ELSEVIER

Toxicology 176 (2002) 175–185

TOXICOLOGY

www.elsevier.com/locate/toxicol

Interactions of flurochemicals with rat liver fatty acid-binding protein

Deanna J. Luebker [a,*], Kris J. Hansen [b], Nathan M. Bass [c], John L. Butenhoff [a], Andrew M. Seacat [a]

[a] *3M Medical Department, Corporate Toxicology, 3M Center Building 220-2E-02, Saint Paul, MN 55144, USA*
[b] *3M Environmental Technology and Safety Services, Building 02-3E-09, Saint Paul, MN 55144, USA*
[c] *School of Medicine, University of California, San Francisco, CA 94143, USA*

Received 5 December 2001; received in revised form 27 February 2002; accepted 28 February 2002

Abstract

Liver-fatty acid binding protein (L-FABP) is an abundant intracellular lipid-carrier protein. The hypothesis that perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and certain related perfluorooctanesulfonamide-based fluorochemicals (PFOSAs) can interfere with the binding affinity of L-FABP for fatty acids was tested. The relative effectiveness of PFOA, PFOS, N-ethylperfluorooctanesulfonamide (N-EtFOSA), N-ethylperfluorooctanesulfonamido ethanol (N-EtFOSE), and of the strong peroxisome proliferator Wyeth-14 643 (WY) to inhibit 11-(5-dimethylamino-napthalenesulphonyl)-undecanoic acid (DAUDA) binding to-L-FABP was determined. The dissociation constant (Kd) of the DAUDA-L-FABP complex was 0.47 nM. PFOS exhibited the highest level of inhibition of DAUDA-L-FABP binding in the competitive binding assays, followed by N-EtFOSA, WY, and, with equal IC_{50}s, N-EtFOSE and PFOA. The in vitro data presented in this study support the hypothesis that these fluorochemicals may interfere with the binding of fatty acids or other endogenous ligands to L-FABP. Furthermore, this work provides evidence to support the hypothesis that displacement of endogenous ligands from L-FABP may contribute to toxicity in rodents fed these fluorochemicals. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Liver fatty acid-binding protein; Perfluorooctanesulfonate; Perfluorooctanoate; N-ethylperfluorooctanesulfonamide; N-ethylperfluorooctanesulfonamido ethanol; Relative dissociation constants

1. Introduction

Perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and related perfluorooctanesulfonamide-based fluorochemicals (PFOSAs) consist of a perfluorinated seven- or eight-carbon tail and either a carboxyl or sulfonic-acid derivatized head group. PFOS and PFOA resemble fatty

* Corresponding author. Tel.: +1-651-737-1374; fax: +1-651-733-1773.

E-mail addresses: djluebker@mmm.com (D.J. Luebker), kjhansen@mmm.com (K.J. Hansen), tony@itsa.ucsf.edu (N. Bass), jlbutenhoff@mmm.com (J.L. Butenhoff), amseacat1@mmm.com (A.M. Seacat).

0300-483X/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0300-483X(02)00081-1

acids with highly hydrophobic and rigid perfluorinated carbon tails, and strongly polar sulfonyl or carboxyl head groups, respectively. PFOS is the terminal metabolite of N-ethylperfluorooctanesulfonamido ethanol (N-EtFOSE) (Gibson et al., 1983) and potentially of all compounds in the perfluorooctanesulfonamide family, a group of compounds that have been utilized in a wide range of industrial and consumer applications (Banks et al., 1994). Treatment of rodents with PFOS, PFOA, or N-EtFOSE causes decreased body weight, increased liver weight, liver peroxisome proliferation, reduced serum cholesterol and triacylglycerol, and increased intracellular free fatty acids and free cholesterol in the liver (Butenhoff and Seacat, 2001; Ikeda et al., 1985, 1987; Kennedy, 1987; Haughom and Spydevold, 1992). These effects are also observed following administration of certain hypolipidemic drugs, such as clofibrate (Haughom and Spydevold, 1992), and the non-β-oxidizable fatty acid analogues, long-chain 3-thia fatty acids (Berge et al., 1989; Vaagenes et al., 1998), which are established peroxisome proliferators in rodents. Decreased hepatic cholesterol ester content, due to decreased 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) and acyl CoA cholesterol acyltransferase (ACAT) activities may be responsible for the observed hypolipidemia, via inhibition of very low-density lipoprotein (VLDL) formation (Haughom and Spydevold, 1992). PFOS, PFOA, and N-EtFOSE are established peroxisome proliferators in rodents (Ikeda et al., 1987; Haughom and Spydevold, 1992; Sohlenius et al., 1993; Kennedy et al., 1998).

Liver-fatty acid binding protein (L-FABP) is a 14 000 Da cytosolic protein that binds endogenous fatty acids (Appelkvist and Dallner, 1980). Hepatic levels of L-FABP correlate with the rate of peroxisomal β-oxidation (Appelkvist and Dallner, 1980). Many peroxisome proliferators, including certain fluorochemicals, bind L-FABP in vitro (Issemann et al., 1992; VandenHeuvel, 1996), with relative strengths of binding that parallel their ability to elicit peroxisome proliferation (Appelkvist and Dallner, 1980; Brandes et al., 1990; Kanda et al., 1989; Kawashima et al., 1983; Cannon and Eacho, 1991). Induction of L-FABP by peroxisome proliferators has been reported (Kawashima et al., 1983).

Many peroxisome proliferators, including PFOA, have been shown to activate a family of nuclear receptor transcription factors, called peroxisome proliferator activated receptors (PPARs), which regulate the expression of genes involved in lipid metabolism and are under investigation for their role in certain chronic diseases (Biegel et al., 1995; Desvergne and Wahli, 1999; Kersten et al., 2000; Lake and Gray, 1985). Activation of PPARα is necessary for the induction of peroxisome proliferation in rats (Cattley et al., 1998). Some peroxisome proliferators, including PFOA, have been shown to activate PPARα by an apparent direct binding mechanism (Ellinghaus et al., 1999; Maloney and Waxman, 1999).

This study was designed to test the hypothesis that PFOS, PFOA, and certain PFOSAs can interfere with the binding affinity and/or capacity of L-FABP for fatty acids. The characteristics of fatty acid and fluorochemical binding to L-FABP at equilibrium were investigated with an in vitro fluorescent probe displacement assay using the fluorescent fatty acid analogue 11-(5-dimethylaminonapthalenesulphonyl)-undecanoic acid (DAUDA). PFOS, PFOA, N-EtFOSE, and N-ethylperfluorooctanesulfonamide (N-EtFOSA) were used as competitors. Wyeth-14 643 (WY), a non-fluorochemical ligand and well-known potent peroxisome proliferator and hepatocarcinogen (Biegel et al., 1995) was used as a comparator compound. These studies were carried out with L-FABP isolated from livers of untreated male rats. Rats were chosen as the model species because they are strong responders to peroxisome proliferators (Elcombe and Mitchell, 1986). The maximum binding capacity of the L-FABP sample, the dissociation constant (Kd) of DAUDA binding to L-FABP, and the concentration of oleic acid which could inhibit fifty percent of specific DAUDA-L-FABP binding (IC_{50}) were determined. The IC_{50}s of the various fluorochemicals and WY were also determined to examine the relative potencies of each for inhibiting DAUDA-L-FABP binding. These experiments were intended to provide evidence to support the hypothesis that displacement of endogenous ligands from L-FABP may contribute to the mechanism of toxicity in rodents fed these fluorochemicals.

2. Materials and methods

2.1. Animals

Male rats, 6–8 weeks of age and weighing between 150 and 250 g were purchased from Charles River Labs, (Wilmington, MA). All animals were housed individually in controlled environments.

2.2. Materials

WY was obtained from ChemSyn Science Laboratories, Lexena, KS. PFOS, PFOA, N-EtFOSA, and N-EtFOSE were provided by the 3M Company, St. Paul, MN. The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Chemical Company, Rockford, IL; and DAUDA was purchased from Molecular Probes, Eugene, OR. All other chemicals were obtained from VWR Scientific, West Chester, PA. Structures of the test compounds and DAUDA are shown in Fig. 1.

2.3. Protein purification and characterization

Protein purification, performed at the University of California San Francisco (UCSF) Liver Research Center, is briefly described as follows. Approximately 10 g of liver were homogenized in 30% (w/v) ice-cold potassium phosphate (KH_2PO_4) homogenization buffer (10 mM, pH 7.4) using a Teflon-glass Potter-Elvehjem tissue homogenizer. The homogenate was then centrifuged for 20 min at 10 000 × g (4 °C). The supernatants were collected and centrifuged for 1 h at 210 000 × g (4 °C) in a Beckman L7 Ultracentrifuge. The supernatant was labeled with 0.5 μCi of 1-^{14}C oleate and applied to a Sephadex G50 M column (5 × 60 cm). The column was equilibrated with 10 mM KH_2PO_4 buffer (pH 7.4), eluted at a flow rate of 1.4 ml/min, and collected in one-hundred 14.5 ml fractions. The radioactivity of a 20 μl aliquot of each fraction was counted in a Packard Tri-carb 4530 scintillation counter (Packard Instrument Co. Meridan, CT) in 5 ml of Optifluor LSC-cocktail. Fractions containing ^{14}C activity were pooled and concentrated to approximately 5 ml using an AMICON YM-5 membrane (Amicon Corp., Lexington, MA). The concentrated fractions were then separated on a Sephadex G50 (fine) gel filtration column (2.5 × 45 cm) equilibrated with 10 mM KH_2PO_4 buffer (pH 7.4) at a flow rate of 0.9 ml/min. Fractions (3.5 ml) containing radioactivity were pooled, concentrated to approximately 5 ml, and dialyzed overnight (3500 molecular weight (MW) cut-off Spectrapore Membrane) against 30 mM Tris–HCl buffer (pH 9). The sample was then applied to a Whatman DE-52 DEAE-cellulose column (1.25 × 15 or 2.5 × 15 cm). The column was equilibrated and then eluted with 30 mM Tris–HCl (pH 9) followed by a linear gradient of NaCl (0–0.2 M) in 30 mM Tris–HC1 (pH 9) at 1 ml/min. Fractions (7.8 ml)

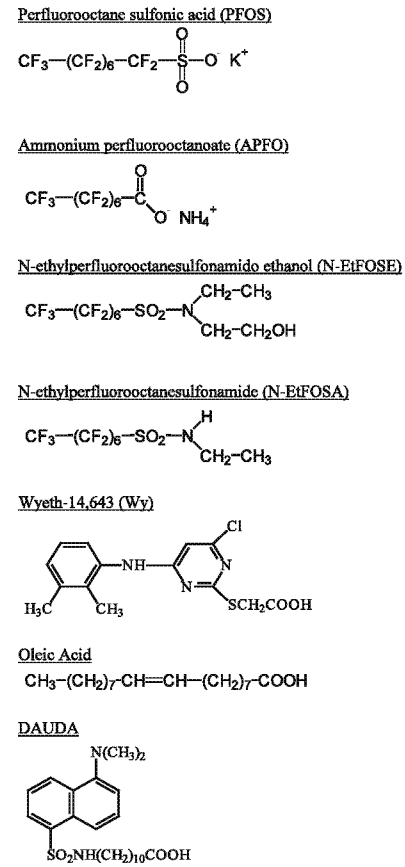

Fig. 1. Compound structures.

containing radioactivity were pooled, concentrated and characterized by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis analysis (SDS-PAGE) on 15% polyacrylamide gels and by liquid chromatography/electrospray tandem mass spectrometry (LC/ESMSMS).

Aliquots of the purified protein were analyzed using a Micromass Quattro II electrospray tandem mass spectrometer (Beverly, MA). The L-FABP was infused into the mass spectrometer at an approximate concentration of 400 pmol/μl in a 50/50 solution of methanol/2.5% acetic acid. The capillary voltage was 2.99 kV and the cone was maintained at 25 V. Data were collected in the continuum mode and several scans were summed to produce an ion series. The average protein concentration of the L-FABP samples was determined to be 123 ± 13.7 μg/ml using the Pierce BCA protein assay kit with BSA as the standard.

2.4. Fluorescence emission spectroscopy

Fluorescence measurements were performed according to previously published techniques (Thumser et al., 1994a,b, 1996; Thumser and Wilton, 1994, 1995; Wilkinson and Wilton, 1986) at room temperature in a SPEX 1681 0.22 m spectrometer (SPEX Industries, Inc. Edison, NJ) using a slit width of 5 nm. A stock solution of DAUDA, 0.1 mM, was prepared by slowly adding nine volumes of 50 mM KH_2PO_4 buffer (pH 7.2) to a stock solution of 1 mM DAUDA in methanol.

Maximum excitation and emission wavelengths (nm), and maximum fluorescence intensity (FI) were determined by adding L-FABP (0.1–3.0 μM) to 1 μM DAUDA until no further change in emission wavelength (EX 350 nm, EM 350–600 nm), excitation wavelength (EX 250–400 nm, EM 500 nm) or FI (EX 350 nm, EM 500 nm) were detected. The average maximum FI ($n = 3$ trials) was determined by plotting FI versus L-FABP concentration.

Total binding of DAUDA to L-FABP was determined by adding aliquots of 0.1 mM DAUDA to 1 μM L-FABP and measuring FI in arbitrary fluorescence units (FU). Non-specific binding/fluorescence was assessed by saturating L-FABP binding sites with excess oleic acid, titrating with DAUDA, and measuring the FI following each addition of DAUDA. Specific binding/fluorescence was determined by subtracting non-specific binding from total binding.

Fluorescence values, determined by titration of 1 μM L-FABP with increasing concentrations of DAUDA, were corrected for non-specific DAUDA fluorescence and fitted to a hyperbolic curve for evaluation using the Michaelis–Menton equation:

$$FI_S = \frac{\beta_{max}\, x[S]}{(Kd + [S])}. \quad (1)$$

In this equation, FI_S is the specific fluorescence, $[S]$ is the total DAUDA concentration (μM), β_{max} is the calculated maximum fluorescence, and Kd is the calculated Michaelis dissociation constant (μM). Specific binding was transformed to units of bound DAUDA (μM) by dividing FI_S by β_{max} per 1 μM DAUDA using the following equation:

$$\text{Bound DAUDA } (\mu M) = \frac{FI_S}{(\beta_{max}/1\ \mu M\ \text{DAUDA})}. \quad (2)$$

2.5. Competitive binding experiments

The concentration of oleic acid that inhibited 50% of specific DAUDA binding (IC_{50}) was determined for the L-FABP sample. IC_{50} values for DAUDA binding to the L-FABP sample were calculated for each fluorochemical, WY, and methanol. The competitors, prepared as 1 mM stock (oleic acid in 10% methanol, fluorochemicals, and WY in 100% methanol, and methanol in 50 mM KH_2PO_4, pH 7.2), were added in 0.4–20 μl aliquots to cuvettes containing 2 ml of 1 μM L-FABP and 1 μM DAUDA, and FI was measured as described above. The percent inhibition of specific DAUDA binding by each competitor (three to six trials) was corrected for the effect of methanol and each IC_{50} was then calculated.

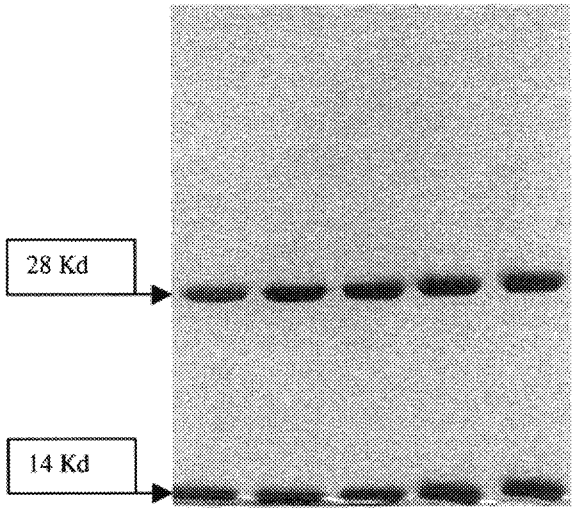

Fig. 2. SDS-PAGE analysis. Fractions following purification of L-FABP from rat liver cytosol. Representative of fractions pooled for experimentation. The band at MW 14 000 Da is L-FABP and the band at MW 28 000 Da is a presumed dimer.

3. Results

3.1. Homogeneity

Homogeneity of the purified L-FABP was confirmed by SDS-PAGE showing a dominant protein band at MW 14 000 Da (Fig. 2). A second protein band appeared at approximately 28 000 Da, which was believed to be a dimer of L-FABP. The LC/ESMSMS spectra showed a multiply charged ion series of ten peaks, relatively free of background noise (Fig. 3), from which a measured mass consistent with previously published results (Gordon et al., 1983) of 14 315 Da was calculated.

3.2. Fluorescence characterization

The maximum excitation and emission wavelengths of the L-FABP sample were approximately 340 and 500 nm, respectively, and in agreement with previously published results (Wilkinson and Wilton, 1986; Thumser et al., 1996). Shifting of emission wavelength from 330 to 340 nm and of excitation wavelength from 550 to 500 nm was seen upon addition of DAUDA to L-FABP. Maximum FI (962 199 ± 14 686 FU) was achieved upon addition of 1 μM DAUDA to 1 μM L-FABP. Specific binding of DAUDA to L-FABP represented approximately 90% of total binding.

3.3. Affinity binding constant (Kd)

The average *Kd* for DAUDA–L-FABP binding was 0.35 ± 0.02 μM ($n = 3$) and the data suggest that DAUDA binds only one site on L-FABP. These findings are in agreement with previous work (Thumser et al., 1994b, 1996; Thumser and Wilton, 1994, 1995).

3.4. IC_{50}'s

Each of the fluorochemical competitors, as well as oleic acid and WY, lowered the maximum FI and shifted the emission wavelength towards 550 nm, representing an inhibition of DAUDA–L-FABP binding, as exemplified with PFOA as the competitor (Fig. 4). Oleic acid (2 μM) inhibited 91% of specific DAUDA–L-FABP binding. The IC_{50} of oleic acid was determined to be 0.01 μM. Curves of percent inhibition of specific DAUDA binding versus increasing competitor concentrations show that PFOS was the strongest fluorochemical inhibitor of specific DAUDA binding (Fig. 5). Upon addition of 10 μM PFOS, 69% of specific DAUDA–L-FABP binding was inhibited. The calculated IC_{50} for PFOS was 4.9 μM. N-Et-FOSA (10 μM) inhibited 51% of specific DAUDA–L-FABP binding. The calculated IC_{50} for N-EtFOSA was 9.7 μM. WY was comparable, inhibiting 50% of specific DAUDA–L-FABP binding at 10 μM and having an IC_{50} of 10 μM. Upon addition of 10 μM of either N-EtFOSE or PFOA, 43% of specific DAUDA–L-FABP binding was inhibited. The calculated IC_{50}s for these compounds were greater than 10 μM. A comparison of IC_{50} values can be found in Table 1.

4. Discussion

The LC/ESMSMS and SDS-PAGE results indicate that the isolation method yielded a relatively

pure sample of L-FABP. The presence of the dimer is not believed to have had any adverse effect on the results.

The *Kd* for DAUDA–L-FABP binding was equivalent to the *Kd*s of 0.46 ± 0.04 μM (Thumser et al., 1994a) and 0.38 ± 0.02 μM (Thumser et al., 1996) reported previously for recombinant wild type rat L-FABP. The shifting of wavelength seen upon addition of DAUDA to L-FABP is characteristic of DAUDA binding to the secondary (non-polar) site and not the primary binding site on L-FABP (Wilkinson and Wilton, 1986; Thumser et al., 1994a). In addition, precise conformational requirements are necessary for primary site binding (Thompson et al., 1997). Thus, DAUDA is believed to bind exclusively to the secondary binding site on L-FABP.

The maximum percent inhibition of DAUDA–L-FABP binding achieved with oleic acid under the conditions used in this study was 91%. This is slightly higher than the previously reported values of 81, 87, and 76% for undetermined reasons (Thumser et al., 1994b, 1996; Thumser and Wilton, 1995). Two fatty acid binding sites exist on L-FABP that interact allosterically. Oleic acid is capable of binding to both the primary and secondary L-FABP binding sites; and it is thought that once a ligand has bound the primary site, binding to the secondary site is facilitated (Thompson et al., 1997). Crystal structures of L-FABP prepared with oleic indicate that the primary binding site is a completely internalized site. When binding to this site, the carboxylate head group of oleic acid forms hydrogen bonds

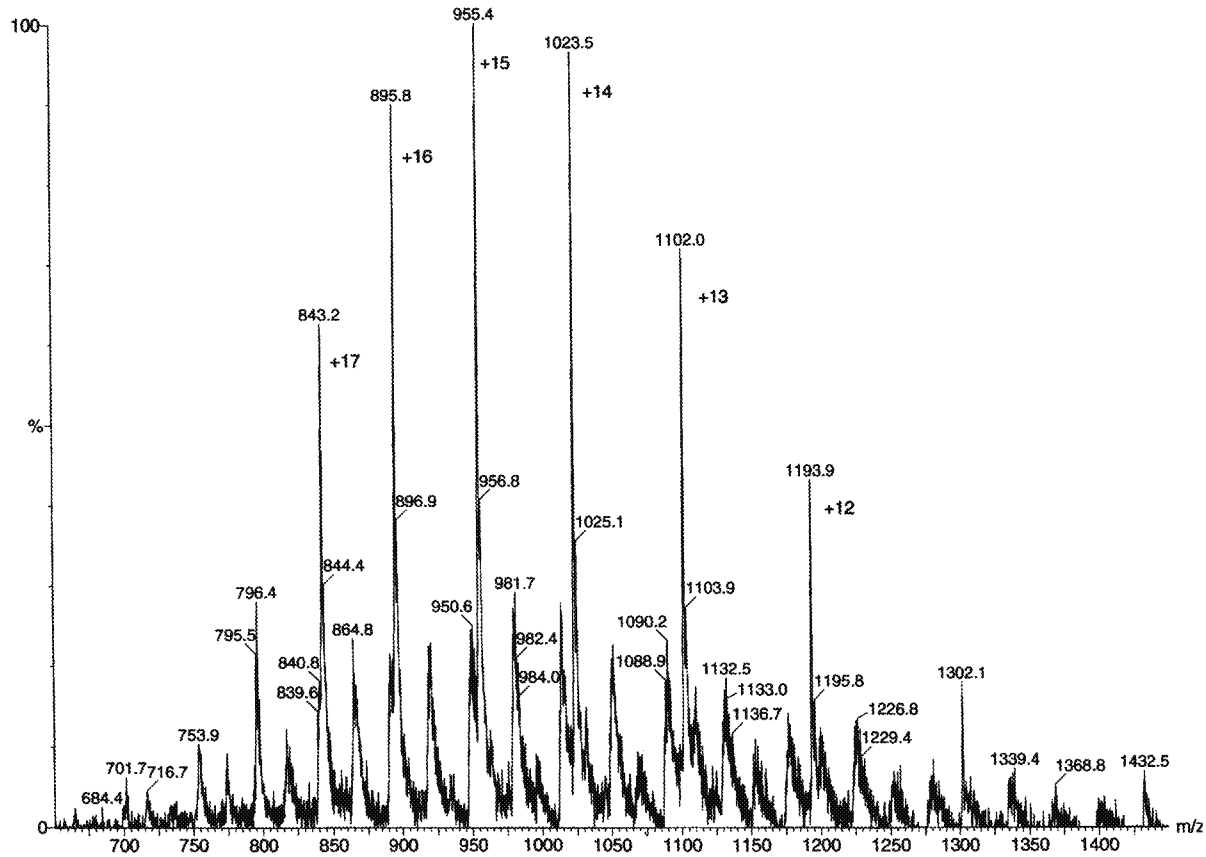

Fig. 3. Electrospray mass spectrum for control rat L-FABP. Charge states are indicated next to the major peaks. Measured mass of 14 315 Da calculated for L-FABP.

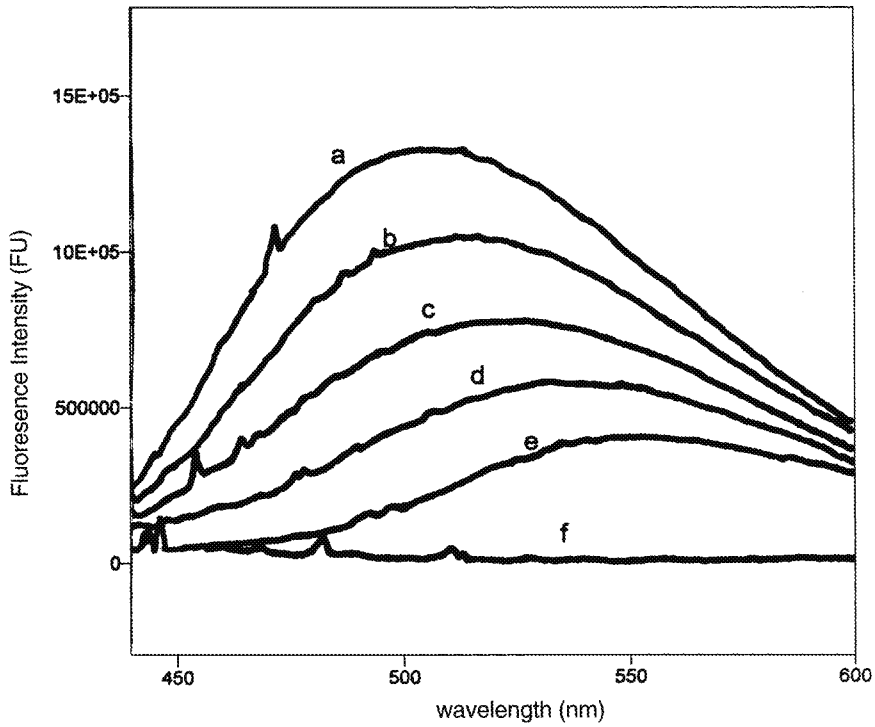

Fig. 4. Competitive inhibition of DAUDA–L-FABP binding by PFOA. PFOA(1 mM) was aliquoted into a solution of 1 μM L-FABP and 1 μM DAUDA. Lowering of the maximum FI and shifting of the emission wavelength towards 550 nm represents an inhibition of DAUDA–L-FABP binding. Arbitrary fluoresence units (FU). Key, 0 μM PFOA (a); 10 μM PFOA (b); 15 μM PFOA (c); 20 μM PFOA (d); DAUDA only (e); buffer only (f).

with Ser^{39}, Arg^{122}, and Ser^{124}, and the hydrocarbon tail loops into a U-shape around an external hydrophobic region of L-FABP. A second oleic acid is able to bind to the secondary binding site with its hydrocarbon tail internalized and its carboxylate head group solvent-accessible. The oleic acid head group at this secondary site is involved in a network of hydrogen bonds with residues, such as Lys^{31}, Tyr^{54}, Ser^{56}, and Asp^{88}, that form the entrance to the primary binding cavity.

The relative potencies of L-FABP binding exhibited by each fluorochemical cannot readily be explained by differences in polarity and hydrogen bonding ability. For example, PFOA, having a very polar carboxylate head group, and capable of extensive hydrogen bonding, had a higher IC_{50} than N-EtFOSA, which has a less polar head group and less ability to hydrogen bond. It is thought that ligands for L-FABP need to have both a hydrophobic and a hydrophilic domain (Thumser et al., 1996). Each fluorochemical and WY fits this description to varying degrees. It is unlikely that the rigid fluorocarbon tail of the fluorochemicals used in this study (Zisman, 1964) would allow for the U-shape conformational requirement for primary site binding (Thompson et al., 1997), unless formed by branching of the fluorocarbon tail. Interestingly, the fluorocarbon tails of the fluorochemicals used in this study consisted of approximately 20–30% mixed branched isomers (Kestner T., unpublished). Furthermore, oral dosing with mixed branched and linear fluorochemicals has been shown to result in a larger proportion of branched PFOS recovered from liver and sera than in the starting material (Hansen K.J., personal communication). Thus, preferential absorption and/or retention of branched fluorochemicals may occur, possibly due to the binding of fluorochemicals to specific carrier proteins.

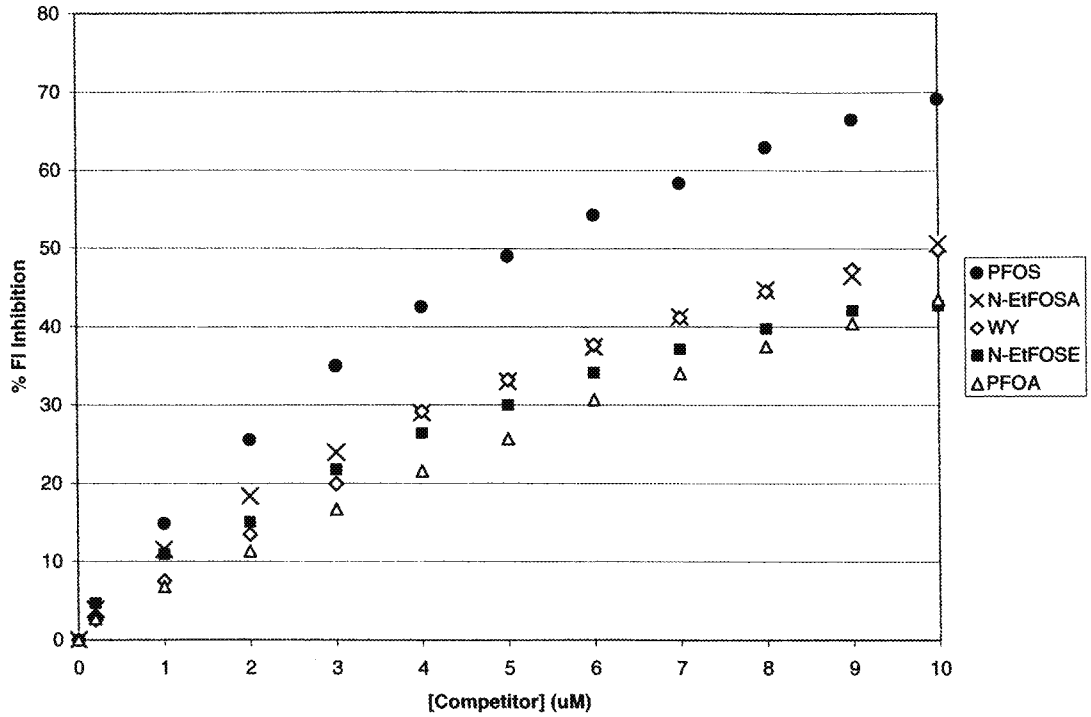

Fig. 5. Inhibition of specific DAUDA–L-FABP binding by fluorochemicals and WY. Each competitor (1 mM) was aliquoted into a solution of 1 μM L-FABP and 1 μM DAUDA. Curves represent an average of three to six trials corrected for the effect of methanol which ranged from 2% inhibition at 1 μM to 21% inhibition at 10 μM.

Binding of the highly polar sulfonate head group of PFOS with the positively charged arginines (Arg^{122} and Arg^{126}) of the primary binding cleft of L-FABP cannot be ruled-out by the present study. A mutation of the conserved internal Arg^{122} at the primary binding site has previously been shown to result in insignificant changes in binding of DAUDA to L-FABP (Thumser et al., 1994a). This suggests a lack of notable ionic involvement of the internal Arg^{122} residue in ligand binding. Therefore, polar interactions may not predominate over the conformational requirements of the hydrophobic region of the L-FABP primary binding site. The shifting of wavelength and decrease in FI seen in the competition assays are indicative of DAUDA being displaced from the non-polar secondary binding site (Thumser et al., 1994a; Wilkinson and Wilton, 1986). Thus, secondary site binding of the fluorochemicals and WY is supported by these data. However, to investigate binding of fluorochemicals to the primary site and draw conclusions about in vivo binding, a probe known to bind both sites, i.e. radiolabeled oleic acid, would need to be used.

Table 2 compares the fluorescence inhibition found with oleic acid, WY, and the fluorochemi-

Table 1
IC_{50} values

Competitor	IC_{50} value (μM)
Oleic acid	0.01
PFOS	4.9
N-EtFOSA	9.7
WY	10
N-EtFOSE	>10
PFOA	>10

The IC_{50} of oleic acid for L-FABP was calculated by adding aliquots of oleic acid (1 mM in 10% methanol) to a solution of 1 μM L-FABP and 1 μM DAUDA. The IC_{50} of each fluorochemical and WY for L-FABP was calculated by adding aliquots of competitor (1 mM) to 1 μM L-FABP and 1 μM DAUDA.

Table 2
Comparison of fluorescence inhibition

Competitor	Competitor concentration (μM)	Fluorescence inhibition (%)
Qleic acid[a]	2	91
Oleate[b]	5	87
Oleate[c]	2	76
Arachidonate[c]	2	73
PFOS[a]	10	69
Cholesteryl sulfate[b]	5	57
Taurolithocholate 3-sulfate[b]	5	56
Lithocholate[b]	5	54
Oleoyl-CoA[c]	2	52
N-EtFOSA[a]	10	51
WY[a]	10	50
PFOS[a]	5	49
Bilirubin[c]	2	43
N-EtFOSE[a]	10	43
PFOA[a]	10	43
N-EtFOSA[a]	5	33
WY[a]	5	33
LysoPE[c]	2	31
N-EtFOSE[a]	5	30
Cholesteryl glucuronide[b]	5	28
PFOA[a]	5	26
Cholate[b]	5	13
Deoxycholate[b]	5	11
Chenodeoxy-cholate[b]	5	10
4-Cholesten-3-one[b]	5	6
25–hydrocholesterol[b]	5	5
Ergosterol[b]	5	4
Cholesterol[b]	5	3

Comparison of fluorescence inhibition by oleic acid, fluorochemicals, and WY to that of endogenous ligands. All assay systems contained 1 μM L-FABP and 1 μM DAUDA.
[a] Current study.
[b] Thumser and Wilton, 1996.
[c] Thumser et al., 1996.

cal competitors in the current study to that of endogenous L-FABP ligands under similar assay conditions. According to these data, 5–10 μM of the fluorochemicals can displace an equivalent percentage of DAUDA from L-FABP as can 2–5 μM of some endogenous ligands. These fluorochemicals may, therefore, be able to successfully compete with some natural ligands, i.e. cholesterol and cholesterol derivatives, for L-FABP binding. Fatty acids with less than 15 carbons are known to have a very weak affinity for L-FABP (Bass et al., 1993); and thus, it is conceivable that they too may be displaced by fluorochemicals in vivo. Other natural L-FABP ligands, i.e. long chain fatty acids, bind with a very high affinity (Bass et al., 1993) and thus are unlikely to be successfully displaced by the compounds investigated in the current study. Direct investigation of fluorochemical displacement of long, medium, and short-chained fatty acid and possibly other ligands from L-FABP may be the focus of future research. The reversibility of fluorochemical binding to L-FABP (the ability of DAUDA to displace fluorochemical from L-FABP) was not investigated in the current study. This too may be the focus of future research and would help to characterize the potential interactions of fluorochemicals with L-FABP in vivo.

The relevance of these data to the mechanism of peroxisome proliferation-induced by these fluorochemicals is unclear. However, total liver PFOS concentrations at which altered lipid metabolism occurs are approximately 600 ppm ($\sim$1.8 mM) in male rats and approximately 400 ppm ($-$1.3 mM) in male cynomolgus monkeys (Butenhoff and Seacat, 2001; Seacat et al., 2002). The concentration of free PFOS in the cytosol is unknown. The estimated solubility of PFOS in a physiological environment, however, based on studies of PFOS in seawater, is 12.4 μg/ml or approximately 24.8 μM (Ellefson, 2001), five times the calculated IC_{50} for PFOS. L-FABP represents about $-$2.5% of the cytosolic protein in an adult rat liver (Sheridan et al., 1987). Based on these data, it is likely that interactions of PFOS with L-FABP would occur at these concentrations in vivo.

The recent evidence indicating that certain fatty acids and eicosanoids activate PPARα in vitro supports the idea that PPARα is activated by endogenous cellular fatty acids (Maloney and Waxman, 1999). Thus, displacement of endogenous fatty acids from carrier proteins such as

L-FABP is one proposed mechanism for PPARα activation. Interestingly, PFOA was shown to activate PPARα but not PPARγ (Maloney and Waxman, 1999), which is involved in adipocyte differentiation and in lipid metabolism in several tissues. Further investigation into the possibility of direct activation of PPARs by PFOS and certain PFOSAs is underway.

In summary, the results of this study support the hypothesis that PFOS, PFOA, and certain PFOSAs can interfere with the binding affinity and/or capacity of L-FABP for fatty acids. The data indicate secondary site binding of the chemicals to L-FABP; however, binding to the primary site cannot be ruled out at this time. The relative effectiveness of the various fluorochemicals to inhibit DAUDA binding to control L-FABP was examined and compared with the potent peroxisome proliferator; WY. These competitive binding assays indicated that the most potent fluorochemical competitor of L-FABP-DAUDA binding was PFOS, followed in potency by the competitors N-EtFOSA, WY, and with equal IC_{50}s, N-EtFOSE and PFOA. These in vitro data support the theory that displacement of endogenous ligands from rat L-FABP could contribute to the mechanism of peroxisome proliferation observed following administration of these fluorochemicals to rats in vivo. Future work may investigate the reversibility of fluorochemical binding to L-FABP and the ability of fluorochemicals to bind the primary L-FABP binding site. Examination of long, medium, and short-chained fatty acid binding to L-FABP and the ability of fluorochemicals to displace such ligands may also be the focus of future research. This work will help determine the relevance of these in vitro data to the hepatotoxic and hypolipidemic effects of PFOS, PFOA, and certain related PFOSAs in vivo.

Acknowledgements

We would like to thank Dr Woubalem W. Birmachu of 3M Pharmaceuticals for the generous use of her equipment and for her extensive guidance in the fluorescence data analysis.

References

Appelkvist, E.L., Dallner, G., 1980. Possible involvement of fatty acid binding protein in peroxisomal β-oxidation of fatty acids. Biochim. Biophys. Acta 617, 156–160.

Banks, R.R., Smart, B.E., Tatlow, J.C., 1994. Organofluorine Chemistry Principles and Commercial Applications. Plenum Press, New York.

Bass, M.M., Kaikaus, R.M., Ockner, R.O., 1993. Physiology and molecular biology of hepatic cytosolic fatty acid-binding protein. In: Tavoloni, N., Berk, P.D. (Eds.), Hepatic Transport and Bile Secretion: Physiology and Pathophysiology. Raven Press, New York, pp. 421–446.

Berge, R.K., Aarsland, A., Kryvi, H., Bremer, J., Aarsaether, N., 1989. Alkylthioacetic acid (3-thia fatty acids)—a new group of non-β-oxidizable, peroxisome-inducing fatty acid analogues. I. A study on the structural requirements for proliferation of peroxisomes and mitochondria in rat liver. Biochim. Biophys. Acta 1004, 345–356.

Biegel, L.B., Liu, R.C., Hurtt, M.E., Cook, J.C., 1995. Effects of ammonium perfluorooctanoate on Leydig cell function: in vitro, in vivo, and ex vivo studies. Toxicol. Appl. Pharmacol. 134, 18–25.

Brandes, R., Kaikaus, R.M., Lysenko, N., Ockner, R.K., Bass, N.M., 1990. Induction of fatty acid binding protein by peroxisome proliferators in primary hepatocyte cultures and its relationship to the induction of peroxisomal β-oxidation. Biochim. Biophys. Acta 1034, 53–61.

Butenhoff, J.L., Seacat, A.M., 2001. Comparative sub-chronic toxicity of perfluorooctane sulfonate (PFOS) and *N*-ethyl perfluoroctanesulfonamidoethanol (*N*-EtFOSE) in the rat. In: Society of Toxicology, vol. 60 Toxicologist, San Francisco, CA.

Cannon, J.R., Eacho, P.I., 1991. Interaction of LY171883 and other peroxisome proliferators with fatty-acid-binding protein isolated from rat liver. Biochem. J. 280, 387–391.

Cattley, R.C., DeLuca, J., Elcombe, C., Fenner-Crisp, P., Lake, B.G., Marsman, D.S., Pastoor, T.A., Popp, J.A., Robinson, D.E., Schwetz, B., Tugwood, J., Wahli, W., 1998. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans. Regul. Toxicol. Pharmacol. 27, 47–60.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.

Elcombe, C.R., Mitchell, A.M., 1986. Peroxisome proliferation due to di(2-ethylhexyl) phthalate (DEHP): species differences and possible mechanisms. Environ. Health Perspect. 70, 211–219.

Ellefson, M.E., 2001. Characterization Study of PFOS, Primary Standard-Test Control Reference # TCR-00017-046 Phase: solubility of PFOS in natural seawater and aqueous solution of 3.5% sodium chloride. 3M, Saint Paul, pp. 1–138.

Ellinghaus, P., Wolfrum, C., Assmann, G., Spener, F., Seedorf, U., 1999. Phytanic acid activates the peroxisome proliferator-activated receptor α (PPARα) in sterol carrier

protein 2-/ sterol carrier protein x-deficient mice. J. Biol. Chem. 274, 2766–2772.

Gibson, S.J., Johnson, J.D., Ober, R.E., 1983. Absorption and biotransformation of *N*-ethyl FOSE and tissue distribution and elimination of carbon-14 after administration of *N*-ethyl FOSE-14C in feed. Riker Laboratories, St. Paul, MN.

Gordon, J.I., Alpers, D.H., Ockner, R.K., Strauss, A.W., 1983. The nucleotide sequence of rat liver fatty acid binding protein mRNA. J. Biol. Chem. 258, 3356–3363.

Haughom, B., Spydevold, O., 1992. The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid. Biochim. Biophys. Acta 1128, 65–72.

Ikeda, T., Aiba, K., Fukuda, K., Tanaka, M., 1985. The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids. J. Biochem. (Tokyo) 98, 475–482.

Ikeda, T., Fukuda, K., Mori, I., Enomoto, M., Komai, T., Suga, T., 1987. Induction of cytochrome P-450 and peroxisome proliferation in rat liver by perfluorinated octanesulfonic acid. In: Fahimi, H.D., Sies, H.E. (Eds.), Peroxisomes in Biology and Medicine. Springer, New York, pp. 304–308.

Issemann, I., Prince, R., Tugwood, J., Green, S., 1992. A role for fatty acids and liver fatty acid binding protein in peroxisome proliferation. Biochem. Soc. Trans. 20, 824–827.

Kanda, T., Iseki, S., Hitomi, M., Kimura, H., Odani, S., Kondo, H., Matsubara, Y., Muto, T., Ono, T., 1989. Purification and characterization of a fatty-acid-binding protein from the gastric mucosa of rats. Possible identity with heart fatty-acid-binding protein and its parietal cell localization. Eur. J. Biochem. 185, 27–33.

Kawashima, Y., Nakagawa, S., Tachibana, Y., Kozuka, H., 1983. Effects of peroxisome proliferators on fatty acid-binding protein in rat liver. Biochim. Biophys. Acta 754, 21–27.

Kennedy, G.L.J., 1987. Increase in mouse liver weight following feeding of ammonium perfluorooctanote and related fluorochemicals. Toxicol. Lett. 39, 5–30.

Kennedy, G.L.J., Keller, D.A., Biegel, L.B., Brock, W.J., 1998. Repeated dose toxicity of fluorinated acids in rats. In: Society of Toxicology International Conference, vol. 42.

Kersten, S., Desvergne, B., Wahli, W., 2000. Roles of PPARs in health and disease. Nature 405, 421–424.

Lake, B.G., Gray, T.J., 1985. Species differences in hepatic peroxisome proliferation. Biochem. Soc. Trans. 13, 859–861.

Maloney, E.K., Waxman, D.J., 1999. Trans-activation of PPARα and PPARγ by structurally diverse environmental chemicals. Toxicol. Appl. Pharmacol. 161, 209–218.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Olsen, G.W., Butenhoff, J.L., 2002. Investigation of the no observable effect level for perfluorooctane sulfonate in cynomolgus monkeys after 26 weeks of oral dosing and 1 year of recovery. Toxicol. Sci., in press.

Sheridan, M., Wilkinson, T.C., Wilton, D.C., 1987. Studies on fatty acid-binding proteins. Changes in the concentration of hepatic fatty acid-binding protein during development in the rat. Biochem. J. 242, 919–922.

Sohlenius, A.K., Eriksson, A.M., Hogstrom, C., Kimland, M., DePierre, J.W., 1993. Perfluorooctane sulfonic acid is a potent inducer of peroxisomal fatty acid β-oxidation and other activities known to be affected by peroxisome proliferators in mouse liver. Pharmacol. Toxicol. 72, 90–93.

Thompson, J., Winter, N., Terwey, D., Bratt, J., Banaszak, L., 1997. The crystal structure of the liver fatty acid-binding protein. A complex with two bound oleates. J. Biol. Chem. 272, 7140–7150.

Thumser, A.E., Wilton, D.C., 1994. Characterization of binding and structural properties of rat liver fatty-acid-binding protein using tryptophan mutants. Biochem. J. 300, 827–833.

Thumser, A.E., Wilton, D.C., 1995. The binding of natural and fluorescent lysophospholipids to wild-type and mutant rat liver fatty acid-binding protein and albumin. Biochem. J. 307, 305–311.

Thumser, A.E., Wilton, D.C., 1996. The binding of cholesterol and bile salts to recombinant rat liver fatty acid-binding protein. Biochem. J. 320, 729–733.

Thumser, A.E., Evans, C., Worrall, A.F., Wilton, D.C., 1994a. Effect on ligand binding of arginine mutations in recombinant rat liver fatty acid-binding protein. Biochem. J. 297, 103–107.

Thumser, A.E., Voysey, J.E., Wilton, D.C., 1994b. The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. Biochem. J. 301, 801–806.

Thumser, A.E., Voysey, J., Wilton, D.C., 1996. Mutations of recombinant rat liver fatty acid-binding protein at residues 102 and 122 alter its structural integrity and affinity for physiological ligands. Biochem. J. 314, 943–949.

Vaagenes, H., Madsen, L., Asiedu, D.K., Lillehaug, J.R., Berge, R.K., 1998. Early modulation of genes encoding peroxisomal and mitochondrial β-oxidation enzymes by 3-thia fatty acids. Biochem. Pharmacol. 56, 1571–1582.

VandenHeuvel, J., 1996. Perfluorodecanoic acid as a useful pharmacologic tool for the study of peroxisome proliferation. Gen. Pharm. 27, 1123–1129.

Wilkinson, T.C., Wilton, D.C., 1986. Studies on fatty acid-binding proteins. The detection and quantification of the protein from rat liver by using a fluorescent fatty acid analogue. Biochem. J. 238, 419–424.

Zisman, W., 1964. Relation of equilibrium contact angle to liquid and solid constitution. In: Gould, R. (Ed.), Contact Angle Wettability and Adhesion—Advances in Chemistty. American Chemical Society, Washington, DC.