Biochem. J. (1991) 280, 387–391 (Printed in Great Britain)

Interaction of LY171883 and other peroxisome proliferators with fatty-acid-binding protein isolated from rat liver

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Fatty-acid-binding protein (FABP) is a 14 kDa protein found in hepatic cytosol which binds and transports fatty acids and other hydrophobic ligands throughout the cell. The purpose of this investigation was to determine whether LY171883, a leukotriene D_4 antagonist, and other peroxisome proliferators bind to FABP and displace an endogenous fatty acid. [^{3}H]Oleic acid was used to monitor the elution of FABP during chromatographic purification. [^{14}C]LY171883 had a similar elution profile when substituted in the purification, indicating a common interaction with FABP. LY171883 and its structural analogue, LY189585, as well as the hypolipidaemic peroxisome proliferators clofibric acid, ciprofibrate, bezafibrate and WY14,643, displaced [^{3}H]oleic acid binding to FABP. Analogues of LY171883 that do not induce peroxisome proliferation only weakly displaced oleate binding. [^{3}H]LY171883 bound directly to FABP with a K_d of 10.8 μM, compared with a K_d of 0.96 μM for [^{3}H]oleate. LY171883 binding was inhibited by LY189585, clofibric acid, ciprofibrate and bezafibrate. These findings demonstrate that peroxisome proliferators, presumably due to their structural similarity to fatty acids, are able to bind to FABP and displace an endogenous ligand from its binding site. Interaction of peroxisome proliferators with FABP may be involved in perturbations of fatty acid metabolism caused by these agents as well as in the development of the pleiotropic response of peroxisome proliferation.

INTRODUCTION

Peroxisome-proliferating agents induce numerous alterations in hepatic lipid metabolism. Initially, the compounds cause a transient lipid accumulation in the liver (Elcombe & Mitchell, 1986; Foxworthy & Eacho, 1988; Lock *et al.*, 1989; Foxworthy *et al.*, 1990*b*). This may be related to the ability of the agents to inhibit mitochondrial fatty acid oxidation (Elcombe & Mitchell, 1986; Eacho & Foxworthy, 1988; Foxworthy & Eacho, 1988; Lock *et al.*, 1989; Foxworthy *et al.*, 1990*b*). With continued administration, peroxisome proliferators cause dramatic induction of fatty acid metabolism. Peroxisomal β-oxidation and microsomal ω-oxidation of fatty acids can be induced 20-fold or more (Reddy & Lalwani, 1983; Hawkins *et al.*, 1987). Mitochondrial β-oxidation is also increased, as is its rate-limiting enzyme, carnitine palmitoyltransferase I (Markwell *et al.*, 1977; Mannaerts *et al.*, 1979; Brady *et al.*, 1989; Foxworthy *et al.*, 1990*b*). Among the other effects of peroxisome proliferators on fatty acid metabolism is elevated expression of hepatic fatty-acid-binding protein (FABP) (Renaud *et al.*, 1978; Appelkvist & Dallner, 1980; Kawashima *et al.*, 1983; Bass *et al.*, 1985; Paulussen *et al.*, 1986).

The focus of the current investigation is the hepatic FABP. This 14 kDa cytosolic protein binds endogenous fatty acids (Appelkvist & Dallner, 1980), including arachidonic acid and its metabolites 5-hydroxyeicosatetraenoic acid, 5- and 15-hydroperoxyeicosatetraenoic acid, and prostaglandin E_1 (Dutta-Roy *et al.*, 1987; Raza *et al.*, 1989). It is believed to be involved in the uptake and intracellular transport of fatty acids (Glatz & Veerkamp, 1985; Peeters *et al.*, 1989; Glatz & van der Vusse, 1990). There is also evidence that FABP interacts with the carcinogens 2-acetylaminofluorine (Bassuk *et al.*, 1987; Blackburn *et al.*, 1982) and aminoazo dyes (Ketterer *et al.*, 1976). The purpose of the present investigation was to characterize the interaction of hepatic FABP with LY171883, a leukotriene D_4 receptor antagonist that induces peroxisomal proliferation in the rodent liver (Eacho *et al.*, 1986, 1989). Analogues of LY171883 that are devoid of peroxisome-proliferating activity, as well as hypolipidaemic peroxisome proliferators, were included in order to examine the relationship between binding to FABP and peroxisome proliferation.

EXPERIMENTAL

Chemicals

1-[2-Hydroxy-3-propyl-4-[4-(1*H*-tetrazol-5-yl)butoxy]phenyl]-ethanone (LY171883), 1-[2-hydroxy-3-propyl-4-[4-(1-methyl-1*H*-tetrazol-5-yl)butoxy]phenyl]ethanone (LY213768), 1-[2-hydroxy-3-propyl-4-[[4-(1*H*-tetrazol-5-ylmethyl)phenoxy]-methyl]phenyl]ethanone (LY163443), 1-[2-hydroxy-3-propyl-4-[[3-(1*H*-tetrazol-5-ylmethyl)phenoxy]methyl]phenyl]ethanone (LY189585), and [*tetrazol*-^{14}C]LY171883 (3 mCi/mmol) were synthesized at Lilly Research Laboratories. Their structures are shown in Fig. 1. [*propyl*-^{3}H(n)]LY171883 (67.3 Ci/mmol) was synthesized by New England Nuclear (Boston, MA, U.S.A.). [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (WY14,643) was obtained from ChemSyn Laboratories (Lenexa, KS, U.S.A.). Ciprofibrate was provided by the Sterling–Winthrop Research Institute (Rensselaer, NY, U.S.A.), bezafibrate by Boehringer–Mannheim GmbH (Mannheim, Germany), and clofibric acid by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sources of other chemicals are listed below.

Purification of FABP

Male Fischer-344 rats (Harlan Sprague–Dawley, Indianapolis, IN, U.S.A.), weighing 225–250 g, were individually housed in wire-bottomed cages and allowed unlimited access to food and water before experiments. Animals were anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and livers were removed, placed in ice-cold isolation buffer (10 mM-sodium phosphate, 250 mM-sucrose, 5 mM-EGTA and 1 mM-phenylmethanesulphonyl fluoride, pH 7.5), minced and

Abbreviation used: FABP, fatty-acid-binding protein.
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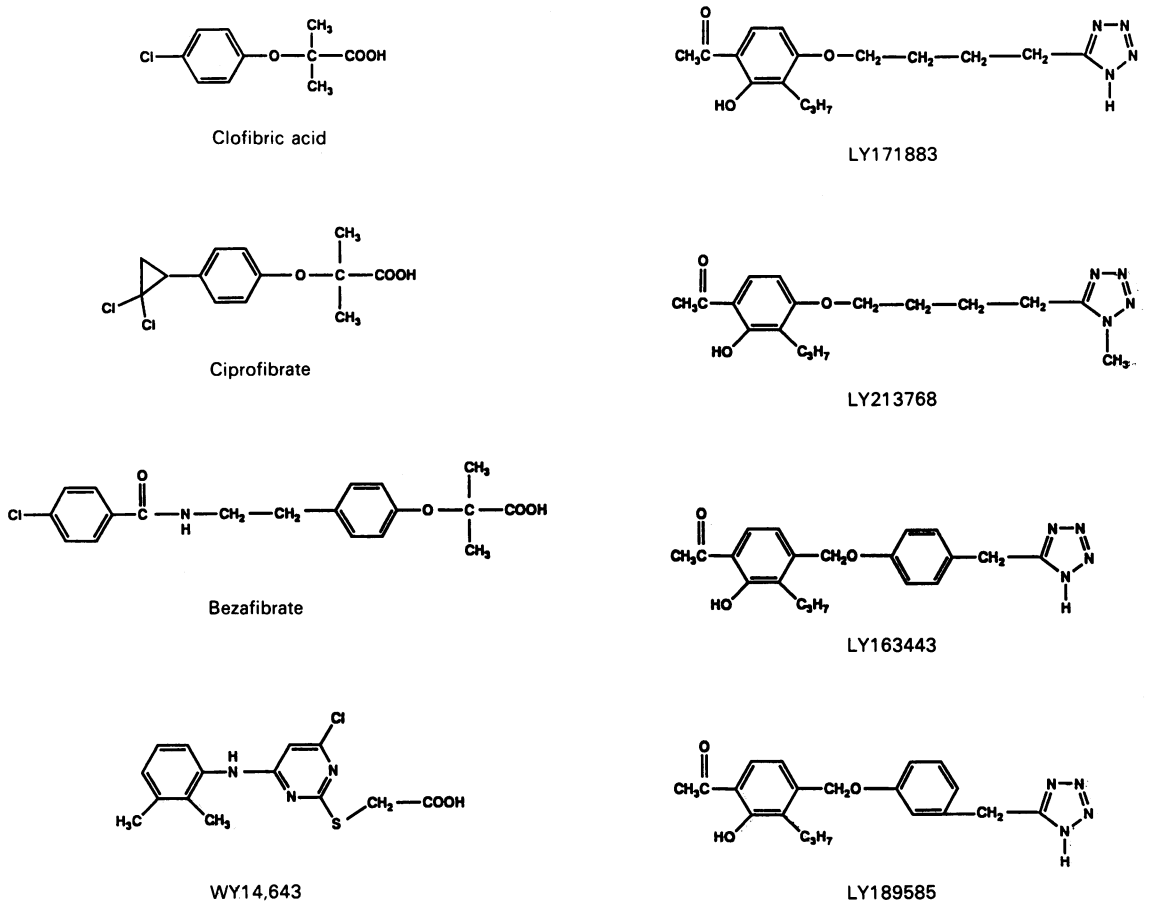

Fig. 1. Chemical structures of the tetrazole-substituted acetophenone leukotriene D_4 antagonists and hypolipidaemic peroxisome proliferators

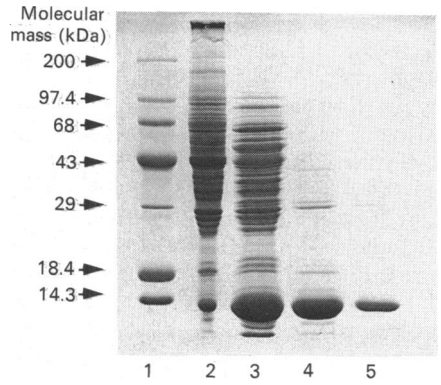

Fig. 2. SDS/PAGE of fractions collected during purification of FABP from rat liver cytosol

Shown is a linear gradient gel of 5–15% acrylamide with a 4% stacking gel run at 200 V constant voltage. Lane 1, high molecular mass standards; lane 2, cytosol (65 μg); lane 3, DEAE fractions (30 μg); lane 4, G-75 Sephadex fractions (15 μg); lane 5, naphthylaminodecyl–agarose affinity column fractions (2 μg).

homogenized with 5–6 strokes of a Teflon–glass Potter–Elvehjem homogenizer. The liver homogenate was centrifuged for 30 min at 9000 *g* to remove cellular debris and mitochondria, and the supernatant was centrifuged for 60 min at 105000 *g* to obtain the cytosol.

Hepatic cytosol was concentrated by ultrafiltration (Amicon YM 10) and incubated for 15 min at 4 °C with [9,10(n)-^{3}H]oleic acid (57 Ci/mmol; Amersham Corporation, Arlington Heights, IL, U.S.A.), which served as a marker for FABP. The concentrated cytosol was applied to a DEAE-cellulose (Sigma) anion-exchange column (2.5 cm × 40 cm) and eluted with 10 mM-sodium phosphate buffer, pH 7.4, containing 0.02% NaN_3 (Trulzsch & Arias, 1981). Fractions of cytosol corresponding to peaks of radioactivity were concentrated and applied to a G-75 Sephadex (Pharmacia–LKB, Piscataway, NJ, U.S.A.) column (2.5 cm × 90 cm). The column was eluted with sodium phosphate buffer and the single radioactive peak was isolated and concentrated. The concentrate was applied to a naphthylaminodecyl–agarose (Pharmacia) affinity column and eluted with sodium phosphate buffer in a procedure modified from that of Wilton (1989). Protein associated with the single peak was collected, concentrated and subjected to SDS/PAGE on a 5–15% linear gradient acrylamide gel according to the method of Laemmli (1970). The procedure yielded highly purified FABP with an electrophoretic mobility corresponding to a molecular mass of 14 kDa (Fig. 2).

Ligand binding to FABP

Endogenous fatty acids bound to the purified FABP were removed prior to binding assays by incubation of FABP with Lipidex-1000 (Sigma) for 10 min at 37 °C (FABP/Lipidex-1000 ratio of 3:1, v/v). Both bound and unbound fatty acids are adsorbed by Lipidex-1000 at 37 °C (Dahlberg *et al.*, 1980; Glatz & Veerkamp, 1985).

Binding of ligands to FABP was determined by a modification of the method of Glatz & Veerkamp (1985). Unless otherwise stated, the reactions contained 10 mM-potassium phosphate, pH 7.4, 1.5 μg of delipidated FABP, and [^{3}H]oleate or [^{3}H]LY171883. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled ligand. Test com-

pounds were dissolved in ethanol and added to the binding assay in small volumes to give a final ethanol concentration of not greater than 0.5%. Delipidated FABP was incubated in the presence of a radiolabelled ligand for 10 min at 37 °C. The binding reaction was terminated by immersing the samples in an ice bath cooled to 4 °C. Ice-cold Lipidex-1000 (1:1, v/v, with 10 mM-potassium phosphate, pH 7.4) was added to the tubes to remove unbound ligand, and the tubes were incubated with shaking for 5 min at 4 °C and then centrifuged at 15000 *g* for 2 min at 4 °C to separate the Lipidex-1000 from the incubation media. An aliquot of supernatant was removed and assayed by scintillation spectroscopy.

Inhibition studies were conducted under similar experimental conditions, except that the reactions contained a fixed concentration of radiolabelled ligand and increasing concentrations of competitors dissolved in ethanol. The inhibition was further analysed by incubating a fixed concentration of competitor and increasing concentrations of radiolabelled ligand. Scatchard analysis was used to characterize the type of inhibition (Scatchard, 1949; Rosenthal, 1967).

Analysis of binding data

Binding data were analysed by linear and non-linear regression using LUNDON analysis. Data are expressed as means ± S.E.M.

RESULTS AND DISCUSSION

The interaction of LY171883 with FABP was initially demonstrated during chromatographic purification of the protein. The elution profile of [^{14}C]LY171883 during three steps of the purification corresponded with that of [^{3}H]oleic acid (Fig. 3), suggesting a common interaction of the two ligands with FABP. Using [^{3}H]LY171883, specific binding to FABP was demonstrated with an apparent K_d of 10.8 ± 0.9 μM (Fig. 4). This binding was inhibited by unlabelled LY171883, bezafibrate, ciprofibrate, clofibric acid and WY14,643 (Table 1).

Oleic acid bound specifically and saturably to purified FABP, with an apparent K_d of 0.96 ± 0.09 μM (Fig. 5). Scatchard analysis of [^{3}H]oleic acid binding in the presence of LY171883 indicated competitive inhibition. Oleate binding was also inhibited by peroxisome proliferators of the hypolipidaemic class (Table 2). The order of potency of the hypolipidaemic agents was WY14,643 > bezafibrate > ciprofibrate > clofibric acid, which was consistent with the displacement of [^{3}H]LY171883 shown by these agents.

LY163443 was a 10-fold less active inhibitor of oleate binding to FABP than was its isomer LY189585 (Table 2). Likewise, LY163443 was less potent at displacing [^{3}H]LY171883 (Table 1). LY163443 differs from LY189585 only in the position of the methylene tetrazole on the phenyl ring (*para* versus *meta* substitution, Fig. 1). This structural difference has a major effect on the peroxisome-proliferating activity. LY189585 has strong peroxisomal activity *in vivo* and *in vitro*, whereas LY163443 is inactive (Table 2; Eacho *et al.*, 1989). The difference in peroxisome-proliferating activity may be related to differences in the tertiary conformations of the molecules (Eacho *et al.*, 1989; Foxworthy *et al.*, 1990*a*). LY189585 and LY171883 assume energetically favourable conformations in which the acidic tetrazole moiety is in close apposition with the hydrophobic acetophenone function. However, the tetrazole in LY163443 does not become as closely oriented to the acetophenone, due to the *para* positioning of the methylene tetrazole.

Sacchettini *et al.* (1988, 1990) have shown that fatty acids

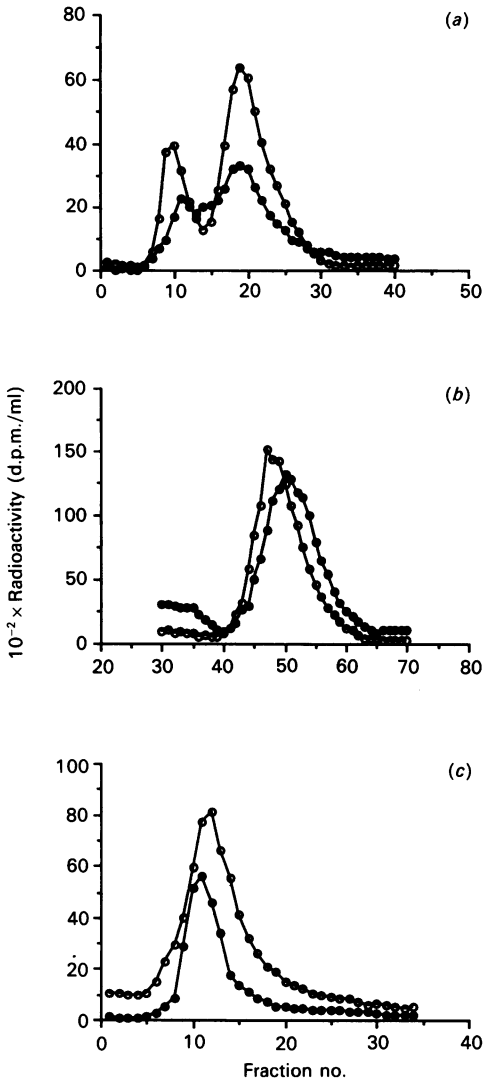

Fig. 3. Purification sequence for isolation of FABP

(*a*) DEAE anion-exchange column (2.5 cm × 40 cm); (*b*) G-75 Sephadex (2.5 cm × 90 cm); (*c*) naphthylaminodecyl–agarose affinity column (1.6 cm × 20 cm). Rat hepatic cytosol (100 mg) was incubated with either 1 μCi of [^{3}H]oleic acid (○) or 0.5 μCi of [^{14}C]LY171883 (●) prior to purification. All fractions collected represent a 5 ml volume.

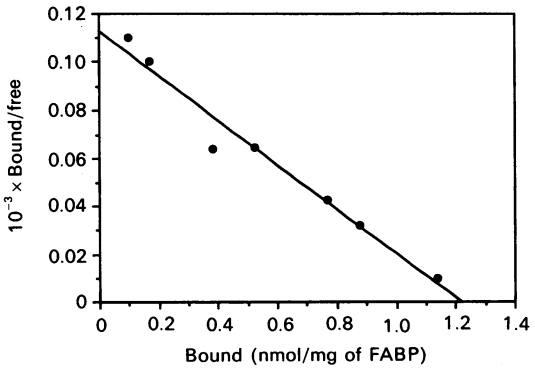

Fig. 4. Scatchard analysis of [^{3}H]LY171883 binding to FABP

Apparent K_d and $B_{max.}$ values are 10.8 ± 0.9 μM and 1.22 ± 0.12 nmol/mg respectively. The experiment shown is representative of triplicate determinations.

Table 1. Displacement of [^{3}H]LY171883 from FABP by peroxisome proliferators

Purified FABP (1.5 μg) was incubated with 10 μM-[^{3}H]LY171883 plus 0–750 μM competitor as described in the Experimental section. The IC_{50} is defined as the concentration of competitor required to cause 50% inhibition of [^{3}H]LY171883 binding. The data are expressed as means ± S.E.M. of three independent determinations.

Competitor	IC_{50} (μM)
WY14,643	12.9 ± 3.7
Bezafibrate	18.4 ± 0.6
Ciprofibrate	50.3 ± 13.8
Clofibric acid	266.0 ± 6.8
LY189585	23.6 ± 1.3
LY163443	92.9 ± 2.4

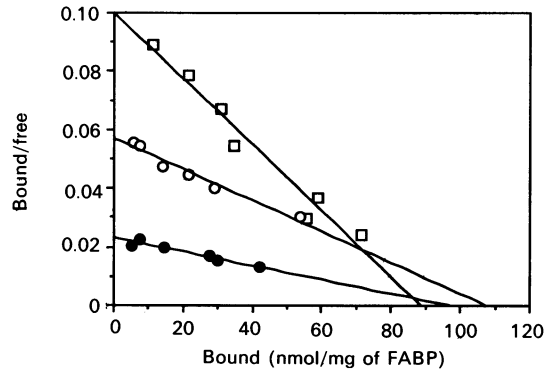

Fig. 5. Scatchard analysis of the inhibition by LY171883 of [^{3}H]oleic acid binding to FABP

Apparent K_d and $B_{max.}$ are: 0.96 ± 0.09 μM and 88.6 ± 7.9 nmol/mg for [^{3}H]oleic acid alone (□), 1.98 ± 0.22 μM and 107.6 ± 11.4 nmol/mg for [^{3}H]oleic acid in the presence of 100 μM-LY171883 (○), and 4.37 ± 0.44 μM and 96.6 ± 8.6 nmol/mg for [^{3}H]oleic acid in the presence of 200 μM-LY171883 (●). Each line is representative of three independent determinations.

bound to intestinal FABP are in a bent configuration in which the carboxylic acid moiety is oriented in proximity to the hydrophobic tail. This is similar to the configuration that is proposed to be required for the peroxisome-proliferating activity of LY171883 and its analogues (Eacho *et al.*, 1989; Foxworthy *et al.*, 1990*a*). Thus the greater affinity of LY189585 for FABP relative to LY163443 may be due to the ability of the former to assume conformations that more closely resemble those of endogenous fatty acids. The data suggest that the structural traits required for the chemicals to interact with FABP overlap with those required for peroxisome proliferation.

LY213768, an analogue of LY171883 in which the acidic nitrogen of the tetrazole is blocked with a methyl group, does not elicit peroxisome proliferation *in vivo* or *in vitro* (Eacho *et al.*, 1989). This lack of activity seems to be due to the absence of an acidic function, which is common to most peroxisome proliferators. LY213768 was a less potent inhibitor of oleic acid binding to FABP than was LY171883 (Table 2). The absence of an acidic moiety in LY213768 may account for the relative weakness of its interaction with FABP. The carboxylate of fatty acids is proposed to interact with a basic amino acid which projects into the hydrophobic core of the FABP (Sacchettini *et al.*, 1988, 1990).

Table 2. Displacement of [^{3}H]oleic acid from FABP by peroxisome proliferators

The IC_{50} values were determined by incubating FABP (1.5 μg) with 1 μM-[^{3}H]oleic acid in the presence of 0–750 μM peroxisome proliferator as described in the Experimental section. The IC_{50} is defined as the concentration of competitor required to inhibit oleate binding by 50%. The data represent the means ± S.E.M. of three independent experiments. The peroxisomal response represents the minimum concentration (μM) of compound required to elicit a 4–6-fold increase in peroxisomal β-oxidation in cultured rat hepatocytes (Foxworthy & Eacho, 1986; Eacho *et al.*, 1989; Foxworthy *et al.*, 1990; P. S. Foxworthy, unpublished work). Values in parentheses indicate the change in peroxisomal β-oxidation activity as percentage of the control. LY213768 and LY163443 did not cause a 4–6-fold increase in peroxisomal β-oxidation at any concentration (Eacho *et al.*, 1989).

Compound	IC_{50} (μM)	Peroxisomal response (μM) (%)
WY14,643	9.7 ± 0.05	1.6 (543)
Bezafibrate	10.4 ± 0.6	10 (478)
Ciprofibrate	32.4 ± 1.3	5 (462)
Clofibric acid	114 ± 2.3	100 (371)
LY189585	24.3 ± 1.4	50 (558)
LY171883	135.3 ± 5.6	50 (504)
LY213768	239.7 ± 10.1	50 (106)
LY163443	300.0 ± 12.7	50 (131)

The absence of the acid in LY213768 would preclude a strong interaction with FABP if it interacts at the same site as the fatty acids.

The results indicate that the peroxisome proliferator LY171883, its analogues and several hypolipidaemic agents interact in a similar manner with FABP. These data are supported by the recent finding that bezafibrate inhibits oleate binding to FABP (Brandes *et al.*, 1990) and that di-(2-ethylhexyl)phthalate is a ligand for rat hepatic and intestinal FABP *in vivo* (Kanda *et al.*, 1990).

The significance of the displacement of fatty acids from FABP by peroxisome proliferators is not clear. One possibility is that peroxisome proliferators disrupt cellular functions normally regulated by FABP (Peeters *et al.*, 1989; Brandes *et al.*, 1990; Kahn & Sorof, 1990). Alternatively, peroxisome proliferators may interact with other proteins whose ligand-binding sites have a similar structure to that of FABP (Takahashi *et al.*, 1982; Sacchettini *et al.*, 1988, 1990; Calvo & Ena, 1989). We speculate that peroxisome proliferators interact with fatty-acid-dependent transcription factors, resulting in altered gene expression. Thus the ability of peroxisome proliferators to compete with fatty acids for binding to a physiological target site may be relevant to the stimulation of the peroxisomal response.

REFERENCES

Appelkvist, E. L. & Dallner, G. (1980) Biochim. Biophys. Acta **617**, 156–160

Bass, N. M., Manning, J. A. & Ockner, R. K. (1985) J. Biol. Chem. **260**, 9603–9607

Bassuk, J. A., Tsichlis, P. N. & Sorof, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7547–7551

Blackburn, G. R., Schnabel, S. J., Danley, J. M., Hogue-Angeletti, R. A. & Sorof, S. (1982) Cancer Res. **42**, 4664–4672

Brady, P. S., Marine, K. A., Brady, L. J. & Ramsey, R. R. (1989) Biochem. J. **260**, 93–100

Brandes, R., Kaikaus, R. M., Lysenko, N., Ockner, R. K. & Bass, N. M. (1990) Biochim. Biophys. Acta **1034**, 53–61

Calvo, M. & Ena, J. M. (1989) Biochem. Biophys. Res. Commun. **163**, 14–17
Dahlberg, E., Snochowski, M. & Gustafsson, J. (1980) Anal. Biochem. **106**, 380–388
Dutta-Roy, A. K., Gopalswamy, N. & Trulzsch, D. V. (1987) Eur. J. Biochem. **162**, 615–619
Eacho, P. I. & Foxworthy, P. S. (1988) Biochem. Biophys. Res. Commun. **157**, 1148–1153
Eacho, P. I., Foxworthy, P. S., Johnson, W. D., Hoover, D. M. & White, S. L. (1986) Toxicol. Appl. Pharmacol. **83**, 430–437
Eacho, P. I., Foxworthy, P. S., Dillard, R. D., Whitesitt, H. D. K. & Marshall, W. S. (1989) Toxicol. Appl. Pharmacol. **100**, 177–184
Elcombe, C. R. & Mitchell, A. M. (1986) EHP Environ. Health Perspect. **70**, 211–219
Foxworthy, P. S. & Eacho, P. I. (1986) Toxicol. Lett. **30**, 189–196
Foxworthy, P. S. & Eacho, P. I. (1988) Biochem. J. **252**, 409–414
Foxworthy, P. S., Herron, D. K., Dillard, R. D., Whitesitt, C. A., Marshall, W. S. & Eacho, P. I. (1990*a*) The Toxicologist **10**, 141
Foxworthy, P. S., Perry, D. N., Hoover, D. M. & Eacho, P. I. (1990*b*) Toxicol. Appl. Pharmacol. **106**, 375–383
Glatz, J. F. C. & van der Vusse, G. J. (1990) Mol. Cell. Biochem. **98**, 237–251
Glatz, J. F. C. & Veerkamp, J. H. (1985) Int. J. Biochem. **17**, 13–22
Hawkins, J. M., Jones, W. E., Bonner, F. W. & Gibson, G. G. (1987) Drug Metab. Rev. **18**, 441–515
Kahn, S. H. & Sorof, S. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9401–9405
Kanda, T., Ono, T., Matsubara, Y. & Muto, T. (1990) Biochem. Biophys. Res. Commun. **168**, 1053–1058
Kawashima, Y., Nakagawa, S., Tachibana, Y. & Kozuka, H. (1983) Biochem. Biophys. Res. Commun. **754**, 21–27
Ketterer, B., Tipping, E., Hackney, J. F. & Beale, D. (1976) Biochem. J. **155**, 511–521
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lock, E. A., Mitchell, A. M. & Elcombe, C. R. (1989) Annu. Rev. Pharmacol. Toxicol. **29**, 145–163
Mannaerts, G. P., Debeer, L. J., Thomas, J. & DeSchepper, P. J. (1979) J. Biol. Chem. **254**, 4585–4595
Markwell, M. A. K., Bieber, L. L. & Tolbert, N. E. (1977) Biochem. Pharmacol. **26**, 1697–1702
Paulussen, R. J. A., Jansen, G. P. M. & Veerkamp, J. H. (1986) Biochim. Biophys. Acta **877**, 342–349
Peeters, R. A., Veerkamp, J. H. & Demel, R. A. (1989) Biochim. Biophys. Acta **1002**, 8–13
Raza, H., Pongubala, J. R. & Sorof, S. (1989) Biochem. Biophys. Res. Commun. **161**, 448–455
Reddy, J. K. & Lalwani, N. D. (1983) CRC Crit. Rev. Toxicol. **12**, 1–58
Renaud, G., Foliot, A. & Infante, R. (1978) Biochem. Biophys. Res. Commun. **80**, 327–334
Rosenthal, H. E. (1967) Anal. Biochem. **20**, 525–532
Sacchettini, J. C., Gordon, J. I. & Banaszak, L. J. (1988) J. Biol. Chem. **263**, 5815–5819
Sacchettini, J. C., Banaszak, L. J. & Gordon, J. I. (1990) Mol. Cell. Biochem. **98**, 81–93
Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51**, 660–672
Takahashi, K., Odani, S. & Ono, T. (1982) Biochem. Biophys. Res. Commun. **106**, 1099–1105
Trulzsch, D. & Arias, I. M. (1981) Arch. Biochem. Biophys. **209**, 433–440
Wilton, D. C. (1989) Biochem. J. **261**, 273–276

Received 15 March 1991/18 June 1991; accepted 9 July 1991