\alpha$ -helices (Hr and Hd) concomitant with a 5.9% increase in total  $\beta$ -sheets (Sr and Sd), 2.3% increase in  $\beta$ -turns, and 1.9% increase in unordered structures (Table 2). These were the largest CD changes elicited by any of the polyunsaturated VLCFA in the order: n-6 20:4 (arachidonic acid, Fig. 6B *filled circles*, Table 2) > n-3 20:5 (eicosapentaenoic acid, Fig. 6C *filled circles*, Table 2) >>> n-3 22:5 (docosapentaenoic acid, Fig. 6E *filled circles*, Table 2), n-3 22:6 (docosahexaenoic acid, Fig. 6F *filled circles*, Table 2). The presence of a single double bond in the 24-carbon nervonic acid altered the CD spectrum of PPAR $\alpha$  (Fig. 6H) to alter the proportions of secondary structures (Table 2) very modestly. In summary, with the exception of the 20-carbon VLCFA (20:0 arachidic acid; n-6 20:4 arachidonic acid; n-3 20:5 eicosapentaenoic acid), the longer chain saturated and polyunsaturated VLCFA did not or only slightly altered the secondary structure of PPAR $\alpha$ . These data were not consistent with many of the free VLCFA directly being physiologically significant PPAR $\alpha$  ligands.

### Effect of Very Long Chain Fatty Acyl-CoAs on PPAR $\alpha$ Secondary Structure: Circular Dichroism

In contrast to the free acids, the CoA thioesters of all the VLCFA altered the secondary structure of PPAR $\alpha$  as shown by significant changes in CD spectra (Figs. 6A-H, *empty triangles*). This qualitative observation was confirmed by quantitative analysis of multiple replicates (Table 2). However, the nature of the circular dichroic alterations induced by the VLCFA-CoAs was distinct for each type of VLCFA-CoA as follows: 20-carbon (20:4-CoA, 20:5-CoA, 20:0-CoA), some 22-carbon (22:5-CoA), and some 24-carbon (24:0-CoA) decreased the proportion of PPAR $\alpha$   $\alpha$ -helices, while others among the 22-carbon (22:0-CoA; 22:6-CoA) and 24-carbon

(24:1-CoA) VLCFA-CoAs increased the proportion of PPAR $\alpha$   $\alpha$ -helices (Table 2). The results showed for the first time, contrary to the free VLCFA, the CoA thioesters of all the VLCFA altered the structure of PPAR $\alpha$ . However, the type of structure change (i.e. increase or decrease in PPAR $\alpha$ 's proportion of  $\alpha$ -helices) was highly dependent on the nature of the VLCFA-CoA. Again, since the polyunsaturated VLCFA (both n-6 and n-3) are known to activate PPAR $\alpha$  in cells and animals (3), these data suggest that the respective VLCFA-CoAs, rather than the free acids, may be the active endogenous ligands that alter PPAR $\alpha$  conformation.

### Effect of Branched-chain Fatty Acids and Acyl-CoAs on PPAR $\alpha$ Secondary Structure: Circular Dichroism

Since phytanic acid and even more so pristanic acid are the most potent known naturally-occurring PPAR $\alpha$  transcriptional activators—much more potent than even the unsaturated VLCFA (rev. in (38-40)—and both the free acids and their CoA thioesters were bound with high affinity by PPAR $\alpha$  (Table 1), it was predicted that both these branched-chain fatty acids would also elicit the largest alterations in PPAR $\alpha$  secondary structure. Both phytanic acid (Fig. 7A, *filled circles*) and pristanic acid (Fig. 7B, *filled circles*) elicited significant changes in molar ellipticity of PPAR $\alpha$ . Although similar effects were noted for the free acid (*filled circles*) and the CoA thioester form (*open triangles*), the effects of the CoA were much more pronounced, especially for phytanoyl-CoA. These results were consistent with phytanic acid and phytanoyl-CoA significantly reducing the relative proportions of  $\alpha$ -helical structures by 6.5% and 11.8%, respectively, and increasing the proportion of  $\beta$ -sheets by 4.3% and 7.1%, respectively, with smaller increases in  $\beta$ -turns and unordered structures (Table 2). Pristanic acid and pristanoyl-CoA also reduced the relative proportions of PPAR $\alpha$   $\alpha$ -helices by 8% and 8.6%, respectively, and increased the proportion of  $\beta$ -sheets by 4.1% and 5.1%, respectively, with smaller increases in  $\beta$ -turns and unordered structures (Table 2). In summary, the data indicated that phytanic acid did not need to be metabolized to pristanic acid to significantly alter PPAR $\alpha$  structure. In addition, unlike most of the VLCFA, both the free acids and CoA thioesters were very potent in inducing PPAR $\alpha$  structural changes. The phytanoyl-CoA induced changes in PPAR $\alpha$  structure were the strongest changes reported for any of the endogenous PPAR $\alpha$  ligands examined.

### Effect of Non-metabolizable Fatty Acids and Non-hydrolyzable Fatty Acyl-CoAs on PPAR $\alpha$ Secondary Structure: Circular Dichroism

Despite their relatively weak binding to PPAR $\alpha$ , the non-metabolizable 2-bromopalmitic acid and 2-fluoropalmitic acid significantly altered the CD spectra of PPAR $\alpha$  (Fig. 7C, *filled circles*, *filled inverted triangles*), resulting in a 6-7% decrease in total  $\alpha$ -helices (Hr and Hd) concomitant with a 3-4% increases in total  $\beta$ -sheets (Sr and Sd), with smaller increases in  $\beta$ -turns and unordered structures (Table 2). Consistent with the possibility of added substituents to the LCFA altering saturated LCFA binding to PPAR $\alpha$ , adding an NBD- group to stearic acid increased its binding by PPAR $\alpha$  more than 100-fold (30). The fact that 2-bromopalmitic acid and 2-fluoropalmitic acid both resulted in almost identical CD changes and were exceptions to the correlation of binding affinity with CD change may be due to the addition of the bromo and fluoro groups altering size, pKa values, stereo-structure, or a variety of other factors. Although addition of either a bromo or fluoro group to palmitic acid resulted in weak binding by PPAR $\alpha$ , addition of the larger bromo group elicited weaker affinity than addition of the smaller fluoro group. However, despite its weaker affinity, the bigger size of the bromo- group added to palmitate resulted in similar overall CD changes as elicited by 2-fluoropalmitic acid. Interestingly, the non-metabolizable *S*-hexadecyl-CoA elicited the largest changes in PPAR $\alpha$  CD spectra (Fig. 7C, *empty triangles*), indicating that the ability of the fatty acyl-CoA to alter PPAR $\alpha$ 's structure did not require hydrolysis to the free acid.



### Effect of Endogenous Very-Long-Chain Fatty Acids on co-factor Recruitment to PPAR $\alpha$ : Co-Immunoprecipitation

While it is generally accepted that ligand activation of a nuclear receptor stimulates recruitment and interaction of co-activators while decreasing interactions with co-repressors (rev. in (44)), there are exceptions and new co-activators and co-repressors are continually being identified (rev. in (45)). To begin to examine the effect of VLCFA on PPAR $\alpha$  co-factor recruitment, co-immunoprecipitation with the PPAR $\alpha$  antibody was used to test the ability of native PPAR $\alpha$  to bind co-activators (Fig. 8; SRC-1, *black bars*; CBP, *white bars*) by precipitation of both proteins from mouse liver homogenate in the presence or absence of select representatives of the three groups of PPAR $\alpha$  ligands examined above: (i) saturated and unsaturated VLCFAs; (ii) VLCFA-CoAs; (iii) BCFAs and BCFA-CoAs.

To test the effect of VLCFA which are poorly bound and only weakly alter structure of PPAR $\alpha$ , the following were examined: saturated arachidic acid (C20:0), monounsaturated nervonic acid (C24:1), and polyunsaturated eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6). Among these only the polyunsaturated C22:6 caused any significant change to co-activator recruitment, and this resulted in a 2-fold increase in co-immunoprecipitation of SRC-1 (Fig. 8A). Since most of these ligands only weakly bound to PPAR $\alpha$ , it was expected that there would be very little change to co-activator recruitment. However, the change in co-activator recruitment by the addition of C22:6 could not be correlated with high affinity binding of the free acid form of this ligand. Thus, the effect of the thioesters of these ligands was tested on co-activator recruitment.

### Effect of Endogenous Very-Long-Chain Fatty Acyl-CoAs on Co-factor Recruitment to PPAR $\alpha$ : Co-Immunoprecipitation

Although the preceding section showed the addition of C22:6 seemed to enhance activation, even though this ligand only weakly binds to PPAR $\alpha$ , it must be considered that liver homogenates contain fatty acyl-CoA synthase activity (46,47). Therefore, the effect of the CoA thioesters of the above representative VLCFA on co-factor recruitment was examined. The saturated C20:0-CoA showed a 60% reduction in co-immunoprecipitation of both co-activators, while the monounsaturated C24:1-CoA resulted in a 50% decrease in CBP co-recruitment (Fig. 8B). Although the polyunsaturated C20:5-CoA did not result in any significant changes, the addition of C22:6-CoA increased co-immunoprecipitation of both co-activators by about 2.5-fold (Fig. 8B). The increase in co-activator recruitment of C22:6-CoA correlated with that of the free acid, although the effect was much stronger for the CoA thioester, suggesting that the effect of the free acid may be due to acylation of the free acid to its corresponding acyl-CoA. Further, the data suggests that saturated and monounsaturated VLCFA-CoAs may elicit repression of PPAR $\alpha$ , while some polyunsaturated VLCFA-CoAs may induce strong activation, dependent upon the affinity of PPAR $\alpha$  for each ligand. Since PPAR $\alpha$  had a weaker affinity for C20:5-CoA (Table 1) than any of the other examined CoA thioesters, it was expected that this ligand would have a smaller effect on co-activator recruitment.

### Effect of Branched-Chain Fatty Acids on Co-factor Recruitment to PPAR $\alpha$ : Co-Immunoprecipitation

Since both the free acid form and the CoA thioesters of branched-chain fatty acids are high affinity PPAR $\alpha$  ligands and alter PPAR $\alpha$  conformation, the possibility that these ligands will also alter co-factor recruitment was examined. Phytanic acid significantly increased co-immunoprecipitation of SRC-1 from liver homogenates, approximately 2-fold, while no effect was seen on CBP (Fig. 8C). To determine if these effects might in part be due to the phytanoyl-CoA thioester, the studies were repeated with phytanoyl-CoA which decreased the co-immunoprecipitation of both co-activators more than 50% (Fig. 8C). Thus, as compared to the

free phytanic acid, the phytanoyl-CoA exhibited opposite effects on co-activator recruitment. Thus, both the free acid and CoA thioester forms of the branched-chain phytanic acid alter co-factor recruitment, each in unique fashion.

### Effect of Non-metabolizable Fatty Acids and Non-hydrolyzable Fatty Acyl-CoAs on Co-factor Recruitment to PPAR $\alpha$ : Co-Immunoprecipitation

Since the effect of VLCFA in recruitment assays may be due to metabolism to their CoA thioesters (i.e. docosahexaenoic acid), the effect of the non-metabolizable fatty acids, 2-bromopalmitic acid and 2-fluoropalmitic acid, in co-activator recruitment was examined. No changes were noted to co-immunoprecipitation of either SRC-1 or CBP upon addition of either 2-bromopalmitic acid (Fig. 8C) or 2-fluoropalmitic acid (data not shown). However, addition of the non-hydrolyzable fatty acid analogue, *S*-hexadecyl-CoA, resulted in a 1.8-fold increase to SRC-1 and a 1.5-fold increase in CBP co-immunoprecipitation with PPAR $\alpha$ . These data again substantiate the role of CoA thioesters as the active form of PPAR $\alpha$  ligands.

## DISCUSSION

Although many of the xenobiotics that undergo  $\beta$ -oxidation are PPAR $\alpha$  activators, the nature of the endogenous ligands for PPAR $\alpha$  is not completely resolved. While there is evidence indicating that VLCFA and BCFA are potent naturally-occurring peroxisome proliferators and may be PPAR $\alpha$  ligands, it is not completely clear whether the unesterified VLCFA and BCFA directly, and/or metabolites thereof, represent the endogenous PPAR $\alpha$  ligands/activators. For example, early transactivation assays show: (i) PPAR $\alpha$  is activated by polyunsaturated VLCFA (C20:4, C20:5, C22:6), but not monounsaturated VLCFA (C24:1, C22:1) (3), (ii) n-3 polyunsaturated VLCFA elicited similar PPAR $\alpha$  activation levels as n-6 polyunsaturated VLCFA (3). These findings of VLCFA being potential PPAR $\alpha$  ligands were supported by gel-shift assays (14) and co-activator-dependent receptor ligand assays (15). Likewise, transactivation studies with branched-chain fatty acids also show PPAR $\alpha$  activation by BCFA (11). It must be noted that most of these assays utilized very high concentrations of VLCFA (i.e. 30-100  $\mu$ M) and BCFA (20  $\mu$ M)—several orders of magnitude higher than concentrations of unesterified fatty acids detected in nuclei of living cells (16,17). These data suggested that affinities of PPAR $\alpha$  for these ligands are very weak, i.e.  $K_d$ s in the  $\mu$ M range, much lower than the concentrations of free fatty acids in nuclei of living cells (16,17), and thus the free acid forms of the VLCFA and BCFA may not represent the endogenous ligands. Since unesterified fatty acids are rapidly activated/metabolized to their CoA thioesters in cells and tissues (rev. (18-20), metabolites such as the CoA thioesters may represent endogenous PPAR $\alpha$  ligands. Unfortunately, there is almost no data resolving whether VLCFA, BCFA, and/or their CoA thioesters exhibit the classic hallmarks of ligands that activate a nuclear receptor such as PPAR $\alpha$ : (i) high affinity binding (i.e. nM  $K_d$ s, in the range of nuclear concentrations of these ligands), (ii) alter conformation of the nuclear receptor, and (iii) alter co-factor recruitment to the nuclear receptor. The data presented herein help to clarify our understanding of these issues.

First, the current work presented evidence that the free acid forms of BCFA and only one free acid form of VLCFA bind to PPAR $\alpha$  with high affinity. PPAR $\alpha$  bound phytanic acid, pristanic acid, and some 20-carbon VLCFA with  $K_d$ s of 19-76nM, well within the physiological range of free fatty acids detected in the nucleoplasm of living cells (16,17). Further, these data showed for the first time that phytanic acid did not need to be metabolized to pristanic acid to be a high affinity PPAR $\alpha$  ligand. Both phytanic acid and pristanic acid, but not shorter chain metabolites, are the highly potent naturally-occurring PPAR $\alpha$  activators in transactivation assays (rev. in (38-40) and in animals (12,13,48). The  $K_d$  values for both BCFA and 20-carbon VLCFA (arachidic acid, arachidonic acid) were in the same range as PPAR $\alpha$  exhibited for unsaturated long chain fatty acids (LCFA) and peroxisome proliferators (bezafibrate) (29,30)—suggesting

that BCFA, arachidic acid, and arachidonic acid are high affinity PPAR $\alpha$  ligands that may be physiologically significant endogenous ligands.

Second, in contrast to the free acid forms, nearly all of the examined BCFA-CoAs and VLCFA-CoAs bind to PPAR $\alpha$  with high affinities (i.e. 2-30nM  $K_{ds}$ ). This was not due to hydrolysis of bound CoA thioesters to the respective free acids since: (i) most of the free acid forms of VLCFA were not or only weakly bound by PPAR $\alpha$ ; (ii) PPAR $\alpha$  bound the non-hydrolyzable *S*-hexadecyl-CoA with very high affinity (i.e. 10nM  $K_d$ ) similar to that observed for the VLCFA-CoAs and BCFA-CoAs. Although nuclear fatty acyl-CoA concentration has been estimated to be less than 10nM, early methodology made it difficult to accurately measure acyl-CoA concentration in organelles (49). More recent data with fluorescent fatty acyl-CoAs in living cells has confirmed the earlier prediction that nuclearplasmic concentrations of fatty acyl-CoAs were indeed less than 10nM (17). However, this concentration can be increased several-fold by expression of cytoplasmic fatty acyl-CoA binding proteins (49,50). Thus, the CoA thioesters of VLCFA and BCFA are nearly all bound with affinities in the physiological range of fatty acyl-CoA concentrations in the nucleoplasm of living cells. The  $K_d$  values for all of the examined VLCFA-CoAs and BCFA-CoAs were in the same range as PPAR $\alpha$  exhibited for long-chain fatty acyl-CoAs (LCFA-CoAs) and peroxisome proliferator drug-CoA thioesters (22,30). The finding that PPAR $\alpha$  exhibits very high affinity (3-29nM  $K_{ds}$ ) for VLCFA-CoAs may help to explain the effects of n-3 and n-6 fatty acid supplementation on PPAR $\alpha$  transactivation. In transactivation assays wherein cultured cells are supplemented with exogenous VLCFA, both n-3 and n-6 fatty acids enhance PPAR $\alpha$  transactivation nearly equally well (3,14,15,40,51-53). Since PPAR $\alpha$  exhibits high affinity (low nM  $K_{ds}$ ) for n-6 unsaturated fatty acids (29,30), as well as their CoA derivatives (30), either or both these ligands could contribute to PPAR $\alpha$  activation. In contrast, since PPAR $\alpha$  exhibits high affinity (low nM  $K_{ds}$ ) for n-3 fatty acyl-CoAs, but not for n-3 fatty acids, the CoA thioesters formed by intracellular fatty acyl-CoA synthases from dietary n-3 fatty acids may represent the high affinity endogenous ligands accounting for PPAR $\alpha$  transactivation by these VLCFA. Thus, the ligand binding data obtained herein may reconcile the PPAR $\alpha$  transactivation data obtaining similar transactivation effects with added n-3 and n-6 fatty acids. Taken together, these data suggest that VLCFA-CoAs and BCFA-CoAs are high affinity PPAR $\alpha$  ligands and thus represent endogenous ligands. These data suggest that the VLCFA-CoAs and BCFA-CoAs, as well as some 20-carbon VLCFA and both BCFA, satisfy the high affinity criterion of physiologically significant ligands for PPAR $\alpha$ .

Third, all the high affinity PPAR $\alpha$  ligands (VLCFA-CoAs, BCFA-CoAs, BCFA, and some 20-carbon VLCFA) significantly altered the structure of PPAR $\alpha$ . This was evidenced by two observations: (i) Binding of these ligands significantly quenched the PPAR $\alpha$  aromatic 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) These ligand-induced structural changes in PPAR $\alpha$  were confirmed by circular dichroism, which resulted in molar ellipticity changes in the presence of compounds demonstrated to bind with high affinity (low nM  $K_{ds}$ ) to PPAR $\alpha$ . Circular dichroic spectra were unaltered in the presence of compounds that did not bind to PPAR $\alpha$ . Interestingly, two types of ligand-induced PPAR $\alpha$  conformational changes were observed; (i) The 20-carbon fatty acids (arachidic, arachidonic) and 20-carbon acyl-CoAs (arachidoyl-CoA, arachidonoyl-CoA), 22-carbon acyl-CoA (docosapentaenoyl-CoA), 24-carbon saturated acyl-CoA (lignoceroyl-CoA), 24-carbon monounsaturated fatty acid (nervonic acid), BCFA, and BCFA-CoAs produced an overall decrease in PPAR $\alpha$  content of  $\alpha$ -helices and a concomitant increase in  $\beta$ -sheets, similar to those obtained previously for LCFA-CoAs and unsaturated LCFA (30). In contrast, binding of a 22-carbon saturated acyl-CoA (behenoyl-CoA), 22-carbon n-3 polyunsaturated acyl-CoA (docosahexaenoyl-CoA), and a 24-carbon monounsaturated CoA (nervonoyl-CoA) increased the proportion of  $\alpha$ -helices and concomitantly decreased  $\beta$ -sheets in PPAR $\alpha$ . These findings

with circular dichroism suggest that the high affinity VLCFA, VLCFA-CoAs, BCFA, BCFA-CoAs, and non-hydrolyzable *S*-hexadecyl-CoA all significantly altered the secondary structure of PPAR $\alpha$ --thereby satisfying the second criterion that these are physiologically significant ligands for PPAR $\alpha$ .

Fourth, the current work presented data demonstrating that high affinity ligands altered co-factor recruitment in a highly selective manner. The effect of ligands on co-activator (SRC-1, CBP) association with PPAR $\alpha$  was examined by co-immunoprecipitation from liver homogenates using an anti-PPAR $\alpha$  monoclonal antibody. The data demonstrated that most of the high affinity ligands significantly altered co-immunoprecipitation of at least one, if not both, of the co-activators, while most of the low affinity ligands did not. An interesting exception was docosahexaenoic acid which increased co-activator recruitment even though this ligand only weakly bound to PPAR $\alpha$ . Furthermore, these data agree with the transactivation data indicating that addition of docosahexaenoic acid or phytanic acid to the medium(11) increases PPAR $\alpha$  activation, while the addition of monounsaturated fatty acids does not (3). A potential explanation for the co-activator recruitment and activation activity of dietary docosahexaenoic acid may come from the observation that the CoA thioester of docosahexaenoic acid was both tightly bound by and increased co-activator recruitment to PPAR $\alpha$ . This suggests that activation of PPAR $\alpha$  in the presence of VLCFA such as docosahexaenoic acid may arise from endogenous fatty acyl-CoA synthase activity present in the homogenates and cells. Liver homogenates and hepatocytes represent a complex milieu wherein multiple endogenous fatty acyl-CoA synthases can convert the VLCFA and/or BCFA to their CoA thioesters (46,47). These data suggest that the VLCFA-CoAs and BCFA-CoAs, as well as some 20-carbon VLCFA and both BCFA, satisfy the co-factor recruitment criterion of physiologically significant ligands for PPAR $\alpha$ .

With regards to ligand-induced conformational change in PPAR $\alpha$  and co-factor recruitment, unique patterns emerged. While the VLCFA-CoAs were all high affinity PPAR $\alpha$  ligands, the dissimilar circular dichroic changes obtained by the addition of behenoyl-CoA, docosahexaenoyl-CoA, and nervonoyl-CoA, as compared to the other high affinity PPAR $\alpha$  ligands, suggested that the binding of these three ligands would elicit different co-factor recruitment than the other examined ligands. Indeed, nervonoyl-CoA binding seemed to decrease co-activator recruitment, similar to arachidoyl-CoA binding. However, docosahexaenoyl-CoA binding increased co-activator recruitment, similar to that seen for other PPAR $\alpha$  ligands (29).

In conclusion, PPAR $\alpha$  binds with highest affinities the VLCFA-CoAs and BCFA-CoAs, slightly weaker affinities the free BCFA and several 20-carbon free VLCFA (arachidic acid, arachidonic acid), but not longer chain free VLCFA. The high affinities (i.e. very low nM  $K_d$ s) were in the range of fatty acyl-CoA concentrations observed in nucleoplasm of living cells (16,17). Furthermore, the high affinity binding of these ligands correlated with ligand-induced conformational changes in PPAR $\alpha$ . Finally, high affinity ligand binding and ligand-induced conformational changes in general, elicited one or more alterations in co-activator recruitment. These data significantly added to our understanding of the action of VLCFA and BCFA on PPAR $\alpha$ .

## Abbreviations

PPAR, peroxisome proliferator-activated receptor  
RXR, retinoid X receptor  
PPRE, peroxisome proliferator response element  
VLCFA, very-long-chain fatty acids  
BCFA, branched-chain fatty acids

CoA, coenzyme A  
 AOX, acyl-CoA oxidase  
 PBE, bifunctional enzyme  
 PTL, 3-ketoacyl-CoA thiolase  
 SCP-x, sterol carrier protein-x  
 LCFA-CoA, long chain fatty acyl-CoA  
 PPAR $\alpha$  $\Delta$ AB, peroxisome proliferator activated receptor  $\alpha$  comprised of amino acids 101-468 (i.e. missing only the amino-terminal A/B domain)  
 SRC-1, steroid receptor co-activator-1  
 CBP, cyclic AMP-responsive enhancer binding protein (CREB) binding protein  
 CD, circular dichroism  
 HPLC, high performance liquid chromatography  
 RAR, retinoic acid receptor  
 LXR, liver X receptor

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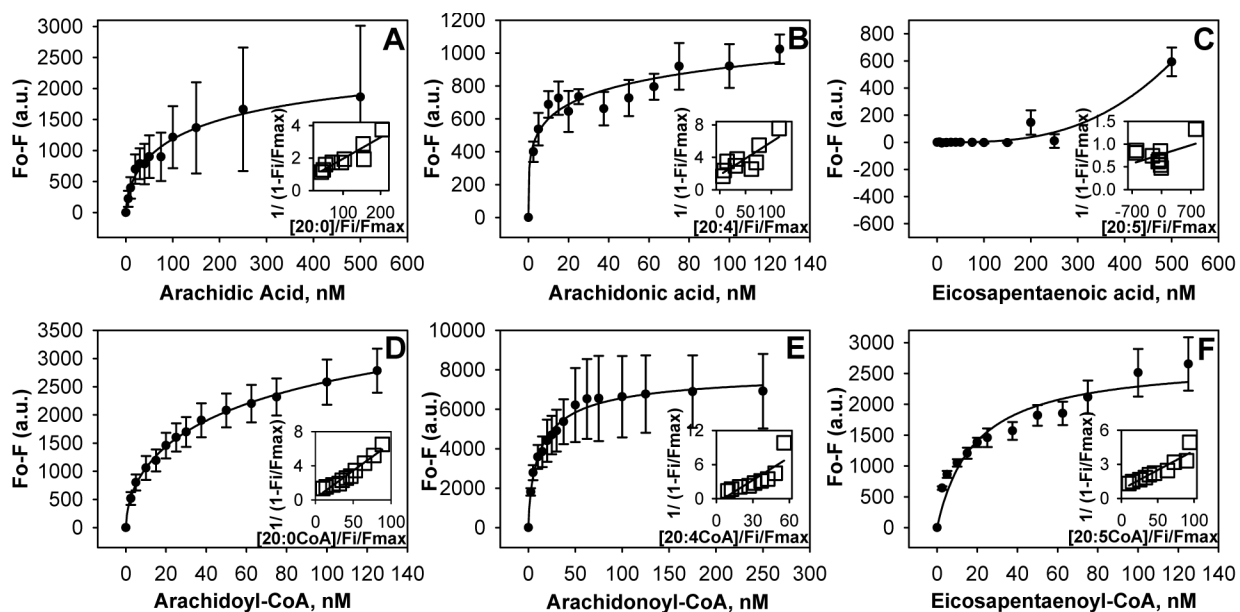
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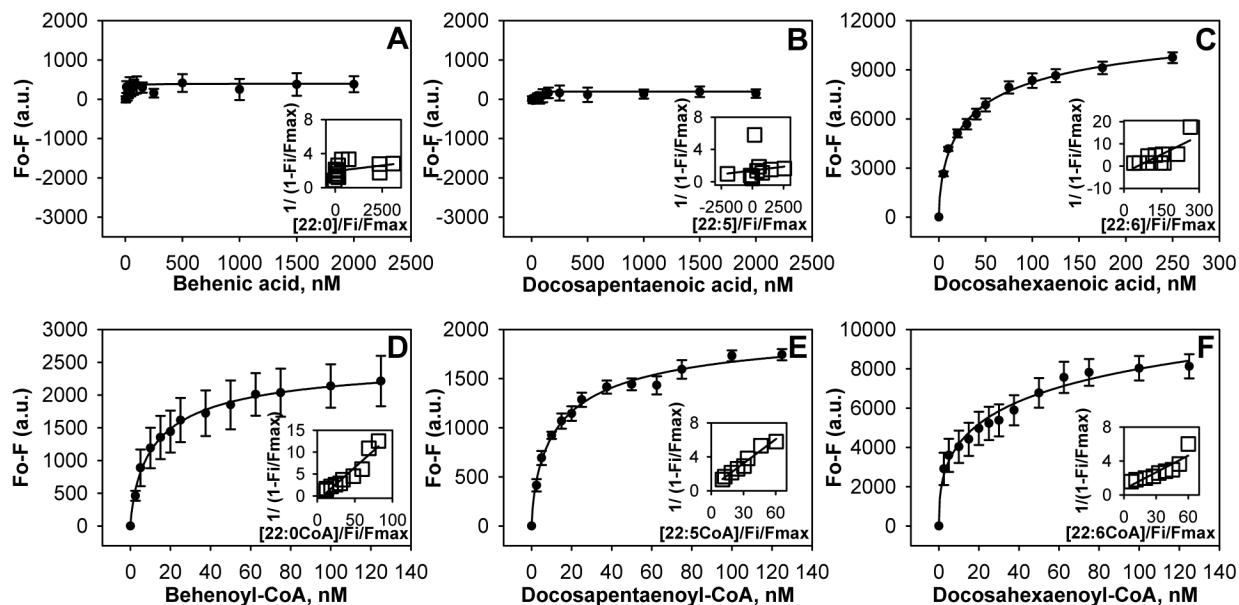
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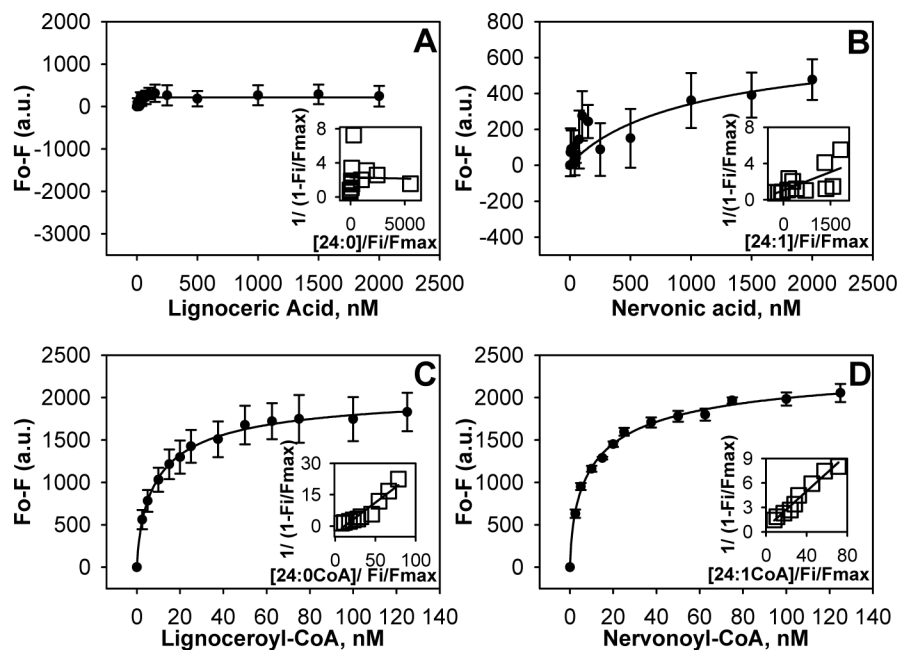
**FIG. 1. Interaction of naturally-occurring C20 fatty acids and fatty acyl-CoAs with PPAR $\alpha$  $\Delta$ AB: Direct binding based on quenching of PPAR $\alpha$  $\Delta$ AB aromatic amino acid fluorescence emission**

The data are presented as the change in fluorescence intensity ( $F_o - F$ ) plotted as a function of ligand concentration. PPAR $\alpha$  $\Delta$ AB (0.1 $\mu$ M) was titrated with 0-600nM of the following ligands: *panel A*, arachidic acid (C20:0); *panel B*, arachidonic acid (C20:4); *panel C*, eicosapentaenoic acid (C20:5); *panel D*, arachidoyl-CoA (C20:0-CoA); *panel E*, arachidonoyl-CoA (C20:4-CoA); *panel F*, eicosapentaenoyl-CoA (C20:5-CoA). Values represent the mean  $\pm$  SE, n=4-5. *Insets*, linear plot of the binding curve from each panel.

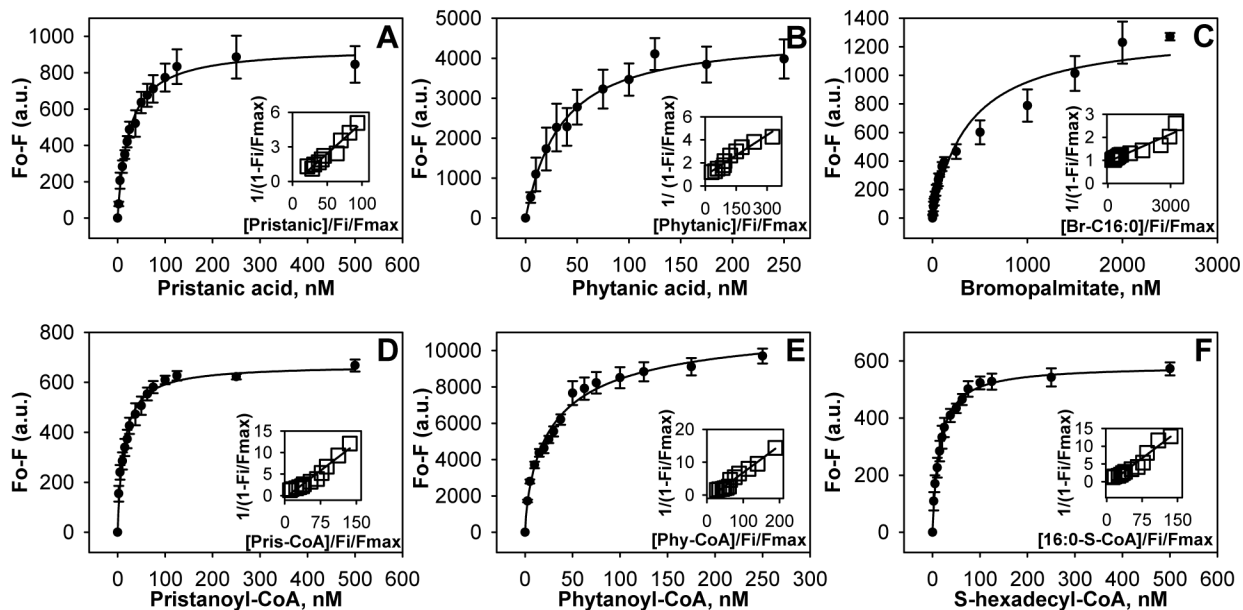


**FIG. 2. Interaction of naturally-occurring C22 fatty acids and fatty acyl-CoAs with PPAR $\alpha$  $\Delta$ AB: Direct binding based on quenching of PPAR $\alpha$  $\Delta$ AB aromatic amino acid fluorescence emission**  
 The data are presented as the change in fluorescence intensity ( $F_o - F$ ) plotted as a function of ligand concentration. PPAR $\alpha$  $\Delta$ AB (0.1 $\mu$ M) was titrated with 0-2500nM of the following ligands: *panel A*, behenic acid (C22:0); *panel B*, docosapentaenoic acid (C22:5); *panel C*, docosahexaenoic acid (C22:6); *panel D*, behenoyl-CoA (C22:0-CoA); *panel E*, docosapentaenoyl-CoA (C22:5-CoA); *panel F*, docosahexaenoyl-CoA (C22:6-CoA). Values represent the mean  $\pm$  SE, n=4-5. *Insets*, linear plot of the binding curve from each panel.

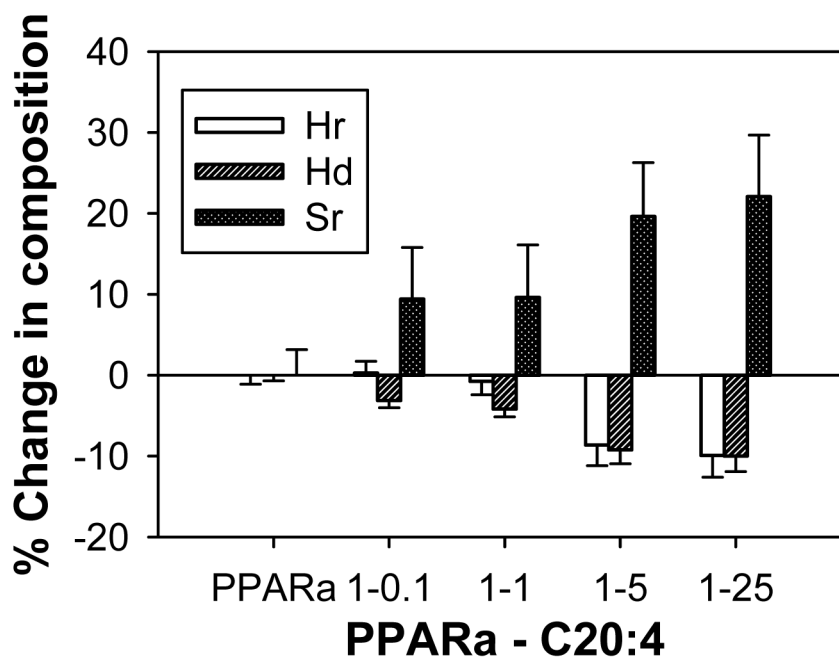




**FIG. 3. Interaction of naturally-occurring C24 fatty acids and fatty acyl-CoAs with PPAR $\alpha$  $\Delta$ AB: Direct binding based on quenching of PPAR $\alpha$  $\Delta$ AB aromatic amino acid fluorescence emission**  
 The data are presented as the change in fluorescence intensity ( $F_o - F$ ) plotted as a function of ligand concentration. PPAR $\alpha$  $\Delta$ AB (0.1 $\mu$ M) was titrated with 0-2500nM of the following ligands: *panel A*, lignoceric acid (C24:0); *panel B*, nervonic acid (C24:1); *panel C*, lignoceroyl-CoA (C24:0-CoA); *panel D*, nervonoyl-CoA (C24:1-CoA). Values represent the mean  $\pm$  SE,  $n=4-5$ . *Insets*, linear plot of the binding curve from each panel.

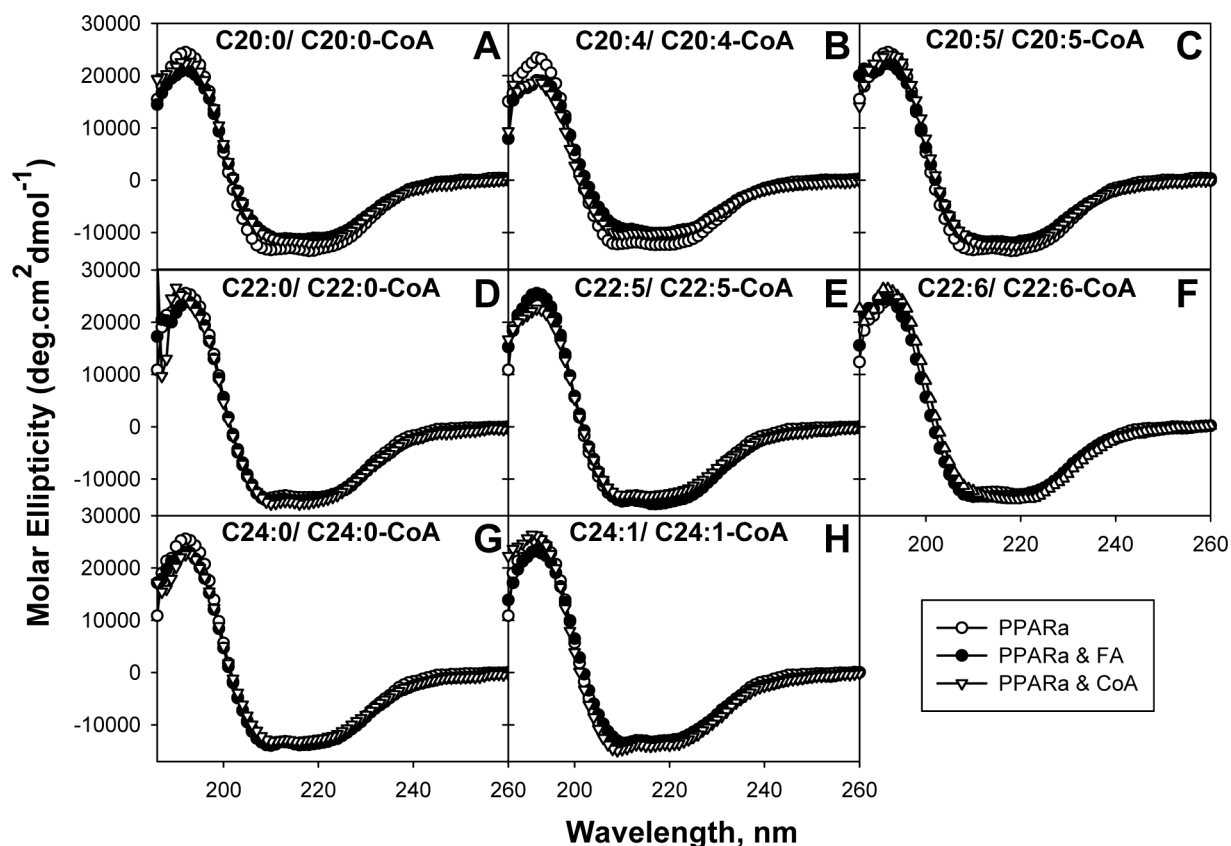


**FIG. 4. Interaction of branched-chain fatty acids and fatty acyl-CoAs with PPAR $\alpha$  $\Delta$ AB: Direct binding based on quenching of PPAR $\alpha$  $\Delta$ AB aromatic amino acid fluorescence emission**  
 The data are presented as the change in fluorescence intensity ( $F_o - F$ ) plotted as a function of ligand concentration. PPAR $\alpha$  $\Delta$ AB (0.1 $\mu$ M) was titrated with 0-2500nM of the following ligands: *panel A*, phytanic acid (C16:0-branched); *panel B*, pristanic acid (C16:0-branched); *panel C*, bromopalmitate (C16:0-Br); *panel D*, phytanoyl-CoA (C16:0-CoA); *panel E*, pristanoyl-CoA (C16:0-CoA); *panel F*, *S*-hexadecyl-CoA (C16:0-S-CoA). Values represent the mean + SE,  $n=4-5$ . *Insets*, linear plot of the binding curve from each panel.



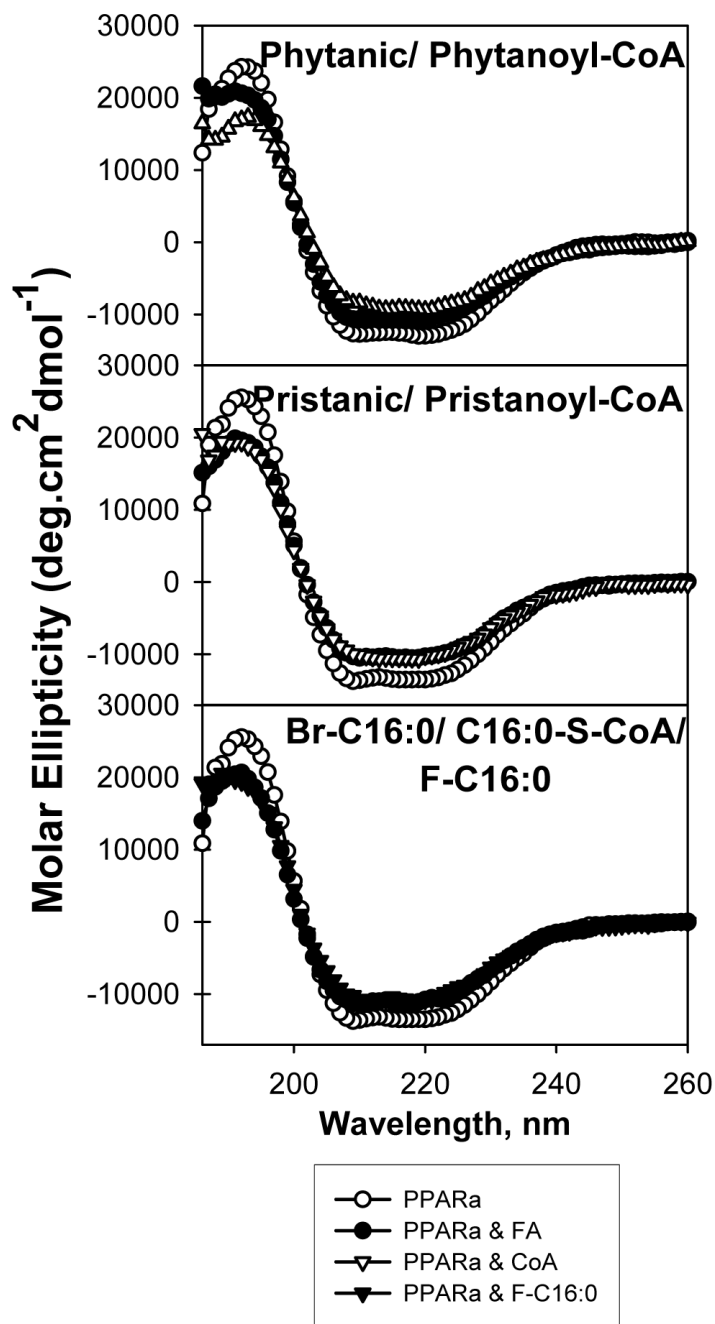
**FIG. 5. Effect of increasing concentrations of ligand on PPARαΔAB conformation: Circular dichroic (CD) spectra**

Far ultraviolet (UV) circular dichroic (CD) spectra of PPARαΔAB in the presence of increasing arachidonic acid concentration were obtained and analyzed for percent composition as described in the “Experimental Procedures”. The effect of increasing PPARαΔAB:C20:4 ratios on regular  $\alpha$ -helices (Hr, *open bars*), distorted  $\alpha$ -helices (Hd, *diagonal bars*), and regular  $\beta$ -sheets (Sr, *cross-hatched bars*) are presented as percent change as compared to that obtained for PPARαΔAB in the absence of ligand.



**FIG. 6. Effect of VLCFA and VLCFA-CoA binding on PPAR $\alpha$  $\Delta$ AB conformation: Circular dichroic (CD) spectra**

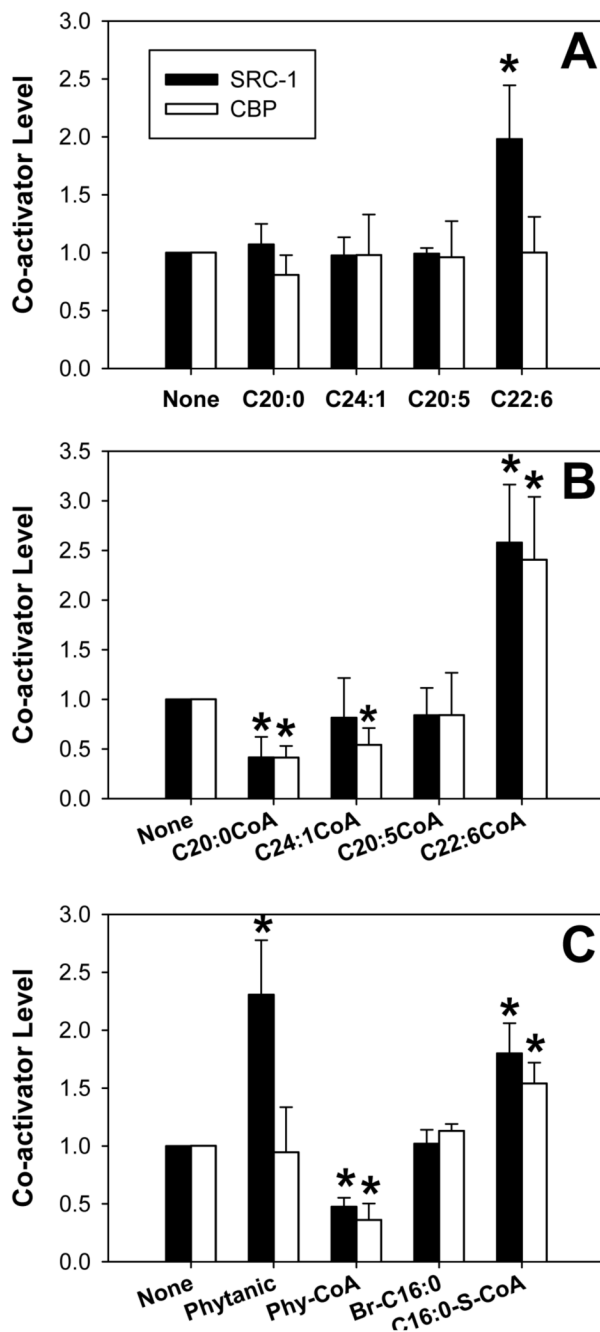
Far ultraviolet (UV) circular dichroic (CD) spectra of PPAR $\alpha$  $\Delta$ AB in the absence or presence of added ligand were obtained as described in the “Experimental Procedures”. *Panel A*, CD spectrum of PPAR $\alpha$  $\Delta$ AB in the absence (*empty circles*) and presence of added ligand: arachidic acid (*filled circles*) or arachidoyl-CoA (*empty triangles*); *panel B*, in the presence of arachidonic acid (*filled circles*) or arachidonoyl-CoA (*empty triangles*); *panel C*, in the presence of eicosapentaenoic acid (*filled circles*) or eicosapentaenoyl-CoA (*empty triangles*); *panel D*, in the presence of behenic acid (*filled circles*) or behenoyl-CoA (*empty triangles*); *panel E*, in the presence of docosapentaenoic acid (*filled circles*) or docosapentaenoyl-CoA (*empty triangles*); *panel F*, in the presence of docosahexaenoic acid (*filled circles*) or docosahexaenoyl-CoA (*empty triangles*); *panel G*, in the presence of lignoceric acid (*filled circles*) or lignoceroyl-CoA (*empty triangles*); *panel H*, in the presence of nervonic acid (*filled circles*) or nervonoyl-CoA (*empty triangles*). Each spectrum represents an average of 10 scans for a given representative spectrum from three replicates.



**FIG. 7. Effect of BCFA and BCFA-CoA binding on PPAR $\alpha$  $\Delta$ AB conformation: Circular dichroic (CD) spectra**

Far ultraviolet (UV) circular dichroic (CD) spectra of PPAR $\alpha$  $\Delta$ AB in the absence or presence of added ligand were obtained as described in the “Experimental Procedures”. *Panel A*, CD spectrum of PPAR $\alpha$  $\Delta$ AB in the absence (*empty circles*) and presence of added ligand: phytanic acid (*filled circles*) or phytanoyl-CoA (*empty triangles*); *panel B*, in the presence of pristanic acid (*filled circles*) or pristanoyl-CoA (*empty triangles*); *panel C*, in the presence of 2-bromopalmitic acid (*filled circles*), 2-fluoropalmitic acid (*filled triangles*) or *S*-hexadecyl-CoA (*empty triangles*). Each spectrum represents an average of 10 scans for a given representative spectrum from three replicates.





**FIG. 8. Effect of select ligands on PPAR $\alpha$  co-factor recruitment: Co-Immunoprecipitation**  
 Mouse liver homogenates were used to co-immunoprecipitate native PPAR $\alpha$  protein with co-activators (SRC-1, black bars and CBP, white bars) in the presence of VLCFA (panel A), VLCFA-CoAs (panel B), and BCFA, BCFA-CoAs, 2-bromopalmitic acid, and S-hexadecyl-CoA (panel C). Protein values were standardized to PPAR $\alpha$  protein levels and normalized for each ligand sample to the level of interaction observed in the absence of ligand (None), which was set equal to 1. Values represent mean protein quantities  $\pm$  SE, n=4. Asterisks (\*) show significant differences between sample with ligand versus no ligand sample (P<0.05).

**Table 1****Affinity of PPAR $\alpha$  $\Delta$ AB for very-long-chain, branched-chain, and non-metabolizable fatty acids and their CoA thioesters as determined by quenching of PPAR $\alpha$  $\Delta$ AB aromatic amino acid fluorescence**

Binding parameters were determined from fluorescent quenching of PPAR $\alpha$  $\Delta$ AB by naturally-occurring, non-fluorescent ligands as described in "Experimental Procedures". The  $K_d$  (dissociation constant) was calculated from the reciprocal plots as described in "Experimental Procedure". Values represent the mean  $\pm$  SE (n = 4-5). Weak refers to detection of very low levels of quenching, but insufficient for determination of binding parameters; --- refers to no noticeable quenching

Ligand	Chain length:double bonds (position)	Kd (nM) FA	Kd (nM) CoA
Arachidic acid/CoA	20:0	76 $\pm$ 12	16 $\pm$ 1
Arachidonic acid/CoA	20:4 (n-6)	20 $\pm$ 5	7 $\pm$ 2
Eicosapentaenoic acid/CoA	20:5 (n-3)	Weak	29 $\pm$ 4
Behenic acid/CoA	22:0	---	6 $\pm$ 1
Docosapentaenoic acid/CoA	22:5 (n-3)	Weak	10 $\pm$ 1
Docosahexaenoic acid/CoA	22:6 (n-3)	145 $\pm$ 48	11 $\pm$ 2
Lignoceric acid/CoA	24:0	Weak	3 $\pm$ 1
Nervonic acid/CoA	24:1 (n-9)	769 $\pm$ 296	9 $\pm$ 1
Pristanic acid/CoA	15:tetramethyl	19 $\pm$ 2	12 $\pm$ 1
Phytanic acid/CoA	16:tetramethyl	34 $\pm$ 4	11 $\pm$ 1
2-Bromopalmitic acid	Br-16:0	808 $\pm$ 195	
2-Fluoropalmitic acid	F-16:0	219 $\pm$ 33	
S-hexadecyl-CoA	S-16:0-CoA		10 $\pm$ 1

**Effect of very-long-chain, branched-chain, and non-metabolizable fatty acids and their CoA thioesters on the relative proportion of PPAR $\alpha$ / $\Delta$ AAB secondary structure determined by circular dichroism (CD)**

The relative proportions of different types of secondary structure for PPAR $\alpha$ / $\Delta$ AAB in the absence and presence of added ligands was calculated as described in "Experimental Procedure". These structures were as follows: H(r) represents regular  $\alpha$ -helices, H(d) represents distorted  $\alpha$ -helices, S(r) represents regular  $\beta$ -sheets, S(d) represents distorted  $\beta$ -sheets, Turns represent  $\beta$ -turns, and Unrd represents unordered structures. Asterisks represent significant differences between PPAR $\alpha$ / $\Delta$ AAB only and PPAR $\alpha$ / $\Delta$ AAB in the presence of added ligand

Table 2

Ligand	length:double bonds (position)	Hr (%)	Hd (%)	Sr (%)	Sd (%)	Turns (%)	Unordered (%)
PPAR $\alpha$ / $\Delta$ AAB		24.2 ± 0.4**	16.0 ± 0.1**	8.0 ± 0.1**	6.5 ± 0.1**	18.7 ± 0.2	26.6 ± 0.2
Arachidic acid	20:0	20.1 ± 0.4**	13.6 ± 0.1**	10.4 ± 0.6***	7.7 ± 0.1**	20.1 ± 0.5**	27.9 ± 0.5*
Arachidoyl-CoA	20:0-CoA	21.5 ± 0.5**	14.3 ± 0.1**	10.0 ± 0.5**	7.2 ± 0.1**	19.3 ± 0.6	28.1 ± 0.8*
Arachidonic Acid	20:4 (n-6)	17.6 ± 0.6***	12.7 ± 0.3***	12.0 ± 0.7***	8.4 ± 0.3***	21.0 ± 0.7***	28.5 ± 0.8***
Arachidonoyl-CoA	20:4-CoA (n-6)	17.4 ± 0.6***	13.0 ± 0.4***	11.1 ± 0.6***	8.5 ± 0.3***	21.5 ± 0.5***	28.8 ± 0.5***
Eicosapentaenoic Acid	20:5 (n-3)	21.1 ± 0.4**	14.5 ± 0.1***	10.0 ± 0.7***	7.2 ± 0.1**	19.4 ± 0.6	27.9 ± 0.7*
Eicosapentaenoyl-CoA	20:5-CoA (n-3)	22.1 ± 0.5**	14.6 ± 0.2**	9.5 ± 0.3**	7.3 ± 0.1**	19.2 ± 0.3	27.5 ± 0.5*
Behenic acid	22:0	24.6 ± 0.8*	16.1 ± 0.2**	8.3 ± 0.2	6.5 ± 0.1*	19.0 ± 0.5	26.3 ± 0.3
Behenoyl-CoA	22:0-CoA	26.0 ± 1.1*	16.6 ± 0.3**	8.5 ± 0.5	5.7 ± 0.4*	17.0 ± 0.9*	26.0 ± 1.3
Docosapentaenoic acid	22:5 (n-3)	24.9 ± 0.7*	16.1 ± 0.2**	8.2 ± 0.1**	6.7 ± 0.1	18.2 ± 0.5	26.5 ± 0.3
Docosapentaenoyl-CoA	22:5-CoA (n-3)	22.6 ± 0.6*	15.3 ± 0.1**	8.9 ± 0.2*	6.7 ± 0.1	18.9 ± 0.5	27.3 ± 0.3
Docosahexaenoic acid	22:6 (n-3)	24.3 ± 0.8*	15.7 ± 0.2	9.0 ± 0.4*	6.6 ± 0.3	17.8 ± 0.7	27.0 ± 0.5
Docosahexaenoyl-CoA	22:6-CoA (n-3)	25.6 ± 0.8*	16.6 ± 0.9	8.7 ± 0.5*	6.0 ± 0.6	16.9 ± 0.8**	26.3 ± 0.4
Lignoceric acid	24:0	23.4 ± 0.6*	15.8 ± 0.2	8.5 ± 0.1*	6.8 ± 0.1*	19.1 ± 0.5	27.0 ± 0.3
Lignoceroyl-CoA	24:0-CoA	22.4 ± 0.6*	15.2 ± 0.2**	9.1 ± 0.2	7.0 ± 0.1**	19.3 ± 0.4	27.2 ± 0.4
Nervonic acid	24:1 (n-9)	22.7 ± 0.8***	15.1 ± 0.1**	9.2 ± 0.2**	7.2 ± 0.1**	19.0 ± 0.5	27 ± 0.3
Nervonoyl-CoA	24:1-CoA (n-9)	26.6 ± 0.6***	17 ± 0.2	6.5 ± 0.3	5.8 ± 0.2	17.7 ± 0.4	26.2 ± 0.3
Pristanic acid	15:tetramethyl	18.7 ± 0.4	13.5 ± 0.1	10.9 ± 0.6***	7.7 ± 0.2	20.7 ± 0.5***	28.8 ± 0.6
Pristanoyl-CoA	15:tetramethyl-CoA	18.2 ± 0.5***	13.4 ± 0.1***	11.9 ± 0.9***	7.7 ± 0.3***	19.9 ± 0.8	28.8 ± 0.9***
Phytanic acid	16:tetramethyl	19.8 ± 0.5***	13.9 ± 0.1***	11.1 ± 0.6***	7.7 ± 0.2	19.8 ± 0.5**	27.6 ± 0.8**
Phytanoyl-CoA	16:tetramethyl-CoA	16.1 ± 0.6***	12.3 ± 0.3***	13.0 ± 1.1***	8.6 ± 0.3***	21.5 ± 0.6	28.3 ± 0.7
2-Bromopalmitic acid	Br-16:0	20.0 ± 0.4	13.9 ± 0.1	10.3 ± 0.4	7.7 ± 0.1	19.9 ± 0.4	28.0 ± 0.8
2-Fluoropalmitic acid	F-16:0	19.1 ± 0.5***	13.5 ± 0.1***	10.9 ± 0.5***	7.4 ± 0.2***	19.9 ± 0.5	29.2 ± 0.7***
S-hexadecyl-CoA	S-16:0-CoA	16.9 ± 0.4	12.6 ± 0.1	12.1 ± 0.8	8.3 ± 0.2	21.2 ± 0.6	28.9 ± 0.8

\* P&lt;0.05

\*\* P&lt;0.01

\*\*\* P&lt;0.001