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cis-Parinaric acid is a ligand for the human peroxisome proliferator activated receptor γ: development of a novel spectrophotometric assay for the discovery of PPARγ ligands

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Abstract Peroxisome proliferator activated receptor γ (PPARγ) is the subject of intense investigation as a target for drugs against diabetes, atherosclerosis and cancer. For this reason there is considerable interest in the spectrum of compounds that bind this receptor. In this paper we have identified *cis*-parinaric acid (CPA) as a novel hPPARγ ligand. The binding of this fatty acid to the receptor increases its fluorescence and causes a shift in the UV spectrum. This spectral shift is reversible by competition with other known ligands for PPARγ. This report represents the first direct demonstration of a fatty acid binding to PPARγ.

Key words: Peroxisome proliferator; Anti-diabetes drug; Fatty acid; Fluorescence; Spectrophotometry; Nuclear receptor

1. Introduction

The peroxisome proliferator activated receptors (PPARs) belong to the nuclear receptor family of ligand activated transcription factors and function as molecular switches, binding fatty acids and a wide range of xenobiotics to activate transcription of responsive genes [1,2]. Several physical changes may occur after ligand binding to such nuclear receptors, and these include increased DNA binding through heterodimerisation with the retinoid X receptor [1] as well as increased interactions with co-activator proteins such as SRC-1 [3].

PPARs have been shown to control target genes involved in lipid metabolism and energy balance in response to fatty acids and eicosanoids [4–7]. One member of this family, PPARα, is activated by the fibrate family of hypolipidemic drugs and is expressed at high levels in the rodent liver [8]. PPARα also mediates the pathological phenomenon known as peroxisome proliferation, that is caused by fibrate drugs in the rodent liver. The absolute requirement for PPARα in these events has been confirmed using mice containing a targeted disruption of the PPARα gene [9]. PPARγ is another member of this subfamily of nuclear receptors, which is expressed at high levels in adipose tissue and has been shown to be an important regulator of adipocyte differentiation [10]. PPARγ is activated by the isoprostanoid 15-deoxy $\Delta^{12,14}$-PGJ_2 and the thiazolidenedione family of insulin sensitising drugs which include troglitazone and BRL 49653 [11–13]. PPARγ is also expressed in other tissues such as colon, bone marrow, leukocytic cell lines and T-cells [14]. The expression of PPARγ in activated macrophages has recently been linked with the anti-inflammatory action of thiazolidenedione drugs and fish oil-derived polyunsaturated fatty acids [15,16]. PPARγ has also been implicated in the anti-neoplastic properties of certain polyunsaturated fatty acids and has been shown to mediate withdrawal from cell cycle in transformed fibroblasts, liposarcoma and human breast cancer cell lines [17–20].

cis-Parinaric acid (CPA) is a naturally occurring polyunsaturated fatty acid which is selectively toxic to tumour cell lines in vitro [21,22]; however, the molecular target for this selective toxicity is not known.

The fluorescent properties of CPA have been used to study its binding to fatty acid binding proteins [23–25]. This fatty acid is relatively non-fluorescent in aqueous environment, however, in a hydrophobic environment, such as in the lipid binding site of a protein, CPA becomes fluorescent. CPA also has a distinct UV spectrum which is shifted by the binding of proteins such as albumin [26]. We have exploited these phenomena in order to demonstrate the binding of this fatty acid to recombinant human PPARγ, and have shown that CPA binds hPPARγ with a high affinity. In addition, the spectral properties of the PPAR/CPA complex have allowed us to develop a simple method for the screening for novel PPAR ligands.

2. Materials and methods

2.1. Isolation of a cDNA human PPARγ ligand binding domain

An 877-bp portion of the human PPARγ cDNA encoding the ligand binding domain (hPPARγLBD, amino acids 195–478) was isolated by PCR from a human kidney cDNA library (Clonetech) using the following primers. Primer A: 5′-CGGGATCCATATGGCGGAGATCTCCAGTGATATCG-3′; primer B: 5′-CGGGATCCCTAGTACAAGTCCTTGTCCTTGTAGATCTCCTGC-3′. The amplification product was cloned into pET15b. Primer A generated an *Nde*I site/ATG initiation codon at the 5′ end of the product and primer B introduced a *Bam*HI site immediately after the terminator codon. The amplified products were digested with *Nde*I/*Bam*HI and ligated into *Nde*I/*Bam*HI digested pET15b. This ligation was transformed into *E. coli* strain XL-1 Blue. Complete sequencing of the cloned DNA revealed that it encodes a protein that is identical to those encoded by previously published cDNAs.

2.2. Expression of hPPARγ ligand binding domains in E. coli

The pET15b-hPPARγLBD plasmid was transformed into *E. coli* strain BL21(DE3) pLYSs. Expression of the His-tagged hPPARγLBD was induced with 0.5 mM IPTG at 30°C for 3 h. Lysates from these cultures were prepared by previously described methods [5] in PBS containing glycerol (10%), phenylmethylsulfonyl fluoride (0.2 mM) and β-mercaptoethanol (5 mM) and then loaded onto a nickel agarose column (1 ml resin per litre of culture) at a flow rate of 1–2 ml/min. The column was then washed with 30 column volumes of loading buffer containing 10 mM imidazole (pH 8.8) followed by 10 column volumes of loading buffer containing 25 mM imidazole (pH 8.8). The

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hPPARγLBD protein was eluted with loading buffer containing 250 mM imidazole (pH 8.8). The imidazole was removed and the protein was concentrated using a Centricon concentrator (10 K cut off). Any aggregated protein was then removed by ultracentrifugation at $100\,000 \times g$ for 1 h. The glycerol concentration in the final protein preparation was increased to 50% (v/v) and aliquots were stored at −20°C. The protein preparations stored in this manner were active for at least nine months. Most of the data presented in this manuscript are, however, derived from freshly prepared protein, stored at 4°C for less than 3 days.

2.3. Handling and storage of CPA

CPA was obtained from Molecular Probes, Eugene, OR, USA. Due to the susceptibility of CPA to oxidation, CPA was purchased in special 10-mg aliquots which were stored at −20°C. Aliquots were resuspended, as required, in DMSO and the concentration of CPA in stock solutions was confirmed before each procedure using the extinction coefficient at 304.2 nm in ethanol of 78 000 [26]. Solutions of CPA were stored in the short term (less than one month) at −20°C under nitrogen.

2.4. CPA fluorescence assays

Purified hPPAR LBD protein was mixed with CPA in 25 mM Tris-HCl, pH 7.5 at room temperature and the resulting fluorescence was measured immediately using a Perkin Elmer LS-3 fluorescence spectrophotometer (excitation wavelength of 318 nm and emission wavelength of 410 nm). The fluorescence resulting from protein alone and CPA acid alone were totalled and deducted from the measured value.

2.5. CPA spectrophotometric assays

CPA was added to 25 mM Tris-HCl, pH 7.5, at room temperature and scanned against buffer between 312 nm and 340 nm using a Shimadzu UV-3000 scanning spectrophotometer. All subsequent additions of protein and competitor compounds were added equally to both the sample and reference cuvettes. Incorporation of up to 2% DMSO did not produce any significant changes in the observed spectra and this was the maximum used in the addition of competitor compounds. Binding constants were calculated using a single binding site curve fitting with Ultrafit for the Macintosh. The resulting K_i values and the standard error of values derived from 2–4 independent experiments is presented in the text. Fatty acids and ibuprofen were obtained from Sigma, and 15-deoxy $\Delta^{12,14}$-PGJ_2 was obtained from the Cayman Chemical Company (Ann Arbor, MI, USA).

3. Results

The cDNA encoding the hPPARγ ligand binding domain (PPARγLBD) was isolated by PCR from a human kidney cDNA library. This cDNA encoded a protein that was identical to the published human PPARγ protein sequences [14].

The pET15b expression vector gave high levels of histidine-tagged recombinant protein in *E. coli* which facillitated the affinity purification of large amounts of highly purified PPARγLBD (~20 mg per litre of culture). These preparations were greater than 90% pure as judged by scanning Coomassie blue-stained SDS-PAGE gels (data not shown).

Affinity purified recombinant protein was assayed for binding to the fluorescent fatty acid CPA (Fig. 1). The PPARγLBD displayed limited intrinsic fluorescence at the protein concentrations studied (Fig. 1A, open squares). However, inclusion of CPA resulted in a much greater fluorescent signal that was dependent on the presence of PPARγLBD and CPA (Fig. 1A, open diamonds). Subtraction of the intrinsic fluorescence of the PPARγLBD demonstrated that the fluorescence of CPA increased with increasing concentrations of PPARγLBD and this observed fluorescence was saturable (Fig. 1B). This revealed that CPA has a K_d of 669 ± 75 nM for binding to PPARγLBD. This is a high affinity interaction that is tighter and therefore more specific than was observed

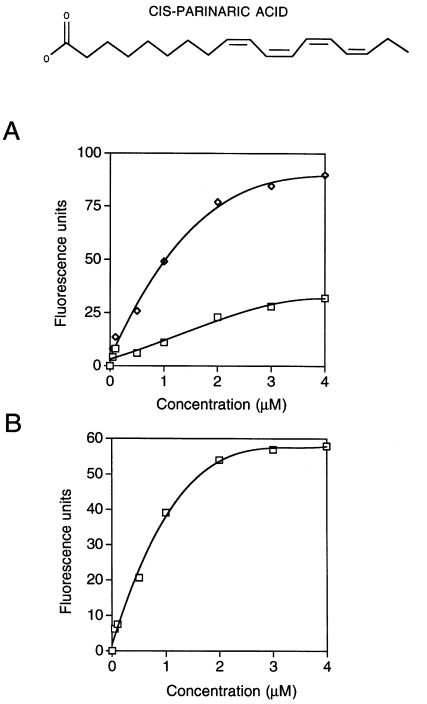

Fig. 1. CPA binds hPPARγ with a high affinity. The structure of CPA is shown. A: Fluorescence of increasing concentrations of purified hPPARγLBD was monitored in the presence (diamonds) and absence (squares) of CPA (300 nM) as described in Section 2. B: Shown is a plot with the intrinsic protein fluorescence subtracted from the fluorescence obtained in the presence of CPA. A K_d of 669 ± 75 nM is observed. As a negative control, trypsinogen (5 μM) was added to 300 nM CPA and this did not result in any increased fluorescence (data not shown). Each point shown is a mean value obtained from 2–4 independent experiments.

for CPA binding to the adipocyte lipid binding protein or bovine serum albumin (both having a K_d ~2 μM) [25,26]. Purified trypsinogen was used as a negative control and no binding of CPA was observed (data not shown).

CPA has a distinct UV spectrum at 319 nm and this is shifted to 324 nm on binding to proteins such as albumin. This phenomenon has been exploited to quantitate free fatty acids in serum as the spectral shift is reversible by competition [26]. Inclusion of PPARγLBD in solutions containing CPA resulted in a shift of the CPA spectrum resulting in a peak at 324 nm (Fig. 2). This shift was dependent on the concentration of both CPA and PPARγLBD in a manner that correlates with the binding relationships observed using the fluorescence assay. The spectral change was also reversible with the addition of the high affinity PPARγ ligand, BRL 49653.

This magnitude of the spectral shift can be quantified by determining the ratio of the absorbancies at 319 nm and 329 nm [26]. The 319/329 ratio of CPA in solution is between 2.6 and 3 and the ratio for the CPA which is fully bound to receptor is 1. Using low concentrations of CPA, that are below the apparent K_d, it can be seen that increasing levels of PPARγLBD reduce the 319/329 ratio from 2.6 to a plateau of 1.6 (Fig. 3A). This curve confirms a K_d of approximately 600 nM. The ratio never reaches 1 in this assay as the low concentration of the CPA does not allow for complete binding of the fatty acid to the PPARγLBD. In assays utilising higher concentrations of both CPA and PPAR a ratio of 1 was

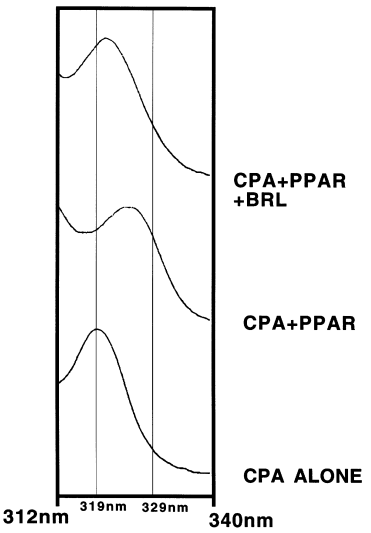

Fig. 2. CPA has a UV spectrum that is shifted by binding to hPPARγ. The sequential UV spectra shown are: CPA alone: CPA (3 μM) in solution. The CPA in solution has a 319 nm/329 nm ratio of 3 due to the peak at 319. CPA+PPAR: hPPARγLBD protein (5 μM) was then added to both the reference and sample cuvettes. This resulted in shifting of the peak to 324 nm and resulted in a 319 nm/329 nm ratio of 1. CPA+PPAR+BRL: A large excess of the thiazolidenedione drug BRL 49653 (50 μM) was then added to both the sample and reference cuvettes. This resulted in the displacement of CPA, shifting the peak back towards 319 nm and producing a ratio of 2.6.

achieved (Fig. 2). In order to establish the specificity of binding and to further validate the assay, we investigated whether known PPARγ ligands would compete for binding. In these assays PPARγLBD (2 μM) and CPA (1.5 μM) were used to give a final 319/329 ratio of approximately 1.2. Using these conditions, competition with BRL 49653 was observed with a K_i of 2.19 ± 0.3 μM. This value is not significantly different from the concentration of the PPARγLBD in the assay (2 μM) and therefore does not reflect the true affinity of BRL 49653, rather this value reflects the stoichiometry of BRL 49653 binding to the PPARγLBD (Fig. 3B, squares). The intrinsic K_d of BRL 49653 for PPARγ is 40 nM [11,13]. In order to accurately measure such a low K_d it would be necessary for both CPA and PPARγLBD to be present in the assay at less

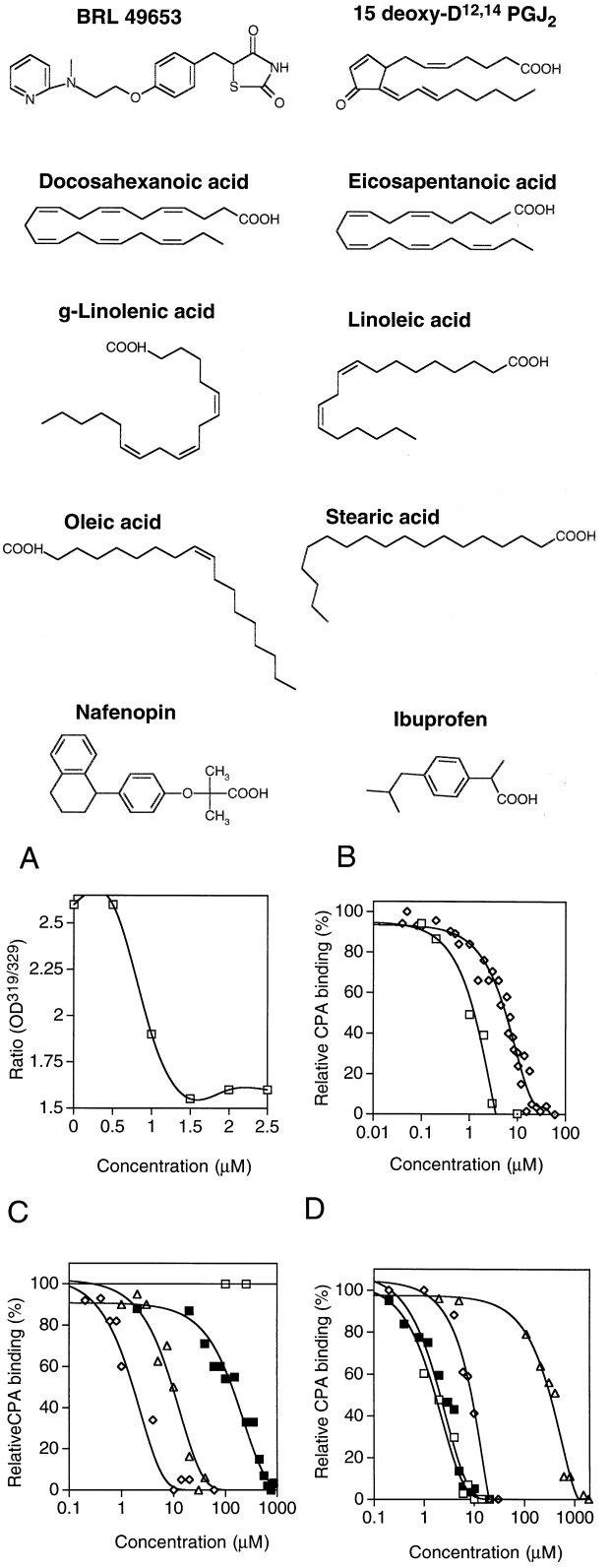

Fig. 3. A displacement assay for PPARγ ligands. The structures of compounds used in the displacement assay are shown. A: Titration of CPA with hPPARγ. Increasing concentrations of hPPARγLBD protein were added to 500 nM CPA and the 319 nm/329 nm ratio determined as described in Section 2. B: Displacement of CPA binding to hPPARγ by the high affinity ligand BRL 49653 and the isoprostanoid 15-deoxy $\Delta^{12,14}$-PGJ_2. Increasing concentrations of BRL 49653 (open squares) and 15-deoxy $\Delta^{12,14}$-PGJ_2 (open diamonds) were added to cuvettes containing 1.5 μM CPA and 2 μM hPPARγ. Shown is the relative drop in 319/329 ratio compared to fully bound. The BRL 49653 plot is a representative experiment of two independent experiments. The 15-deoxy $\Delta^{12,14}$-PGJ_2 shows three independent experiments plotted together to demonstrate the reproducibility of the assay. C: Displacement of CPA binding to hPPARγ by docosahexanoic acid, oleic acid, nafenopin and stearic acid. Increasing concentrations of docosahexanoic acid (diamonds), oleic acid (triangles), nafenopin (filled squares), stearic acid (open squares) were added to cuvettes containing 1.5 μM CPA and 2 μM hPPARγ. Shown is the relative drop in 319/329 ratio compared to CPA fully bound to PPAR. Each plot is a representative experiment of 2–4 independent experiments. D: Displacement of CPA binding to hPPARγ by γ-linolenic acid, eicosapentanoic acid and linoleic acid. Increasing concentrations of γ-linolenic acid (open squares), eicosapentanoic acid (filled squares), linoleic acid (diamonds) and ibuprofen (triangles) were added to cuvettes containing 1.5 μM CPA and 2 μM hPPARγLBD. Shown is the relative drop in 319/329 ratio compared to CPA fully bound to PPAR. Each plot is a representative experiment of 2–4 independent experiments.

than 10 nM. Under these conditions, however, the CPA and PPARγLBD would be completely dissociated and could not serve as a reporter system.

Displacement of CPA was also observed using a proposed endogenous ligand, 15-deoxy $\Delta^{12,14}$-PGJ_2 with a K_i of 6.4 ± 0.9 μM (Fig. 3B, diamonds). The PPARα ligand and peroxisome proliferator nafenopin has a very poor affinity for this receptor of 128 ± 10 μM (Fig. 3C, filled squares). The NSAID, ibuprofen was also a very poor ligand for the PPARγLBD with a K_i of 329 ± 50 μM (Fig. 3D, triangles). This value agrees well with the concentrations required to elicit PPARγ activity in human macrophages (EC_{50} = 140–450 μM) [15]. The saturated fatty acid, stearic acid, did not displace CPA from PPARγLBD at concentrations up to 200 μM (Fig. 3C, open squares). Beyond this concentration stearic acid was insoluble.

Polyunsaturated fatty acids such as docosahexanoic acid (Fig. 3C, diamonds, K_i = 2.93 ± 0.3 μM), γ-linolenic acid (Fig. 3D, open squares, K_i = 2.28 ± 0.1 μM), and eicosapentanoic acid (Fig. 3D, filled squares, K_i = 3.1 ± 0.2 μM) displaced CPA from PPARγLBD at concentrations similar to the concentration of PPARγLBD in the assay; whereas oleic acid and linoleic acid have lower K_is of 11.7 ± 0.5 and 8.37 ± 0.8, respectively (Fig. 3C, triangles and Fig. 3D, diamonds).

4. Discussion

In the present study we have shown CPA to be a novel ligand for human PPARγ that has a high affinity for this receptor with a K_d of 669 nM. This is the first report of fatty acids binding to human PPARγ. The spectral properties of CPA have been used to develop an assay for the binding of fatty acids and drugs to the human PPARγ. Previous ligand binding studies for PPARs have utilised radiolabelled compounds that are not widely available or have utilised indirect methods based on protein/protein interactions [1–3,11]. In contrast to these methods, the assay presented in this report only requires a simple 2 point spectrophotometric determination and utilises reagents and equipment that are readily available. Very high and low affinity ligands can be identified by this assay. This assay therefore serves as a valuable initial screen in the discovery of new ligands.

There are a few reports on fatty acid binding to proteins related to hPPARγ. Previous studies have examined binding of polyunsaturated fatty acids to *Xenopus* and mouse PPARγ using radioligand displacement and indirect protein/protein interaction assays. The affinity of CPA for hPPARγ is slightly higher than that reported for the proposed natural ligand 15-deoxy $\Delta^{12,14}$-PGJ_2 binding to mouse PPARγ as measured by the displacement of radiolabelled BRL 49653 (K_i = 2.5 μM). This study has shown that the human PPARγLBD has a affinity for this ligand of 6.4 μM which is comparable to the concentrations required for the stimulation of lipogenesis in murine fibroblasts (EC_{50} = 7 μM) [12]. In the *Xenopus* system two widely different values have been determined. The affinity of 15-deoxy $\Delta^{12,14}$-PGJ_2 for the xPPARγ was measured to be 500 nM by the displacement of radiolabelled fibrate, GW2331 [2] and approximately 15 μM by the activation of co-receptor binding in vitro [3]. The variations between studies presumably reflect the different assays used in each case.

Species differences have been seen for the activation of other human and *Xenopus* PPARs [27]. This report has demonstrated that hPPARγ has a high affinity for several essential fatty acids such as γ-linolenic acid, eicosapentanoic acid and docosahexanoic acid when compared to the xPPARγ and a lower affinity for fatty acids that are abundant in the human diet such as linoleic acid and oleic acid [2,3]. Such species differences presumably reflect the differences in diet and physiology that occur between frogs and man.

The binding of these dietary fatty acids at low micromolar concentrations is in the same range, or tighter than 15-deoxy $\Delta^{12,14}$-PGJ_2. These essential fatty acids therefore represent high affinity ligands for hPPARγ. The presence of these compounds in the diet would suggest that they may have an impact on the activation status of PPARγ in man. It is therefore important that future studies on essential fatty acid supplementation in man should consider the possibility that any observed effects may be mediated by this novel receptor system.

In conclusion, we have presented the first direct demonstration of fatty acid binding to hPPARγ and have developed a simple assay that allows the identification of potential agonists and antagonists of PPARγ function. This screening method may facilitate the discovery of a large number of novel natural compounds and pharmaceuticals for the treatment of diabetes, atherosclerosis and cancer.

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