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Selectivity of fatty acid ligands for PPARα which correlates both with binding to *cis*-element and DNA binding-independent transactivity in Caco-2 cells

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Abstract

It is thought that peroxisome proliferator-activated receptor α (PPARα) is a major regulator for fatty acid metabolism. Long-chain fatty acids have been shown to induce expression of the genes related to fatty acid metabolism through PPARα. However, it is unclear whether the intensity of PPARα activation is different among various fatty acids. In this study, we compared various fatty acids in the capability of PPARα activation by differential protease sensitivity assay (DPSA), electrophoretic mobility shift assay and GAL4-PPAR chimera reporter assay in intestinal cell line, Caco-2. DPSA revealed that polyunsaturated fatty acids of 18 to 20 carbon groups with 3–5 double bonds strongly induced a PPARα conformational change. The ligand-induced changes in the sensitivity to protease corresponded to the enhancement of the binding of PPARα–RXRα heterodimer to the PPAR-response element (PPRE). The GAL4-PPAR chimera reporter assay revealed that the DNA binding-independent transactivity of PPARα was induced by various fatty acids with a wide spectrum of intensity which correlated with the conformational change of PPARα. These results suggest that PPARα has greater selectivity to certain types of polyunsaturated fatty acids, and that the ligand-induced conformational change of PPARα leads to parallel increases in both DNA binding to the PPAR-response element and the DNA binding-independent transactivity.
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Keywords: PPARα; Polyunsaturated fatty acid; Chimera reporter assay; Caco-2

Introduction

Fatty acids, as major dietary constituents, participate together with several hormones in the regulation of gene expression in response to dietary manipulations. This regulation operates on several metabolic pathways and involves mechanisms that control fuel utilization according to the availability of lipid and glucose and govern the interconversion, transport, storage, mobilization, and use of these nutrients and their metabolites. Together these mechanisms ensure an energy homeostasis, but alteration of this balance can lead to pathological states such as obesity, hyperlipidemia, diabetes, and the resulting cardiovascular diseases (Jump and Clarke, 1999). Recently, several laboratories have shown that peroxisome proliferator-activated receptor α (PPARα), a lipid-activatable transcription factor that belongs to the nuclear hormone receptor superfamily, up-regulates acyl-CoA oxidase, a representative gene activated when the metabolic pathway for fatty acid break down (β-oxidation) is stimulated (Hostetler et al., 2005; Jump and Clarke, 1999; Kota et al., 2005). PPARα is expressed in various tissues including the small intestine (Braissant et al., 1996; Escher et al., 2001).

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Our previous studies indicated that not only acyl-CoA oxidase, but also liver-type fatty acid-binding proteins (L-FABP) and cellular retinol-binding protein, type II (CRBPII), the two major cytosolic binding proteins for lipophilic nutrients in the small intestine were strongly and coordinately up-regulated by dietary fat (Goda et al., 1994; Mochizuki et al., 2001a; Suruga et al., 1999a). It has been reported that polyunsaturated fatty acids and eicosanoids might be potent ligands for PPARs (Forman et al., 1996, 1997; Kliewer et al., 1997). Indeed, previous studies demonstrated that certain types of long-chain unsaturated fatty acids are able to induce the transcription of the PPAR-dependent genes in the intestinal cell line, Caco-2 (Poirier et al., 1997; Suruga et al., 1999b). However, it has been unclear whether the selectivity of various fatty acids as PPARα ligand is determined by the difference in the ability to enhance the binding activity of PPARα to the *cis*-regulatory elements (i. e., PPRE) on the gene, or it might be attributable to the change in structure of the ligand-binding domain of the PPARα which would lead to the modulation of its the DNA binding-dependent transactivity which indicates a transcriptional machinery-recruiting activity to PPARα on the *cis*-element. Therefore, we considered it pertinent to compare various fatty acids from the aspect of the capability to evoke conformational changes in PPARα. To explore the functional significance of the ligand-induced conformational change of PPARα, we employed two currently available assay systems. One is electrophoretic mobility shift assay (EMSA), which monitors in vitro binding activity of PPARα–RXRα heterodimer to the PPRE, and the other is GAL4-PPARα chimera reporter assay, a co-transfection analysis in cells which enables us to quantitatively evaluate the ligand-induced changes in the DNA binding-independent transactivity, presumably evoked by the stimulation of the recruitment of endogenous transcriptional machinery.

Materials and methods

Materials

Stearic acid, arachidic acid, palmitic acid, oleic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and caprylic acid were purchased from Nakarai tesque (Kyoto, Japan). *cis*-8, 11, 14, 17-eicosatetraenoic acid (ETA ($n-3$)), *cis*-7, 10, 13, 16,19-docosapentaenoic acid (DPA ($n-3$)), and *cis*-4, 7, 10, 13, 16-docosapentaenoic acid (DPA ($n-6$)) were provided from Suntory Limited Research Center (Osaka, Japan). WY14,643 and carbaprostacyclin (cPGI) were purchased from Cayman Chemical (Ann Arbor, MI).

Differential Protease Sensitivity Assay (DPSA)

^{35}S-labeled PPARα was produced using T7 Quick TNT in vitro transcription/translation kit (Promega, Madison, WI) as described previously (Mochizuki et al., 2001a). Reactions for DPSA were carried out in a final volume of 10 µl in the buffer containing 20 mM Hepes, pH 7.7, 75 mM KCl, 2.5 mM $MgCl_2$, 2 mM DTT, 0.1% NP-40, and 7.5% glycerol. ^{35}S-labeled PPARα was incubated in the absence or presence of 40 µM various fatty acids or synthetic PPAR ligands (WY14,643, cPGI) for 20 min at room temperature. After this initial incubation period, 1.0 µg of chymotrypsin (sequence grade, Roche Applied Science) was added to allow digestion of the PPARα protein for 20 min at room temperature. The reactions were terminated by addition of an equal volume of stop solution [6% SDS, 22.4% glycerol, 0.02% bromophenol blue, 10% mercaptoethanol and 140 mM Tris–HCl, pH 6.8], and immediate boiling for 3 min, and then the digested products were resolved on SDS-PAGE with 14% acrylamide gels. The gels were fixed with 10% acetic acid and 20% methanol, dried under vacuum, and exposed to an image plate (Fuji Film, Tokyo, Japan) for 12 h.

Electrophoretic Mobility Shift Assay (EMSA)

PPARα and retinoid x receptor α (RXRα) were produced using T7 Quick TNT in vitro transcription/translation kit (Promega, Madison, WI) as described previously (Mochizuki et al., 2001a). Parallel translations of these nuclear receptors were carried out in the presence of [^{35}S] methionine. The assay conditions for EMSA were the same as described previously (Mochizuki et al., 2001b). The double-strand oligonucleotide probe which corresponds to the PPRE on the rat acyl-CoA oxidase (AOX) (Dreyer et al., 1992) was used in this study. The sequence of the oligonucleotide probe was as follows (only underlined strands with basal motifs were shown): AGCTT GGGGACCAGGACAAAGGTCAGGATC-3′.

GAL4-PPARα chimera reporter assay

The plasmid vectors used in GAL4-PPARα chimera reporter assays, pBIND/rat PPARα-LBD were as described previously (Mochizuki et al., 2001 b). Caco-2 cells from the American Type Culture Collection were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 2 mM glutamine, 20 mM Hepes, 50 U/ml penicillin and 50 µg/ml streptomycin sulfate. Cells were plated at a density of 2.5×10^4 cells/well in a 48-well cell culture plate (Iwaki, Tokyo). At 72 h after plating, transfections were performed as described previously (Mochizuki et al., 2001 b). Various fatty acids dissolved in DMSO (final concentration 10 µM) were added 2 h after transfection to the culture medium containing 10% charcoal/dextran treated fetal bovine serum, and then the cells were incubated for 48 h.

Results and discussion

Increasing number of evidence supports the notion that dietary fat modulates PPAR-dependent gene expression in various tissues (Jump and Clarke, 1999; Kota et al., 2005). One of the subtypes, PPARα is known to play a major role in mediating the signal of dietary fat to the modulation of gene expression in various tissues including the liver, kidney, muscle, heart and small intestine (Braissant et al., 1996; Jump and Clarke, 1999).

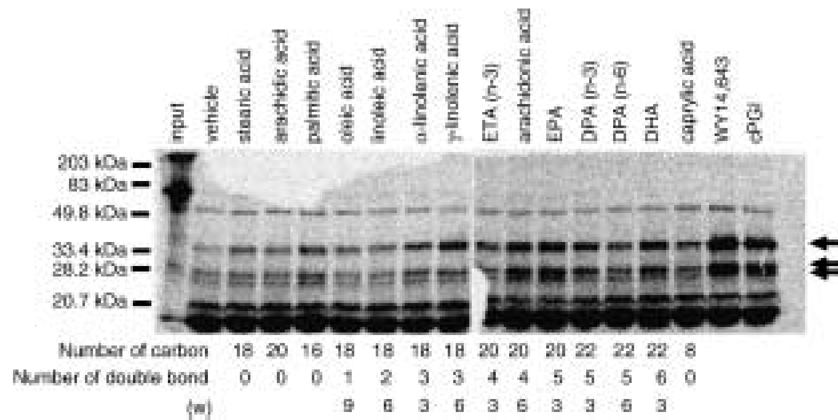

Fig. 1. Fatty acid-induced conformational change of PPARα. ^{35}S labeled-PPARα was subjected to differential protease sensitivity assay. ^{35}S-labeled PPARα was preincubated for 20 min at room temperature with vehicle (2.5% DMSO, lane 2), 40 μM various fatty acids, or 40 μM synthetic ligands (WY14,643, carbaprostacyclin (cPGI)) before addition of chymotrypsin (final concentration, 0.1 mg/ml). Proteolytic digestions were carried out at room temperature for 30 min, and then the samples were subjected to SDS-PAGE on a 14% polyacrylamide gel. Undigested ^{35}S-labeled PPARα was also electrophoresed. The arrowheads indicate the bands specifically protected by the fatty acid ligands. The positions of the molecular weight markers are indicated to the left.

Several studies indicated that certain polyunsaturated fatty acids were directly bound to PPARα, whereby inducing transactivity of PPARα as the endogenous ligands (Forman et al., 1996, 1997; Kliewer et al., 1997). Several laboratories demonstrated that PPARα has a selectivity to certain types of fatty acids from the aspect of its binding affinity (Hostetler et al., 2005; Lin et al., 1999; Pawar and Jump, 2003; Seimandi et al., 2005). However, the intensity of the binding of fatty acid ligands to PPARα does not always correlate with the transcriptional activity of PPARα when it is monitored by a conventional transfection of reporter gene in the cell (Forman et al., 1996; Hostetler et al., 2005; Jump and Clarke, 1999; Kliewer et al., 1997; Lin et al., 1999; Pawar and Jump, 2003; Seimandi et al., 2005). From a theoretical point of view, a ligand-induced conformational change of PPARα may result in the alteration of the binding activity of PPARα to either the DNA element or its DNA binding-independent transactivity which indicates the binding efficiency to other mutually interacting proteins including coactivators and components of basic transcriptional machinery. However, few studies have examined separately the ligand-induced change in DNA-binding activity of PPARα and the change in its DNA binding-independent transactivity. Therefore, in this study, we have employed the GAL4-PPARα chimera reporter assay, in order to distinguish the ligand-induced change of the PPARα ligand-binding domain, which may lead to alteration of its transcriptional machinery-recruiting activity, from the well known ligand-induced change in the PPARα DNA-binding domain resulting in an increase in DNA binding.

First, we performed differential protease sensitivity assays to examine which types of fatty acids are able to induce conformational change in PPARα in the presence of fatty acids or synthetic ligands. A previous study using a similar technique showed that ETYA and WY14,643 were able to induce a conformational change in PPARα (Dowell et al., 1997). As shown in Fig. 1, ^{35}S-PPARα was almost completely digested by chymotrypsin in the absence of fatty acids, producing several bands with small sizes below 20 kDa (Fig. 1). We found that three major bands of approximately 33 kDa, 26 kDa and 24 kDa were protected from protease digestion by almost all fatty acid ligands as well as by the synthetic ligands. ^{35}S-PPARα was protected by some saturated fatty acids that include stearic acid and palmitic acid, as well as by most unsaturated fatty acids. Among unsaturated fatty acids, α-linoleic acid, γ-linoleic acid, arachidonic acid ($n-6$), EPA and DHA were the typical polyunsaturated fatty acids which most strongly protected PPARα from protease digestion (Fig. 1). These results suggest that various fatty acids induce a conformational change in PPARα and that polyunsaturated fatty acids of 18 to 20 carbon groups with 3–5 double bands in general strongly induce a conformational change in PPARα. As shown Fig. 2, bands protected from protease digestion by linoleic acid, WY14,643 and cPGI did not change upon addition of RXRα which is the heterodimer

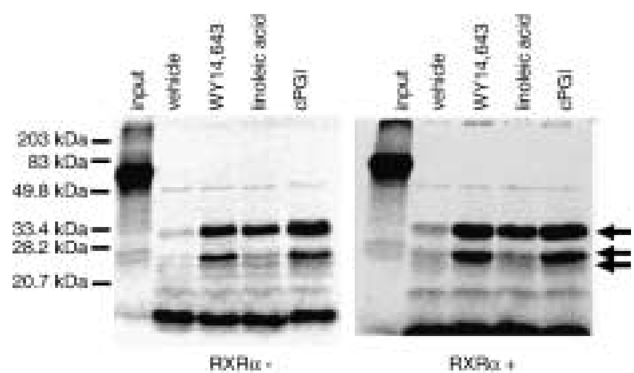

Fig. 2. Effects of RXRα on fatty acid-induced conformational change of PPARα. ^{35}S labeled-PPARα was subjected to differential protease sensitivity assay. ^{35}S-labeled PPARα in the presence or absence of unlabeled RXRα (^{35}S-labeled PPARα:unlabeled RXRα = 1:1) was preincubated for 20 min at room temperature with vehicle (2.5% DMSO, lane 2), 40 μM linoleic acid, or 40 μM synthetic ligands (WY14,643, carbaprostacyclin (cPGI)) before addition of chymotrypsin (final concentration, 0.1 mg/ml). Proteolytic digestions were carried out at room temperature for 30 min, and then the samples were subjected to SDS-PAGE on a 14% polyacrylamide gel. Undigested ^{35}S-labeled PPARα was also electrophoresed. The arrowheads indicate the bands specifically protected by the fatty acid ligands. The positions of the molecular weight markers are indicated to the left.

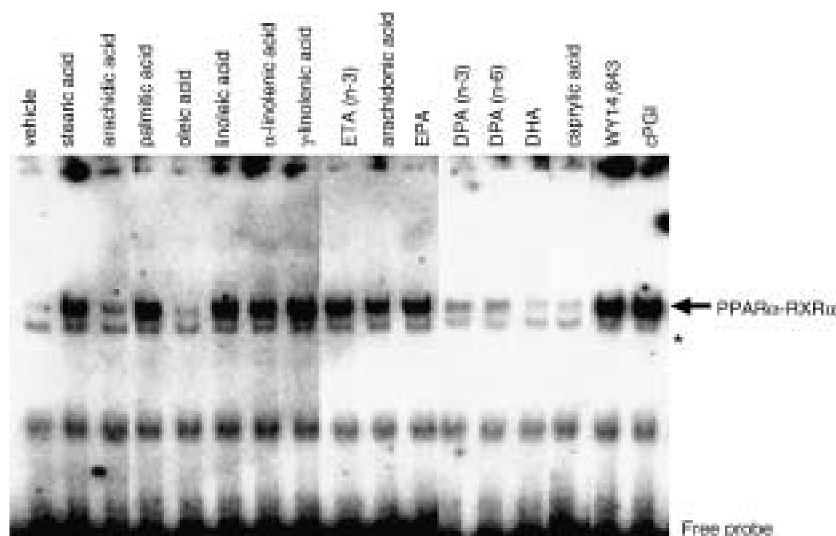

Fig. 3. Effects of fatty acid ligands on the binding activities of PPARα–RXRα heterodimers to AOX–PPRE. In vitro-translated PPARα was mixed with an equivalent mole of RXRα in the absence (2.5% DMSO) or presence of 40 μM various fatty acids or synthetic ligands (WY14,643, cPGI). The formed heterodimers were detected by the electrophoretic mobility shift assays, which were performed using ^{32}P-labeled oligonucleotides representing AOX–PPRE. The asterisk indicates non-specific binding complex.

partner of PPARs. These results indicate that PPARα conformational change by fatty acids in Fig. 1 is able to occur in a RXR-independent manner.

We have previously reported that synthetic ligands and certain fatty acids enhanced the DNA binding activity of PPARα–RXRα heterodimer to the PPRE (Mochizuki et al., 2001a; Suruga et al., 1999a). In this study, we have examined using electrophoretic mobility shift assays whether the conformational change of PPARα by fatty acids was accompanied by a change in binding activity of PPARα–RXRα heterodimer to the AOX–PPRE. The DNA binding of PPARα–RXRα to AOX–PPRE was explicitly enhanced by the saturated fatty acids which were able to protect PPARα from protease digestion, i. e., stearic acid and palmitic acid. Among the unsaturated fatty acids, polyunsaturated fatty acids of 18–20 carbon groups, i. e., linoleic acid, α-linolenic acid, γ-linolenic acid, ETA ($n-3$), arachidonic acid ($n-6$) and EPA, but not polyunsaturated fatty acids of 22 carbon group, i.e., DPA and DHA, strongly enhanced the DNA binding of PPARα–RXRα to PPRE (Fig. 3).

It was reported that a ligand-induced conformational change of PPARs which was detectable by differential protease sensitivity assay was closely associated with the activation of the transcription of the PPRE-linked reporter gene, presumably through the activated formation of the transcriptional machinery (Berger et al., 1999; Dowell et al., 1997). Moreover, it has been suggested that formation of the nuclear receptor-transcriptional complex on chromatin is stimulated by coactivator(s) in a ligand dependent manner (Moras and Gronemeyer, 1998). Also, PPAR is bound to coactivator(s) in a ligand dependent manner (Krey et al., 1997). Therefore, we considered it most likely that by stimulating the conformational change of PPARα, various fatty acids might differentially induce coactivator recruitment and this formation of the transcriptional machinery. Previous studies showed that expressions of PPARα-dependent genes were up-regulated by fatty acids and their analogs in the intestinal cell line, Caco-2 (Suruga et al., 1999b). Recently, we found that one of the major coactivators in the small intestine, p300, was strongly bound to PPARα in a ligand dependent manner in Caco-2 cells (Mochizuki et al., 2002).

In this study, using the Caco-2 cells, we have co-transfected the chimeric receptors composed of the GAL4 DBD and PPAR

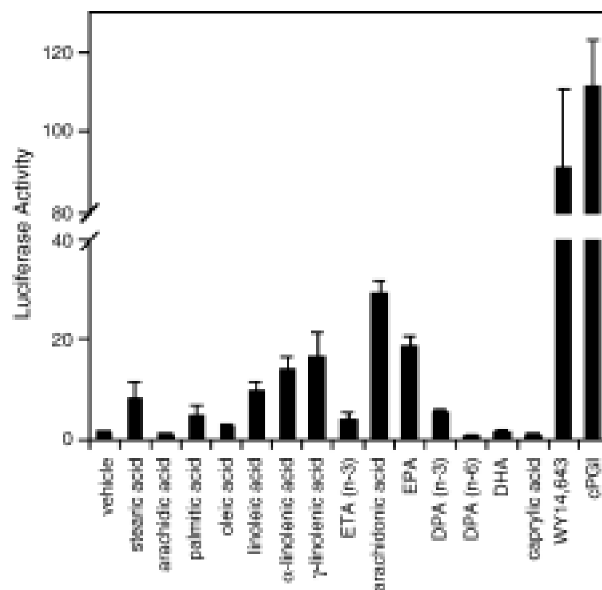

Fig. 4. Effects of various fatty acids on DNA binding-independent transactivity of PPARα in Caco-2 cells. The plasmids containing GAL4-fused ligand-binding domain (LBD) of PPARα were co-transfected into Caco-2 cells with GAL4-responsive luciferase reporter plasmids and internal standard plasmid, pRL-SV40. Transfected cells were cultured in the presence of 10 μM various fatty acids, 10 μM synthetic ligands (WY14,643, cPGI, bromopalmitate), or vehicle (DMSO). Data represent the means+SEM of three independent experiments which were all performed in triplicate.

LBD along with a GAL4-responsive reporter gene. These GAL4-PPAR chimera reporter assays enable us to evaluate the DNA binding-independent transactivity in a quantitative manner. As shown in Fig. 4, luciferase activities of the PPARα-LBD pBIND transfected cells were highly variable among the fatty acids tested. We confirmed that palmitic acid, linoleic acid, arachidonic acid and EPA, which activated PPARα transactivity in Fig. 4, induced PPARα transactivities in a dose-dependent manner in Caco-2 cells (data not shown). Among the saturated fatty acids, stearic acid and palmitic acid caused a detectable increase in luciferase reporter activity over the control. Among the unsaturated fatty acids, luciferase activities of the transfected cells were greatly elevated only by the polyunsaturated fatty acids of 18–20 carbon groups, i.e., linoleic acid (18:2), α-linolenic acid (18:3), γ-linolenic acid (18:3), arachidonic acid (20:4, $n-6$) and EPA (20:5, $n-3$), with an exception of ETA (20:4, $n-3$) which produced only a moderate increase in the reporter activity (Fig. 3). By contrast, polyunsaturated fatty acids of a longer carbon group caused a moderate (DPA ($n-3$)) or little increase (DPA ($n-6$) and DHA) in the reporter activity. Caprylic acid (8:0) did not induce PPARα transactivity. Also, 9-*cis* retinoic acid, which is a ligand of RXR, did not induce the reporter activity in this assay as well as conformational change and DNA binding to AOX–PPRE (data not shown). These results suggest that polyunsaturated fatty acids of 18 to 20 carbon groups with 3–5 double bands with an exception of ETA ($n-3$), promote the DNA binding-independent transactivity which indicates the formation of PPARα-associated coactivator-transcriptional complex. Recently, our study showed that a major coactivator in the small intestine, p300, strongly bound to PPARα in Caco-2 cells in the presence of PPARα ligand (Mochizuki et al., 2002). Moreover, a mammalian two hybrid assay revealed that linoleic acid and arachidonic acid induced the PPARα binding to p300 in Caco-2 cells. Taken together, the signals of DNA binding-independent transactivity of PPARα induced fatty acids in Caco-2 cells might be transmitted by p300. In this study, we did not determine endogenous mRNA levels of PPARα-target genes in Caco-2 cells. Our previous study showed that linoleic acid and α-linolenic acid induced a jejunal mRNA level of CRBPII which is a target gene of PPARα in the small intestine (Suruga et al., 1995). Furthermore, the induction of CRBPII mRNA levels by arachidonic acid and EPA in Caco-2 cells was higher than that by linoleic acid. These results are correlated both with PPARα binding to its *cis*-element and DNA binding-independent transactivity in this study. The sensitivity of each fatty acid to PPARα activation might determine the expression levels of PPARα-target genes in Caco-2 cells.

Conclusion

Our data suggest that the conformational change of PPARα induced by fatty acid ligands leads to a parallel increase in both DNA binding to the PPAR-response element and the DNA binding-independent transactivity of PPARα. This is the first to show evidence that PPARα possesses a great selectivity to certain types of polyunsaturated fatty acids.

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