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INDUCTION OF HEPATIC ACYL-CoA-BINDING PROTEIN AND LIVER FATTY ACID-BINDING PROTEIN BY PERFLUORODECANOIC ACID IN RATS

LACK OF CORRELATION WITH HEPATIC LONG-CHAIN ACYL-CoA LEVELS*

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Abstract—Liver fatty acid-binding protein (L-FABP) and acyl-CoA-binding protein (ACBP) are involved in the intracellular trafficking and compartmentalization of fatty acids and fatty acyl-CoA esters, respectively, in the liver. Both proteins are induced in rat liver by the potent peroxisome proliferator perfluorodecanoic acid (PFDA). While it is believed that the peroxisome proliferator-activated receptor may mediate the responses to peroxisome proliferators by inducing responsive genes, the ligand(s) of this receptor remains unknown. We hypothesized that induction of L-FABP and ACBP in rat liver by PFDA is secondary to accumulation of long-chain acyl-CoA esters. However, neither dose–response nor time–course effects of PFDA on hepatic long-chain acyl-CoA, L-FABP, or ACBP concentrations confirmed this hypothesis. In a dose–response study, PFDA increased hepatic long-chain acyl-CoA concentrations (7 days after treatment) over the dose range of 20–50 mg/kg, whereas it increased ACBP and L-FABP over the wider dose range of 20–65 mg/kg. In the time–course study, PFDA treatment (50 mg/kg) elevated long-chain acyl-CoA esters in the liver beginning on day 3 post-treatment, yet hepatic L-FABP concentrations were increased earlier beginning on day 2 and ACBP was not induced until day 7. To determine if this dissociation of increases in hepatic long-chain acyl-CoA concentrations from increases in hepatic L-FABP and ACBP concentrations could be demonstrated under other conditions, control rats fasted for 24–48 hr were used. Fasting increased hepatic long-chain acyl-CoA levels to a greater extent than PFDA treatment, yet neither L-FABP nor ACBP was induced. We conclude that elevated concentrations of hepatic long-chain acyl-CoAs in PFDA-treated rats are not a major contributor to the induction of L-FABP or ACBP by peroxisome proliferators. A more plausible mechanism is that PFDA induces L-FABP and ACBP by activating the peroxisome proliferator receptor directly rather than indirectly through long-chain acyl-CoA esters.

Key words: perfluorodecanoic acid; acyl-CoA binding protein; liver fatty acid-binding protein; acyl-CoA synthetase; long-chain acyl-CoA esters; peroxisome proliferator-activated receptor; fasting; liver; rats

PFDA** is a fatty acid analog belonging to a structurally diverse group of chemicals that cause peroxisome proliferation in rodent liver [1]. Similar to other peroxisome proliferators, PFDA induces the peroxisomal fatty acid β-oxidation pathway [2, 3] and alters liver lipid metabolism [4, 5]. Two hypotheses have been proposed to explain the pleiotropic effect of peroxisome proliferators. One hypothesis asserts that induction of gene expression is secondary to an accumulation of lipid intermediates in the liver, the so-called "substrate overload" hypothesis. The other hypothesis asserts that the tissue-specific response to these chemicals is mediated by a hepatic peroxisome proliferator receptor [6]. These hypotheses are not mutually exclusive, however, and the recent cloning of peroxisome proliferator-activated receptors, which are activated by both peroxisome proliferators and fatty acids, may support both hypotheses [7–9].

High fat diets [10], diabetes [11] and starvation [12] also induce peroxisomal β-oxidation, although to a lesser magnitude than xenobiotic peroxisome proliferators. Since each of these states is accompanied by an enhanced influx of fatty acids into the liver, a relationship may exist between accumulation of lipid metabolites in the liver and

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** Abbreviations: PFDA, perfluorodecanoic acid; ACBP, acyl-CoA binding protein; L-FABP, liver fatty acid-binding protein; ACS, acyl-CoA synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and PCR, polymerase chain reaction.

subsequent peroxisome proliferation. Two proteins believed to play important roles in regulating intracellular lipid homeostasis in the liver are L-FABP [13] and ACBP [14]. L-FABP is upregulated in response to peroxisome proliferators [15, 16], and PFDA administration can elevate hepatic ACBP as well [17]. Given the significance of ACBP and L-FABP to liver lipid homeostasis, the question arises as to whether increases in long-chain acyl-CoA esters in the liver lead to increases in these hepatic binding proteins. The role of long-chain acyl-CoAs as regulators of fatty acid metabolism in *Escherichia coli* [18], and the ability of fatty acids to activate the peroxisome proliferator-activated receptor in transactivation assays [8, 19], suggest that long-chain acyl-CoAs may, by activating the peroxisome proliferator receptor in rat liver, induce L-FABP and ACBP.

A number of peroxisome proliferators are converted to acyl-coenzyme A thioesters [20, 21], which may interfere with metabolic reactions utilizing acyl-CoA esters. This is not the case with PFDA, as we have been unable to detect or synthesize the coenzyme A ester of PFDA or to identify lipid conjugates of this chemical [22, 23]. This makes PFDA a useful probe for examining the possible role endogenous long-chain acyl-CoAs play in the induction of ACBP and L-FABP without the confounding influence of PFDA entering lipid metabolic pathways as an activated CoA conjugate.

The purpose of the present investigation was to determine if increased concentrations of long-chain acyl-CoAs in the liver lead to increased hepatic L-FABP and ACBP concentrations following exposure of rats to PFDA. It will be shown in dose–response and time–course studies that PFDA treatment did not cause parallel changes in hepatic long-chain acyl-CoA, ACBP and L-FABP concentrations. It will also be shown that increases in hepatic long-chain acyl-CoA concentrations in fasted control rats were not accompanied by induction of ACBP, L-FABP, or ACS (EC 6.2.1.3).

MATERIALS AND METHODS

Materials. PFDA was purified (99% pure) as described previously [24]. Sources of other materials were as follows: [1-^{14}C]palmitic acid (59 mCi/mmol, Amersham, Arlington Heights, IL); [α-^{32}P]dCTP (3000 Ci/mmol, Dupont New England Nuclear Research Products, Boston, MA); Hionic Fluor scintillation fluid (Packard Chemical Co., Downer's Grove, IL); coenzyme A and palmitoyl coenzyme A (Pharmacia, Piscataway, NJ); 96-well polystyrene ELISA plates used for analysis of L-FABP (Corning, Park Ridge, IL); and goat anti-rat albumin (Cappel, Organon Teknika, Durham, NC). Rabbit anti-rat palmitoyl-CoA synthetase was the gift of T. Hashimoto (Shinshu University School of Medicine, Nagano, Japan). E. Costa and A. Guidotti (Fidio-Georgetown Institute for the Neurosciences, Washington, DC) supplied the rabbit anti-rat acyl-CoA binding protein serum used in western blot analysis. The plasmid containing the cDNA for rat long-chain ACS (pRACS15) was provided by T. Yamamoto (Tohoku University, Sendai, Japan). The cDNA for human β-actin, shown to specifically recognize rat β-actin, was provided by W. Mellon (University of Wisconsin, Madison), who also provided the cDNA for rat GAPDH. cDNAs for rat acyl-CoA binding protein and fatty acid binding protein were generated by the reverse transcriptase PCR based on the published sequence of these genes. All primer sequences were chosen using a primer selection program (Oligo™, National Biosciences, Hamel, MN). L-FABP primers (forward 5′ AAACTCACCATCACCTAT 3′ and reverse 5′ TTGTCACCCTCCATCTTA 3′) and ACBP primers (forward 5′ TGTGGAAAAGGTAGAAGAGC 3′ and reverse 5′ AAGGAAGGAGGAGCAGTAAT 3′) did not bare significant homology with any other sequences in the database based on a Pearson–Lippman search of the EMBL/Genebank sequence database using FASTA in GCG (Genetics Computing Group, Madison, WI). Reverse transcription and PCR conditions were essentially as described previously [25]. Aliquots of the PCR reaction were electrophoresed on 4% NuSieve/agarose (BRL, Gaithersburg, MD; 3:1, w/w) gels visualized with ethidium bromide staining. The 122 bp L-FABP and 184 bp ACBP PCR fragments were gel purified and quantitated by absorbance at 260 nm.

Animals. Male Sprague–Dawley rats (200–250 g, Harlan–Sprague Dawley, Madison, WI), were housed individually in suspended, stainless-steel cages in a temperature-controlled room (approximately 21°) with a 12-hr light/dark cycle (lighted 6:00 a.m. to 6:00 p.m.). Unless otherwise noted, rats received ground feed *ad libitum* (Purina Rat Chow, No. 5012, Ralston Purina Co., St. Louis, MO) during the 12-hr dark cycle, and water *ad libitum*. Rats were given single graded doses of PFDA (20, 35, 50, or 65 mg/kg, i.p.) or vehicle (propylene glycol:water, 1:1, v/v, i.p.) at 7:00 a.m. To control for hypophagia induced by PFDA, some vehicle-treated rats were pair-fed to 50 mg/kg PFDA-treated rats and given the amount of feed their PFDA-treated partner consumed during the previous 24 hr. Rats were decapitated between 7:00 a.m. and 9:00 a.m. on days 1, 2, 3, 5, or 7 post-treatment, and livers were quickly excised, weighed, and frozen at −70° pending future analyses. In a second experiment, additional groups of rats were deprived of food for 24 or 48 hr to examine effects of fasting.

Long-chain acyl-CoA ester determination. Preparation of liver samples and subsequent HPLC analysis of long-chain acyl-CoA esters were based on the methods of Ingebretsen *et al.* [26]. Aliquots (100 μL) of CoA standards or samples were injected into the liquid chromatographic system for quantitation. Percent recovery of long-chain acyl-CoA derivatives, determined by utilizing standard solutions of palmitoyl-CoA in the extraction procedure, was 85–90%. The chromatographic system consisted of a Gilson model 302 pump with a model 802B Manometric Module, a model 231 automatic sample injector, and model 116 UV-detector (254 nm) set at 0.5 mV full scale. Peak separation was achieved at room temperature on a microparticulate strong anion exchanger prepacked from Whatman (Partisil-10 SAX), preceded by a short guard column consisting of Whatman Solvecon

silica gel. Peak area integration was achieved with a Gilson model 506C System Interface Module.

Purification of fatty acid-binding protein. L-FABP was isolated from male rat liver using a napthoylaminodecyl-agarose affinity column [27]. L-FABP homogeneity was confirmed by the appearance of a single protein band on SDS–PAGE (15% gels [28]) following staining with Coomassie Blue. This protein band was shown to react with rabbit anti-rat liver L-FABP antibody in western blot analysis.

Purification of rabbit anti-rat liver L-FABP and rabbit anti-rat liver ACBP antisera. Whole rabbit serum containing anti-rat liver L-FABP was generated by the Promega Corp. (Madison, WI). The IgG fraction of this serum was affinity purified with a MAbTrap G II Kit following instructions of the supplier (Pharmacia). Following coupling of purified L-FABP to CNBr-activated Sepharose 4B (Pharmacia), IgG specific for L-FABP was purified based on the method of Börchers *et al.* [29]. The rabbit anti-rat ACBP and biotinylated rabbit anti-rat ACBP antibodies used in the ACBP ELISA were produced as described by Knudsen *et al.* [30].

ELISAs for L-FABP and ACBP. A variation of the sensitive noncompetitive, direct enzyme-linked immunosorbent assay method developed by Paulussen *et al.* [31], as described by Kaikaus *et al.* [32], was used to quantitate liver L-FABP. However, the addition of 100 μL of 1 N H_2SO_4 to each well was used to terminate the final reaction after 5–7 min. Absorbance was read at 450 nm using a Bio-Tek Instruments Microplate Reader, model 312e. ACBP quantitation was performed as described by Hansen *et al.* [33].

SDS–PAGE and immunoblotting analyses. Samples containing 25 μg of homogenate protein were electrophoresed as described by Vanden Heuvel *et al.* [17], with slight modifications for ACBP. For this protein, separation was achieved in a 20.1% T, 0.5% C_{bis}, pH 9.3, separating gel with a 9.4% T, 4.8% C_{bis}, pH 6.8, stacking gel [34]. Following electrophoresis and transfer to nitrocellulose, filters were incubated overnight in blocking buffer (3%, w/v, Blotto and 1.5% normal goat serum in 10 mM sodium phosphate, 0.9% sodium chloride, pH 7.5). Incubation with goat anti-rat albumin serum (dilution 1:10,000), rabbit anti-rat palmitoyl-CoA synthetase (1:5000), rabbit anti-rat ACBP or rabbit anti-rat L-FABP (1:5000) followed. After washing in PBST (10 mM sodium phosphate, 0.9% sodium chloride, pH 7.5, 0.1% Tween 20), nitrocellulose filters were incubated in goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Hyclone, Logan, UT) (1:2500) in blocking buffer followed by washing with PBST. The blots were developed using Western Blue Stabilized Substrate (Promega Corp. Madison, WI).

Assay of ACS enzyme activity. Palmitoyl-CoA synthetase (ACS) activity was measured by the method of Krisans *et al.* [35].

Isolation of RNA and northern-blot analysis. Total cellular RNA was isolated from powdered liver samples using TRI-REAGENT (Molecular Research Center, Cincinnati, OH). Total RNA separation (25 μg/lane) on 1% agarose gels and northern-blot analyses were conducted as described previously [36]. Labeling of cDNA probes with ^{32}P was performed with the Prime-A-Gene labeling system manufactured by the Promega Corp. Following exposure of the membranes to film (Kodak XAR-5 X-OMAT), relative band intensities were analyzed on a Molecular Dynamics ImageQuant 3.0 Computing Densitometer, model 300A. ACS, ACBP, and L-FABP mRNA band intensities were normalized to the intensity of the β-actin message for each particular sample.

Protein determination. Total protein in liver samples used for L-FABP quantitation was determined by the method of Lowry *et al.* [37]. A modification of this technique was used to determine protein content in samples destined for ACBP ELISA analysis [38]. The protein concentration in purified L-FABP as well as in the rabbit anti-rat L-FABP serum was determined by the BCA Protein Assay (Pierce, Rockford, IL) adapted to microtiter plate analysis. ACBP concentrations were determined using $\varepsilon_{280} = 15.4\ mM^{-1} \cdot cm^{-1}$ [14].

Statistical analyses. Statistical analysis was performed using the SAS statistical package (version 6.04; Cary, NC). Dose–response data were analyzed by one-way analysis of variance (ANOVA), while time–course data were analyzed by two-way

Table 1. Dose-related effects of PFDA treatment on hepatic long-chain acyl-CoA synthetase activity and hepatic content of long-chain acyl-CoA esters and lipid binding proteins 7 days post-treatment

Treatment	ACS activity (nmol/min/mg protein)	Long-chain CoA (nmol/g tissue)	Lipid binding proteins (μg/mg protein)	
			ACBP	L-FABP
Vehicle	11.30 ± 0.70	51.0 ± 3.2	1.76 ± 0.13	30.3 ± 3.0
PFDA 20 mg/kg	18.33 ± 0.55*	63.1 ± 4.0*	2.27 ± 0.23*	51.0 ± 4.9*
35 mg/kg	18.56 ± 0.33*	65.8 ± 5.4*	2.16 ± 0.05	53.4 ± 4.6*
50 mg/kg	19.17 ± 0.27*	69.8 ± 4.6*	2.47 ± 0.18*	47.6 ± 3.2*
65 mg/kg	19.73 ± 0.23*	52.6 ± 4.2	2.23 ± 0.19*	51.8 ± 3.9*

Rats were administered a single i.p. dose of PFDA or vehicle and killed 7 days post-treatment. The liver was removed and prepared for analyses as described in Materials and Methods. Values are means ± SEM, N = 3–5.

* Significantly different from vehicle control ($P < 0.05$).

ANOVA. Fisher's LSD was used as a *post-hoc* test when ANOVA was significant [39]. Normality of distribution was observed by plotting residuals, and homogeneity of variance was assessed by Levene's test (part of the SAS statistical package). When necessary, natural log transformations, square-root transformations or rank-ordering of the data were conducted [40]. When the criteria for homogeneity of variance were not satisfied, the Kruskal–Wallis test for non-parametric data was performed followed by a distribution-free multiple comparison test [41]. Significance was set at $P < 0.05$.

RESULTS

Dose–response for PFDA induction of hepatic ACS, long-chain acyl-CoA esters, ACBP, and L-FABP. Rats given single graded doses of PFDA were killed 7 days following treatment to assess effects on hepatic long-chain ACS activity and concentration in the liver of long-chain acyl-CoA esters, L-FABP, and ACBP (Table 1). The doses of PFDA used were shown previously to cause alterations in liver lipid metabolism in rats at 7 days post-treatment [5]. The activity of ACS, utilizing palmitic acid as substrate, was increased about 1.7-fold by all doses of PFDA on day 7 post-treatment. This increase in ACS activity was reflected by long-chain acyl-CoA levels being increased in animals treated with 20, 35, and 50 mg/kg PFDA. However, rats treated with 65 mg/kg PFDA did not have elevated long-chain acyl-CoA levels.

PFDA also affected hepatic concentrations of the intracellular lipid-binding proteins ACBP and L-FABP. All doses of PFDA, except 35 mg/kg, elevated ACBP concentration about 1.5-fold. L-FABP levels were increased about 1.7-fold by all doses of PFDA.

Dose–response for PFDA induction of hepatic ACS, ACBP and L-FABP protein. The dose-related effects of PFDA on the amount of ACS, ACBP and L-FABP immunoreactive protein were determined

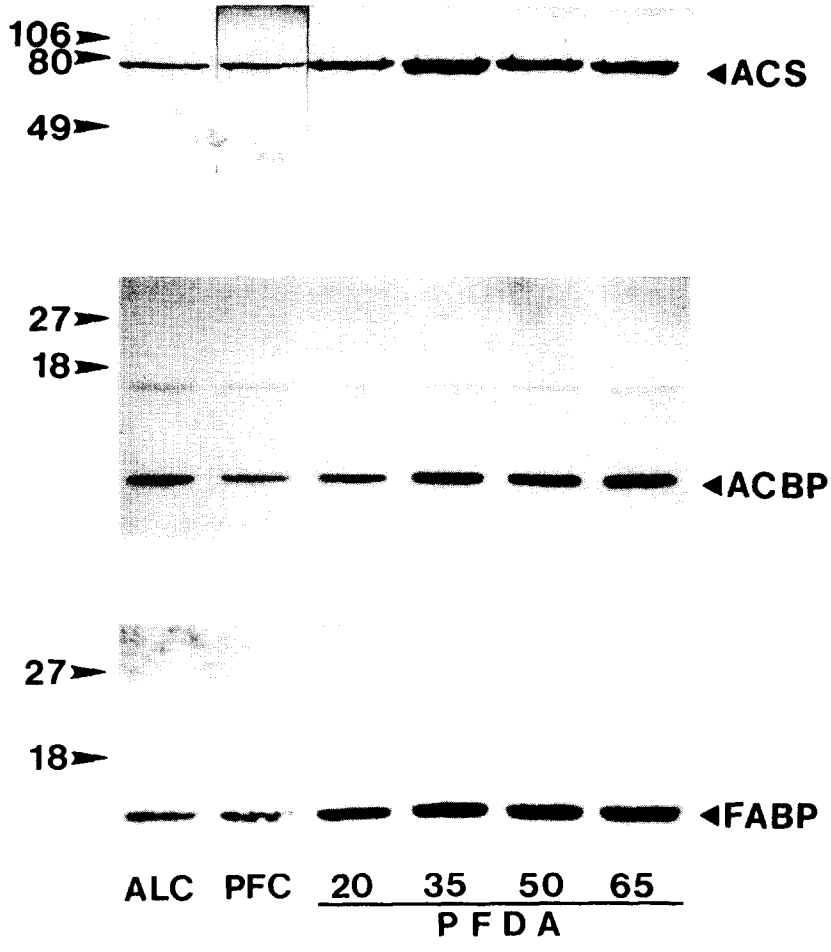

Fig. 1. Immunostaining of hepatic proteins from rats 7 days following treatment with graded single doses of PFDA. Twenty-five micrograms of homogenate protein was separated by SDS-PAGE and transferred to nitrocellulose paper. ACS (top panel), ACBP (middle panel), and L-FABP (bottom panel) detection was performed as indicated in Materials and Methods. Molecular weights (kDa) of protein markers are depicted to the left of each panel. Key: ALC, *ad libitum*-fed control; PFC, pair-fed control; and 20, 35, 50, or 65, dose of PFDA (mg/kg).

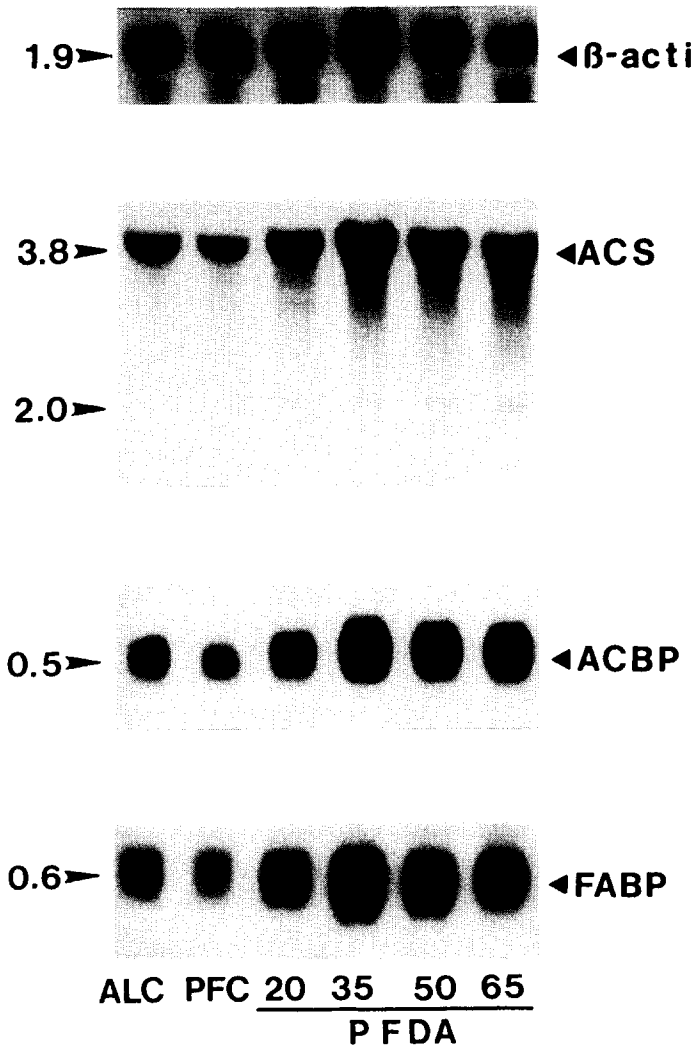

Fig. 2. Hybridization analysis of hepatic mRNA from rats 7 days following treatment with PFDA. Twenty-five micrograms of total RNA was separated by agarose gel electrophoresis, blotted and hybridized with cDNA probes for human β-actin, ACS, ACBP, and L-FABP as described in Materials and Methods. Sizes of identified bands (kilobases) are depicted to the left of each panel. Key: ALC, *ad libitum*-fed control; PFC, pair-fed control; and 20, 35, 50, or 65, dose of PFDA (mg/kg).

using immunoblotting techniques (Fig. 1). The amount of albumin served as a loading control for each western blot and was constant (data not shown). Compared to *ad libitum*-fed control rats, there was an observable increase in levels of ACS, ACBP, and L-FABP proteins 7 days after PFDA at each of the doses used. As observed previously, the rabbit anti-rat ACBP reacted with ACBP (10 kDa) as well as with a slightly larger protein in all treatment groups [17, 42]. Since intensity of the latter band can be abated by altering the electrophoretic conditions, it is believed to be a dimer of ACBP molecules. Presumably, the ELISA for ACBP is measuring only the 10 kDa protein.

Also included in each of the immunoblots is a sample from control rats that were pair-fed for 7 days to rats administered 50 mg/kg of PFDA. It can be seen that pair-feeding the control rats for 7 days had no effect on hepatic ACS or L-FABP concentration compared with *ad libitum*-fed control rats. However, the amount of hepatic ACBP in these pair-fed animals was slightly less than that in *ad libitum*-fed controls.

Dose–response for PFDA induction of hepatic mRNA for ACS, ACBP and L-FABP. The effects of exposure for 1 week to graded doses of PFDA on hepatic mRNA levels of ACS, ACBP, and L-FABP were examined by hybridization analysis using specific ^{32}P-labeled cDNA probes. Following hybridization and autoradiography, the nylon membrane was stripped of radioactive probe and rehybridized with an alternate probe. Thus, all northern blots originated from a single nylon membrane. Normalization of total RNA content/

Table 2. Time–course of PFDA effects on hepatic long-chain acyl-CoA synthetase activity and hepatic concentrations of long-chain acyl-CoA esters and lipid binding proteins

Treatment	Time post-treatment (days)	ACS activity (nmol/min/mg protein)	Long-chain CoA (nmol/g tissue)	Lipid binding proteins (μg/mg protein)	
				ACBP	L-FABP
Vehicle (*ad libitum*-fed)	1	13.21 ± 0.52*†	51.9 ± 2.5	1.77 ± 0.07	28.3 ± 2.8
	2	11.91 ± 0.59†	44.1 ± 0.9*	1.82 ± 0.33†	13.6 ± 5.2*†
	3	12.23 ± 0.64†	46.4 ± 3.9†	1.75 ± 0.08	28.9 ± 2.4†
	5	11.13 ± 1.10†	50.4 ± 1.4	1.68 ± 0.20	29.1 ± 2.5†
	7	11.30 ± 0.70†	51.0 ± 3.2†	1.76 ± 0.13†	30.3 ± 3.0
Vehicle (pair-fed)	1	9.94 ± 0.08†‡	67.5 ± 8.1	1.56 ± 0.13	29.5 ± 4.0
	2	10.35 ± 0.38†	64.1 ± 5.7‡	1.36 ± 0.07	28.2 ± 4.1‡
	3	10.21 ± 0.28†	44.9 ± 2.4†	1.31 ± 0.11	22.9 ± 2.9†
	5	10.79 ± 0.41†	43.9 ± 1.9†	1.32 ± 0.11	29.4 ± 2.4†
	7	10.66 ± 0.50†	50.7 ± 2.0†	1.35 ± 0.07†	25.3 ± 4.3†
PFDA (50 mg/kg, *ad libitum*-fed)	1	16.71 ± 0.62*‡	57.0 ± 3.8	1.37 ± 0.04	40.4 ± 5.5
	2	19.07 ± 0.51*‡	57.3 ± 2.6	1.25 ± 0.10‡	44.4 ± 6.1‡
	3	18.60 ± 0.29*‡	65.8 ± 6.3*‡	1.47 ± 0.14	54.7 ± 5.1*‡
	5	20.89 ± 0.72*‡	60.2 ± 5.2*	1.47 ± 0.23	58.1 ± 6.8*‡
	7	19.17 ± 0.27*‡	69.8 ± 4.6*‡	2.47 ± 0.18*‡	47.6 ± 3.2*

Rats were administered a single i.p. injection of vehicle or 50 mg/kg PFDA. Vehicle-treated, *ad libitum*-fed, control animals and PFDA-treated rats were allowed unlimited access to chow during the 12-hr feeding period; vehicle-treated, pair-fed rats were given the amount of chow that their PFDA-treated partner consumed during the previous 24 hr. Rats were killed and livers were removed either 1, 2, 3, 5, or 7 days post-treatment and prepared for analyses as described in Materials and Methods. Values are means ± SEM, N = 3–5.

* Significantly different from vehicle-treated, pair-fed control group at this time ($P < 0.05$).

† Significantly different from PFDA-treated group at this time ($P < 0.05$).

‡ Significantly different from vehicle-treated, *ad libitum*-fed control group at this time ($P < 0.05$).

lane was achieved by hybridization of the membrane with a specific cDNA probe for β-actin (Fig. 2, top panel). Northern blot analysis, performed with a second set of samples, confirmed results presented in Fig. 2.

The message for ACS was elevated approximately 2-fold by all doses of PFDA, when compared to that of *ad libitum*-fed control animals. mRNAs for ACBP and L-FABP displayed a similar trend, except that the 65 mg/kg PFDA-treated group revealed an approximate 3-fold increase in mRNA for both ACBP and L-FABP. Pair-feeding vehicle-treated rats for 7 days against rats receiving 50 mg/kg of PFDA did not appear to greatly affect mRNA levels for ACS, ACBP, or L-FABP. The cDNA for ACS also hybridized weakly with a message slightly smaller than that identified as the mRNA for ACS. Although recognition of this band has been observed in the past [43], its identity remains unknown.

Time–course for PFDA induction of hepatic ACS, long-chain acyl-CoA esters, ACBP, and L-FABP. Based on results of the dose–response study, 50 mg/kg of PFDA was used in the time–course study. Rats treated with vehicle or 50 mg/kg of PFDA were killed after 1, 2, 3, 5 or 7 days post-treatment, and livers were removed and prepared for analyses. A third treatment group consisting of pair-fed control rats was also used. Results are summarized in Table 2.

ACS activity was slightly higher at earlier than later times after vehicle treatment in the *ad libitum*-fed control group. This initial tendency for ACS activity to be elevated may explain why ACS activity in the pair-fed control group initially was less than that of the *ad libitum*-fed controls. PFDA-treated rats had higher hepatic ACS activity beginning 1 day after treatment, and it remained elevated for 7 days.

Pair-feeding caused an initial elevation in hepatic long-chain acyl-CoA ester concentrations, but by day 3 the levels had decreased to the *ad libitum*-fed control level where it remained through day 7. Rats administered PFDA had slightly higher concentrations of long-chain acyl-CoAs in the liver during the first 2 days post-treatment; the levels increased further on day 3 and peaked at 1.4-fold above the *ad libitum*-fed control group on day 7. Hepatic long-chain acyl-CoA concentrations in the PFDA group were diminished slightly in comparison to their pair-fed counterparts at days 1 and 2 post-treatment. However, a significant elevation relative to the pair-fed control group was observed beginning on day 3, and it continued through day 7.

Pair-fed control rats tended to have diminished hepatic ACBP concentrations compared to *ad libitum*-fed controls. This same pattern was observed through day 5 for PFDA-treated rats. Yet on day 7 hepatic ACBP concentration in the PFDA treatment group increased 1.4- and 1.8-fold above the *ad libitum*-fed and pair-fed control groups, respectively. Hepatic L-FABP concentrations were not altered by pair-feeding, but PFDA treatment increased L-FABP concentrations beginning on day 2 post-treatment, and the levels remained elevated on days 3, 5, and 7.

Time–course of PFDA induction of hepatic ACS, ACBP, and L-FABP protein. Changes in

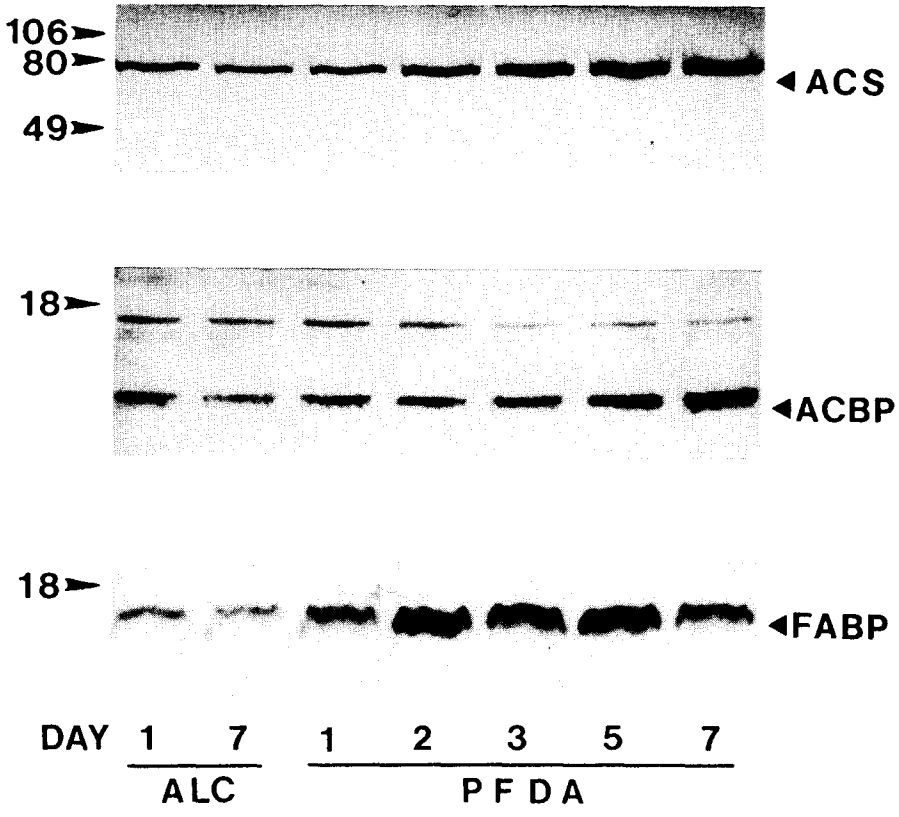

Fig. 3. Immunostaining of hepatic proteins from rats at selected times following treatment with PFDA. Twenty-five micrograms of homogenate protein was separated by SDS–PAGE and transferred to nitrocellulose paper. ACS (top), ACBP (middle), and L-FABP (bottom) detection was performed as indicated in Materials and Methods. Molecular weights (kDa) of protein markers are depicted to the left of each panel. Key: ALC, *ad libitum*-fed control; and PFDA, 50 mg/kg PFDA.

immunoreactive content of ACS, ACBP, and L-FABP in the liver were determined from day 1 to day 7 after PFDA treatment (Fig. 3). Blots were cut in half and exposed to goat anti-rat albumin as a loading control; immunostaining for albumin was constant (data not shown). Compared with *ad libitum*-fed controls, PFDA increased ACS protein by day 2, and it was sustained through day 7 (top panel). An increase in ACBP protein was not apparent until days 5 and 7 post-treatment. An increase in immunostaining for L-FABP was detected on day 1, became more prominent on days 2–5, and remained elevated on day 7.

Time–course of PFDA induction of hepatic mRNA for ACS, ACBP, and L-FABP. Hybridization studies with specific ^{32}P-labeled cDNA probes were used to identify temporal changes in hepatic mRNA content for ACS, ACBP, and L-FABP (Fig. 4). A single nylon membrane was used for all analyses. This served to simplify comparisons between analyses. Slight differences in levels of ACS, ACBP, or L-FABP mRNAs between *ad libitum*-fed controls and pair-fed controls were observed 1 day post-treatment. However, a greater tendency for the message level of these proteins to be decreased by pair-feeding was seen at 7 days post-treatment. In contrast to pair feeding, treatment with 50 mg/kg PFDA increased ACS mRNA at all time points. This augmentation of ACS mRNA appeared to peak at about a 3-fold increase on days 2 and 3 post-treatment. The levels of ACBP and L-FABP mRNA were also increased by PFDA treatment. These increases, about 2-fold, appeared to be relatively constant from days 1 to 7 post-treatment.

Effect of fasting on hepatic ACS, long-chain acyl-CoA esters, ACBP, and L-FABP. Prolonged fasting of rats can elevate long-chain acyl-CoA concentrations in the liver [44, 45]. To determine whether a relationship exists between increased hepatic long-chain acyl-CoA ester concentrations and induction of the hepatic lipid binding proteins, ACBP and L-FABP, vehicle-treated control rats were fasted for 24 or 48 hr, and ACS activity as well as ACBP and L-FABP concentrations in the liver were measured (Table 3). Rats treated with 50 mg/kg of PFDA and fed *ad libitum* were also included. Although ACS activity in vehicle-treated fasted control rats was similar to that of vehicle-treated, *ad libitum*-fed control rats, ACS activity in PFDA-treated animals was increased at both 24 and 48 hr. Fasted control rats had the largest increase in hepatic long-chain acyl-CoA esters, although rats exposed to PFDA also had elevated long-chain acyl-CoA levels.

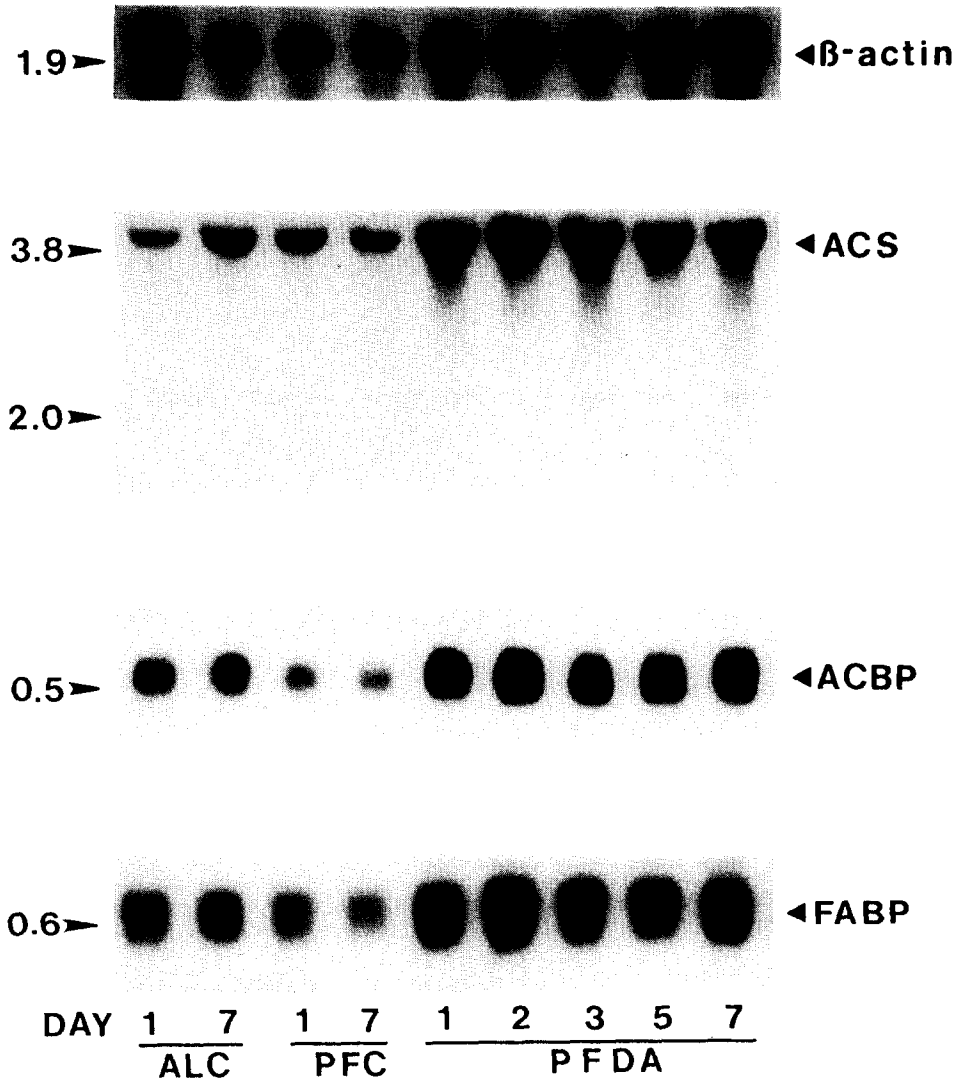

Fig. 4. Hybridization analysis of hepatic mRNA from rats at selected times following treatment with PFDA. Twenty-five micrograms of total RNA was separated by agarose gel electrophoresis, blotted and hybridized with cDNA probes for human β-actin, ACS, ACBP, and L-FABP as described in Materials and Methods. Sizes of identified bands (kilobases) are depicted to the left of each panel. Key: ALC, *ad libitum*-fed control; PFC, pair-fed control; and PFDA, 50 mg/kg PFDA.

A major finding was that the 24- to 48-hr fast in control rats had no effect on ACBP or L-FABP concentrations in the liver as measured by ELISA. In contrast to the previous experiment (Table 2), PFDA treatment caused a slight elevation of hepatic ACBP concentrations compared with the *ad libitum*-fed control group. Hepatic L-FABP concentrations also tended to increase in response to PFDA, but the effect was not significant.

Effect of fasting on hepatic mRNA for ACBP and L-FABP. The time–course study revealed that PFDA elevated hepatic ACBP and L-FABP mRNAs prior to observed changes in ACBP and L-FABP protein. To verify that the absence of change of hepatic ACBP and L-FABP protein in response to fasting was not due to the acute length of this study, northern blot analysis utilizing ^{32}P-labeled cDNA probes was performed on liver tissue derived from rats fasted for 24 and 48 hr (Fig. 5). Since the message for β-actin became a greater proportion of the total mRNA content in fasted rats, the mRNA for GAPDH was monitored as a loading control. In contrast to PFDA-treated rats, hepatic ACBP mRNA was decreased by one-half in rats fasted for 48 hr compared with *ad libitum*-fed control animals. No change in L-FABP mRNA was observed subsequent to 48 hr of fasting.

DISCUSSION

A potential role for elevated hepatic long-chain acyl-CoA esters in the induction of peroxisomal β-

Table 3. Effects of fasting or PFDA treatment on hepatic long-chain acyl-CoA synthetase activity and hepatic concentrations of long-chain acyl-CoA esters and lipid binding proteins

Treatment	Time post-treatment (hr)	ACS activity (nmol/min/mg protein)	Long-chain CoA (nmol/g tissue)	Lipid binding proteins (μg/mg protein)	
				ACBP	L-FABP
Vehicle (*ad libitum*-fed)	24	9.82 ± 0.52*	50.4 ± 0.5*†	1.58 ± 0.08	30.1 ± 3.9
	48	9.72 ± 0.22*	48.0 ± 3.6*†	1.52 ± 0.05	25.6 ± 4.2
Vehicle (fasted)	24	11.27 ± 0.15	101.9 ± 7.5*‡	1.47 ± 0.10	27.1 ± 2.5
	48	10.64 ± 0.34*	130.8 ± 1.4*‡	1.29 ± 0.06*	25.9 ± 2.4
PFDA (50 mg/kg, *ad libitum*-fed)	24	11.87 ± 0.27‡	76.4 ± 1.4†‡	1.73 ± 0.07	35.1 ± 4.6
	48	14.50 ± 0.50†‡	69.2 ± 3.1†‡	1.70 ± 0.13†	40.9 ± 6.2

Rats were administered a single i.p. injection of vehicle or 50 mg/kg PFDA. Vehicle-treated, *ad libitum*-fed, control animals and PFDA-treated rats were allowed unlimited access to chow during the feeding period; vehicle-treated, fasted rats were deprived of chow for the indicated period of time. Rats were killed and livers were removed either 24 or 48 hr post-treatment and prepared for analyses as described in Materials and Methods. Values are means ± SEM, N = 3–5.

* Significantly different from PFDA-treated group at this time ($P < 0.05$).

† Significantly different from vehicle-treated, fasted group at this time ($P < 0.05$).

‡ Significantly different from vehicle-treated, *ad libitum*-fed control group at this time ($P < 0.05$).

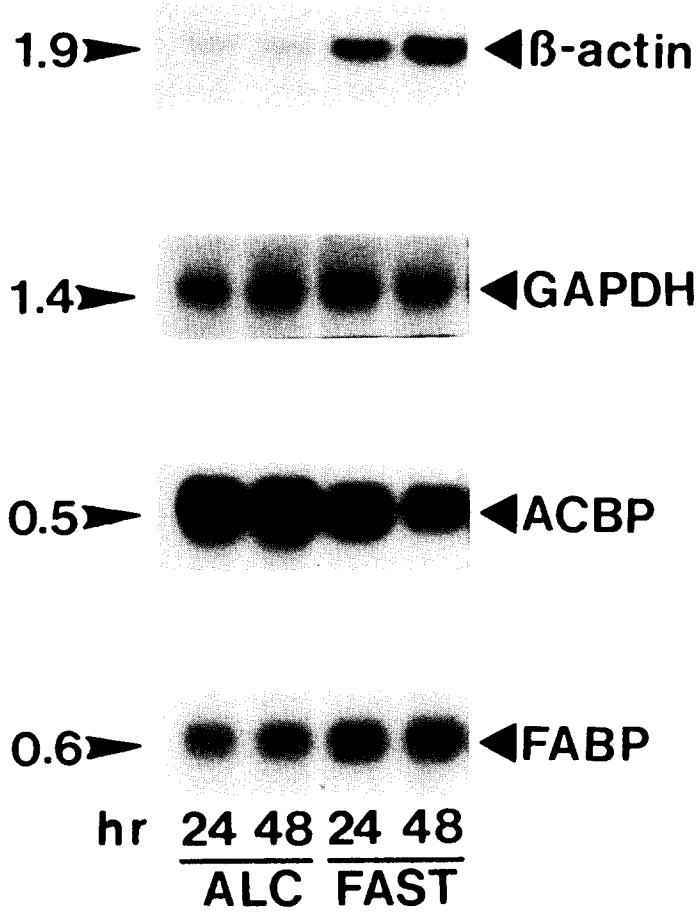

Fig. 5. Hybridization analysis of hepatic mRNA from rats fasted for 24 and 48 hr. Twenty-five micrograms of total RNA was separated by agarose gel electrophoresis, blotted and hybridized with cDNA probes for human β-actin, GAPDH, ACBP, and L-FABP as described in Materials and Methods. Sizes of identified bands (kilobases) are depicted to the left of each panel. Key: ALC, *ad libitum*-fed control; and FAST, fasted.

oxidation, either as a consequence of pathophysiological conditions [11, 46] or treatment with peroxisome proliferators [47, 48], has been suggested. Nevertheless, attempts to relate, in dose–response and time–course studies, changes in hepatic long-chain acyl-CoA concentrations with those of the hepatic intracellular lipid binding proteins, ACBP and L-FABP, have not been done. ACBP binds acyl-CoA esters with very high affinity (K_d = 400 fM [49]), serving as an intracellular pool former and transporter of these fatty acid metabolites [50]. L-FABP, although able to bind acyl-CoA esters, has a greater influence on the storage, transport and utilization of fatty acids [13]. We hypothesized that PFDA treatment or fasting in rats would cause an increase in hepatic long-chain acyl-CoA concentrations, which would be compensated for by the induction of ACBP or L-FABP.

Previous findings suggest that treatment of rats with PFDA may elevate hepatic long-chain acyl-CoAs. Hepatic triacylglycerols and cholesteryl esters are elevated 7 days following treatment with PFDA [5], and it is conceivable that these esterification pathways become available for the removal of excess long-chain acyl-CoAs in response to PFDA. The observed inhibition of mitochondrial fatty acid β-oxidation by PFDA *in vitro* [51] could feasibly contribute to an increase in long-chain acyl-CoA esters as well. In addition, the structural similarity of peroxisome proliferators to fatty acids may explain why many peroxisome proliferators, including PFDA, have the ability to displace fatty acids from L-FABP *in vitro* [17, 52]. Since PFDA readily distributes to rat liver [22], displacement of fatty acids from L-FABP by PFDA could increase levels of hepatic free fatty acids. Esterification or β-oxidation of these fatty acids initially requires activation to coenzyme A esters via the enzyme ACS [43]. Thus, an increase in hepatocellular long-chain acyl-CoAs might be expected to occur in PFDA-treated rats. Alternatively, an increased flux of fatty acids through the microsomal P450IVA1 ω-oxidation pathway, which is also induced after treatment with peroxisome proliferators [53], would not necessitate elevated hepatic long-chain acyl-CoA ester levels. Moreover, a dramatic increase in cytosolic acyl-CoA hydrolase activity in the livers of PFDA-treated rats, as is observed in perfluorooctanoic acid-treated rats [54], may have negated modest increases in long-chain acyl-CoAs. Therefore, the elevation of long-chain acyl-CoA esters in PFDA-treated animals was certainly not a foregone conclusion due to the effects of this peroxisome proliferator on both the synthesis and degradation of acyl-CoAs.

Although an augmentation in hepatic long-chain acyl-CoA levels was detected in PFDA-treated rats, our results refute any cause-and-effect relationship between increased hepatic concentrations of endogenous long-chain acyl-CoAs and induction of ACBP or L-FABP. L-FABP was induced prior to a measurable increase in long-chain acyl-CoAs, whereas ACBP only became elevated significantly on day 7 in response to PFDA. The delayed response by ACBP protein suggests a secondary mechanism for ACBP induction, especially since ACBP mRNA was elevated on day 1 post-treatment. Nuclear run-on experiments are currently being undertaken in our laboratory to elucidate whether the early increase in ACBP mRNA observed is due to an increase in transcription of the ACBP gene, which has been confirmed for other members of the peroxisome proliferator-responsive gene-battery [55]. In addition, hypophagia in control rats pair-fed to PFDA-treated rats was associated with an initial rise in long-chain acyl-CoA levels, but it was not accompanied by corresponding increases in ACBP or L-FABP concentrations in the liver. A significant increase in ACBP, L-FABP, and ACS activity was observed on day 7 following treatment with 65 mg/kg of PFDA, but long-chain acyl-CoA ester levels were not different at this time. In addition, PFDA is more potent than certain fatty acids in activating the peroxisome proliferator-activated receptor in transactivation studies.* Due to the metabolic inertness of PFDA, this indicates that a non-coenzyme A moiety is responsible for peroxisome proliferator-activated receptor activation and ultimately in regulating ACBP and L-FABP gene expression.

Results obtained with fasted rats lend the most compelling evidence to refute the involvement of long-chain acyl-CoA esters in L-FABP or ACBP regulation. In fasted animals, a doubling of long-chain acyl-CoA concentrations in the liver occurred with no concomitant changes in either L-FABP or ACBP concentration. Furthermore, northern blot analysis confirmed a decrease in ACBP mRNA at 24 and 48 hr after fasting, while FABP mRNA was not affected. Since a peroxisome-proliferator response element has been identified upstream of the L-FABP gene [56, 57], L-FABP induction would have been expected to occur in fasted rats by 48 hr if long-chain acyl-CoA esters are the inducing-ligand for the peroxisome proliferator-activated receptor. The lack of response of L-FABP to fasting corroborates previous studies of prolonged fasting, where only a slight decrease [58] or no change [31] in hepatic concentrations of L-FABP was seen. However, we are unaware of previous attempts to measure hepatic ACBP concentrations in rats in response to the fasted state. In the present study, the ratio of long-chain acyl-CoA esters to ACBP (expressed per g liver) was approximately 2 for *ad libitum*-fed control rats, 3–4 for PFDA-treated rats, and 5–6 for fasted control rats. Thus, the observation that hepatic ACBP was not induced in fasted control rats cannot be attributed to a lack of elevation of long-chain acyl-CoA esters in the liver relative to hepatic ACBP. Analysis of the subcellular distribution of long-chain acyl-CoAs might be helpful in further interpreting this result, but this was beyond the scope of the present study. Also, it is emphasized that this experimental design only examined short-term effects related to elevated long-chain acyl-CoAs, and it is not known what responses might occur following extended observations.

Our results provide evidence against a role for long-chain acyl-CoA esters as endogenous inducers of ACBP, L-FABP, or ACS in rat liver. Although

* C. Corton, personal communication. Cited with permission.

PFDA presumably alters gene expression by activating the peroxisome proliferator receptor, the degree to which altered lipid metabolism is involved in modulating such receptor-mediated effects remains to be determined. Thus, the likelihood that a "substrate overload" condition could mediate the observed effects via the peroxisome proliferator-activated receptor must still be considered a legitimate possibility. However, long-chain acyl-CoA esters do not appear to be the key "substrate" involved.

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