Biochemical Pharmacology, Vol. 42, No. 2, pp. 295–302, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITION OF LONG-CHAIN ACYL-CoA SYNTHETASE BY THE PEROXISOME PROLIFERATOR PERFLUORODECANOIC ACID IN RAT HEPATOCYTES

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(*Received* 18 *December* 1990; *accepted* 6 *February* 1991)

Abstract—Perfluorodecanoic acid (PFDA) is a potent peroxisome proliferator and is known to affect hepatic lipid metabolism in rats. The effects of PFDA on fatty acid utilization were examined in isolated rat hepatocyte suspensions and in rat liver mitochondria and microsomes. PFDA inhibited the oxidation of palmitic acid but not octanoic or pyruvic acids when hepatocytes were incubated with 1 mM PFDA. At this PFDA concentration the esterification of palmitic acid into triacylglycerols was also reduced. The activity of long-chain acyl-CoA synthetase (ACS), an enzyme essential for both oxidation and esterification of fatty acids, was reduced in hepatocytes incubated with 1 mM PFDA. Carnitine palmitoyltransferase (CPT), an important enzyme for the oxidation of long-chain fatty acids, was not altered in hepatocytes incubated with this PFDA concentration. In rat liver mitochondria, palmitate oxidation and ACS activity were reduced significantly ($P < 0.01$) at a PFDA concentration that had no effect on CPT activity. The inhibition of ACS by PFDA was similar in liver mitochondria and microsome preparations. In mitochondria incubated with PFDA, the inhibition of ACS appears to be noncompetitive for the substrates palmitic acid and CoA. However, the ACS inhibition by PFDA appeared to be competitive for the ATP binding site of the enzyme. Several chain length perfluorinated fatty acids were examined for their ability to inhibit mitochondrial ACS. Short-chain perfluorinated fatty acids (perfluoropropionic and -butyric acid) did not inhibit ACS activity. However, medium-chain perfluorinated acids (perfluorooctanoic, -ananoic and -decanoic acid) were found to be potent inhibitors of ACS in isolated mitochondria. Whether ACS inhibition is causally related to PFDA-induced peroxisome proliferation and altered lipid metabolism seen *in vivo* is yet to be determined.

Perfluorodecanoic acid (PFDA¶) belongs to a structurally diverse group of chemicals which cause peroxisome proliferation in rodent liver [1, 2]. Similar to several other peroxisome proliferators, PFDA causes alterations in lipid metabolism. A dose-dependent increase in hepatic triacylglycerols, and to a lesser extent in cholesteryl esters, is observed 7 days after treatment with PFDA in rats [3]. It was hypothesized that PFDA causes a diversion of fatty acids from oxidation towards esterification into acylglycerols. Other researchers have observed altered fatty acid profiles following PFDA administration with increases in the long-chain fatty acids palmitic and oleic acids [4]. These effects on lipid metabolism are consistent with the substrate overload hypothesis of peroxisome proliferation [5]. Recently we found that neither PFDA nor perfluorooctanoic acid (PFOA) was metabolized to CoA esters to a detectable extent** nor are they incorporated into lipids [6]. Therefore, the effects of PFDA on fatty acid metabolism appear to be caused by the parent compound and are probably not a result of PFDA entering lipid metabolic pathways as an activated CoA conjugate.

The purpose of the present investigation was to determine the cause of the altered hepatic lipid metabolism seen with PFDA. The oxidation of fatty acids in suspended rat hepatocytes and isolated rat liver mitochondria, as well as the effects of PFDA on fatty acid incorporation into triacylglycerols in isolated rat hepatocytes, was examined. PFDA, similar to the peroxisome proliferators 4-THA [7], POCA [8] and bezafibrate [9], was found to inhibit the oxidation of palmitic acid but not octanoic acid. Further, we demonstrate that PFDA produced these effects in part by inhibiting long-chain acyl-CoA synthetase (ACS), an enzyme which activates long-chain fatty acids to CoA esters prior to their oxidation or esterification.

MATERIALS AND METHODS

Materials. PFDA (99% pure) was purified as described previously [10]. [1-^{14}C]Palmitate

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¶ Abbreviations: PFDA, perfluorodecanoic acid; ACS, acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; PFOA, perfluorooctanoic acid; 4-THA, 2-hydroxy-3-propyl-4-[6-(tetrazol-5-yl)hexyloxy]acetophenone; POCA, 2[5(4-chlorophenyl)pentyl]oxirane-2-carboxylate; BSA, bovine serum albumin; CoA, coenzyme A; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

** Kuslikis BI, Vanden Heuvel JP and Peterson RE, Lack of evidence for perfluorodecanoyl- or perfluorooctanoyl-coenzyme A formation in male and female rats. Manuscript submitted for publication.

(56.5 mCi/mmol) and L-*methyl*-^{3}H]carnitine (70 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). DuPont–New England Nuclear (Boston, MA) was the source for [1-^{14}C]palmitoyl carnitine chloride (55 mCi/mmol), [1-^{14}C]palmitoyl-CoA (60 mCi/mmol), [1-^{14}C]octanoate (53.5 mCi/mmol) and [1-^{14}C]pyruvate (15 mCi/mmol). Percoll was obtained for Pharmacia (Piscataway, NJ). Leibovitz L-15 tissue culture medium, medium supplements, and all cofactors and substrates were purchased from the Sigma Chemical Co. (St. Louis, MO). Perfluorobutyric acid, perfluoroproprionic acid, perfluorooctanoic acid, 2-bromooctanoic acid and 2-bromopalmitic acid were from the Aldrich Chemical Co. Inc. (Milwaukee, WI). Perfluoroananoic acid was from K & K Laboratories (Cleveland, OH). Silica gel (250 μm layer) thin-layer chromatography plates were from J. T. Baker Inc. (Phillipsburg, NJ). Hionic Fluor scintillation fluid was obtained from the Packard Chemical Co. (Downer's Grove, IL). Male Sprague–Dawley rats (200–250 g) obtained from Harlan–Sprague Dawley (Madison, WI) were housed individually in suspended stainless-steel cages in a temperature-controlled room (*ca.* 21°) with a 12-hr light/dark cycle (lighted 5:00 a.m. to 5:00 p.m.). Rats received food (Purina Rat Chow, No. 5012, Ralston Purina Co., St. Louis, MO) and water *ad lib.*

Hepatocyte and subcellular preparations. Hepatocytes were isolated from male rats by a two-step collagenase perfusion technique [11] and purified by Percoll density centrifugation [12]. After Percoll purification, the viability of cells assessed by trypan blue exclusion was greater than 95%. The hepatocytes were suspended in L-15 medium supplemented with 5 μg insulin/mL, 5 μg transferrin/mL and 5 ng selenium/mL, 2 mg albumin (essentially fatty acid free)/mL, 18 mM HEPES, 1 mg galactose/mL, 100 units penicillin/mL and 100 μg streptomycin/mL [13]. The final concentration of hepatocytes was 1×10^6 cells/2 mL. Mitochondria and microsomes were prepared from male rats as described previously [14]. The appropriate dilutions were performed following protein determination using the Bradford dye-binding technique [15].

Fatty acid oxidation in hepatocytes. Hepatocytes were diluted in the L-15 supplemented medium, as described above, and were incubated in small flasks at 37° for 10 min. PFDA in methanol (15 μL) was added to the cells and incubated for 20 min at 37°. The reactions were initiated by adding radiolabeled substrate in 1% BSA and sealing the flasks with rubber stoppers containing a plastic center well. The reaction (2 mL) was terminated after 30 min by adding 2 mL of 6% perchloric acid through the rubber seal, and $^{14}CO_2$ was trapped in the center well which contained 250 μL ethanolamine. The flasks remained at 37° for 1 hr after adding the ethanolamine, at which time the wells were transferred to scintillation vials containing 10 mL Hionic Fluor. The contents of the flask were centrifuged at 2000 *g* for 10 min, and the radioactivity in 1 mL of the acid supernatant was assessed.

Palmitic acid esterification in hepatocytes. The hepatocyte dilutions, PFDA incubations and addition of [^{14}C]palmitate were identical to those described above. However, after the reaction was allowed to proceed for 30 min, it was terminated by placing the flasks on ice. The cells were collected by centrifugation at 500 *g* for 2 min and were washed twice with Hanks' balanced salt solution. To the final cellular pellet 2 mL chloroform/methanol (2:1, v/v) was added and vortexed. The solution remained at ambient temperature for 30 min, and then 0.8 mL of 0.9% NaCl was added, vortexed and centrifuged at 1500 *g* for 20 min. The upper aqueous phase was aspirated carefully. An aliquot of the lower, lipid-containing phase was dried under nitrogen and resuspended in chloroform/methanol. The extract was applied to silica gel thin-layer chromatography (TLC) plates, and the neutral lipids were separated as described previously [16]. The TLC plate was sprayed with 3% cupric acetate in 15% phosphoric acid and charred at 170° for 15 min [17]. The triacylglycerol and free fatty acid spots, as determined using authentic standards, were scraped directly into scintillation vials for radioactivity determination.

Mitochondrial fatty acid oxidation. PFDA in methanol (15 μL) was added to a buffer containing 120 mM KCl, 2.5 mM K_2HPO_4, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 0.4 mM ADP, 1 mM ATP, 0.7 mM carnitine, 0.7 mM CoA, 10 mM malonate, 4 mg BSA/mL and [1-^{14}C]palmitate (0.2 μM, 0.125 μCi/μmol) at pH 7.2. The reaction (1 mL) was incubated at 37° for 5 min prior to addition of 0.25 mg mitochondrial protein. The flasks were sealed and incubated for 10 min at 37°. The reaction was terminated with 1 mL of 6% perchloric acid, and $^{14}CO_2$ and acid soluble products were collected as described above for hepatocytes.

Enzyme assays. Long-chain acyl-CoA synthetase (ACS, EC 6.2.1.3) activity was measured as described by Krisans *et al.* [18]. The final reaction mixture (0.2 mL) contained 150 mM Tris–HCl, 2 mM EDTA, 2.5 mM ATP, 0.6 mM CoA, 1 mM dithiothreitol, [1-^{14}C]palmitic acid (0.01 to 0.2 mM, 6.25 μCi/μmol) and the protein sample (20 μg). The reaction proceeded for 6 min at 37° and was terminated by adding 3.25 mL chloroform/methanol/heptane (1.4:1.25:1, by vol.) and 1.04 mL acetate (pH 4.0). The solution was shaken for 20 min and then centrifuged at 1500 *g* for 10 min, and the radioactivity in the aqueous phase, which contains the [1-^{14}C]palmitoyl-CoA formed, was assessed.

Carnitine palmitoyltransferase (CPT, EC 2.3.1.21) activity was assayed with a butanol extraction procedure [19]. The assay mixture contained, in a total volume of 0.5 mL, 0.2 mM L-[*methyl*-^{3}H]carnitine (6 μCi/μmol), 50 μM palmitoyl-CoA, 20 mM HEPES buffer (pH 7.0), 2% fatty acid free bovine serum albumin, 50 mM KCl and 2 mM KCN. The reaction was initiated upon the addition of 0.25 mg of protein sample.

Statistical analysis. Differences between the means were analyzed using one-way analysis of variance (ANOVA) and least significant difference (LSD) protected for multicomparison [20]. Significance was set at $P < 0.01$. K_m, V_{max} and IC_{50} values were collected as described by Tallarida and Murray [21].

RESULTS

Effects of PFDA on hepatocyte viability. The

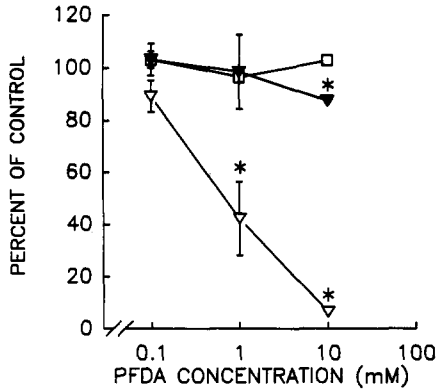

Fig. 1. Effects of increasing PFDA concentration on the oxidation of palmitic (▽), octanoic (▼) and pyruvic (□) acids in suspended rat hepatocytes. Hepatocytes (1×10^6 cells), suspended in Leibovitz L-15 medium, were preincubated with PFDA for 20 min prior to the addition of radiolabeled fatty acids. The flasks were sealed and the reaction proceeded for 30 min at which time radioactive oxidative products were collected. Results are expressed as a percent of the oxidation seen in vehicle-treated hepatocytes (mean ± SEM, N = 4). Mean rates of oxidation for control hepatocytes in 30 min: palmitic acid, 18.1 nmol; octanoic acid, 29.8 nmol; and pyruvic acid, 176 nmol. An asterisk indicates a significant difference from control ($P < 0.01$).

effects of PFDA on hepatocyte viability were assessed by lactate dehydrogenase (LDH) leakage into the medium, as well as trypan blue exclusion. The cells were suspended in L15 supplemented medium as stated for the hepatocyte oxidation studies, and the perfluorinated acid was preincubated with the hepatocytes for 20 min at 37°. PFDA at doses of 0.01 to 10 mM had no effect on either LDH leakage or trypan blue exclusion (data not shown). At 50 and 100 mM PFDA LDH activity in the medium was reduced significantly ($P < 0.01$), possibly due to precipitation of LDH by the perfluorinated acid, and the cells excluded significantly less trypan blue than the control cells. Therefore, all experiments were performed at 10 mM PFDA or less.

Fatty acid oxidation in suspended hepatocytes. Figure 1 shows the effects of various concentrations of PFDA on the oxidation of fatty acids of different chain length in suspended rat hepatocytes. The data are expressed as a percent of the oxidation rates found in hepatocytes incubated with vehicle (methanol). PFDA at 0.1 mM had no effect on the oxidation rate of palmitate, octanoate or pyruvate. However 1 mM PFDA significantly reduced the oxidation of the long-chain fatty acid palmitate (42 ± 14% of control). Neither medium-chain nor short-chain fatty acid oxidation was affected at this PFDA concentration. At 10 mM PFDA, the oxidation of palmitate was reduced to 2% of the control rate, while octanoate oxidation was affected minimally (87 ± 1% of control). The oxidation of pyruvate was unaffected at all PFDA doses.

Palmitate esterification in suspended hepatocytes. The effects of PFDA preincubation on the incorporation of [^{14}C]palmitate into hepatocyte triacylglycerols are shown in Table 1. PFDA at a concentration of 0.1 mM had no effect on the amount of palmitate-derived ^{14}C in triacylglycerol or free fatty acid. The hepatocytes incubated with 1 mM PFDA showed a significant reduction in the amount of [1-^{14}C]palmitate incorporated into triacylglycerol in 30 min, with the PFDA-treated hepatocytes showing a reduction in incorporation to 56% of control. At this dose of PFDA there was a slight increase in the amount of radioactivity present in the free fatty acid form, although this difference was not statistically significant. At the highest concentration of PFDA the triacylglycerol formation was reduced to 11% of control, while the amount of palmitate present as the free fatty acid was increased.

Table 1. Effects of PFDA on fatty acid esterification in isolated hepatocytes

PFDA concentration (mM)	Fate of palmitate-derived ^{14}C	
	Triacylglycerol (% of control)	Free fatty acid (% of control)
0	100 ± 8	100 ± 10
0.1	122 ± 13	104 ± 12
1	56 ± 1*	126 ± 17
10	11 ± 3*	1020 ± 544*

Hepatocytes were preincubated without (control) or with PFDA for 20 min at 37° and then allowed to react with 0.2 mM palmitic acid for 30 min at 37°. The hepatocytes were isolated by centrifugation and the lipids extracted and separated using TLC. The triacylglycerol and free fatty acid spots were quantitated for palmitate-derived ^{14}C. The control value of palmitate-derived ^{14}C in triacylglycerols was 43.1 ± 5.6 nmol/10^6 cells and in free fatty acids was 4.93 ± 0.5 nmol/10^6 cells. Values are means ± SEM, N = 3.

* Significantly different from control at $P < 0.01$.

In experiments where hepatocytes and medium were analyzed for incorporation of [1-^{14}C]palmitic acid into triacylglycerols, results similar to those stated above were obtained (data not shown). That is, PFDA decreased the amount of palmitic acid incorporated into triacylglycerols, both in hepatocytes and in hepatocytes plus medium.

ACS and CPT activity in isolated hepatocytes. Since both oxidation and esterification of palmitic acid were affected by preincubating hepatocytes with PFDA, we hypothesized that long-chain acyl-CoA synthetase (ACS) may be inhibited due to its role in both metabolic pathways. In our examination of ACS we included the study of CPT, a mitochondrial enzyme which affects the partitioning of fatty acids between esterification and oxidation. The whole cell homogenate was examined due to the multiple subcellular distribution of ACS. As shown in Table 2, PFDA at the lowest concentration (0.1 mM) had no effect on either ACS or CPT activity. Preincubation of hepatocytes with 1 mM PFDA reduced the total cellular ACS activity (to 25% of control) while having virtually no effect on CPT. The 10 mM concentration of PFDA caused a significant reduction in both enzyme activities. Thus,

Table 2. Effects of PFDA on long-chain acyl-CoA synthetase and carnitine palmitoyltransferase activities in isolated hepatocytes

PFDA concentration (mM)	ACS activity (nmol/min/mg protein)	CPT activity (nmol/min/mg protein)
0	2.45 ± 0.35 (100)	1.78 ± 0.04 (100)
0.1	2.35 ± 0.10 (96)	1.78 ± 0.06 (100)
1	0.60 ± 0.28* (25)	1.60 ± 0.06 (90)
10	−0.02 ± 0.01* (0)	0.06 ± 0.02* (3)

Hepatocytes were incubated with PFDA or with vehicle for 20 min, at which time the hepatocytes were isolated by centrifugation, resuspended and briefly sonicated. The enzyme activities were assessed in this sonicate and expressed relative to the amount of protein present. Values are means ± SEM, N = 3–4. Numbers in parentheses give the percent of control.

* Significantly different from control at $P < 0.01$.

Table 3. Effects of PFDA on palmitate oxidation and long-chain acyl-CoA synthetase and carnitine palmitoyltransferase activities in rat liver mitochondria

PFDA concentration (mg/mg*)	Palmitate oxidation (nmol/min/mg protein)	ACS activity (nmol/min/mg protein)	CPT activity (nmol/min/mg protein)
0	0.548 ± 0.040 (100)	2.85 ± 0.065 (100)	0.526 ± 0.013 (100)
0.06	0.568 ± 0.012 (104)	2.85 ± 0.11 (100)	0.680 ± 0.050 (129)
0.6	0.086 ± 0.016† (16)	0.15 ± 0.03† (5)	0.656 ± 0.056 (125)
6	0.041 ± 0.011† (8)	0.01 ± 0.005† (0.3)	0.010 ± 0.002† (2)

Mitochondria were preincubated with vehicle or with PFDA for 10 min at 37°. Palmitate oxidation as well as enzyme activities were measured as described in Materials and Methods. Values are means ± SEM, N = 3–4. Numbers in parentheses are the percent of control.

* Milligrams PFDA per milligram mitochondrial protein.

† Significantly different from control at $P < 0.01$.

1 mM PFDA caused a selective loss of ACS activity, whereas both enzymes activities were reduced at 10 mM PFDA.

Mitochondrial palmitate oxidation and enzyme activities. The inhibition of fatty acid oxidation by PFDA was studied in isolated rat liver mitochondria. The amount of PFDA added to the incubations is expressed as milligrams PFDA per milligram of mitochondrial protein such that the assays could be directly compared to each other. As shown in Table 3, palmitate oxidation was unaltered at 0.06 mg PFDA/mg mitochondrial protein but was reduced significantly at 0.6 mg PFDA/mg protein (to 16% of control) and at 6 mg PFDA/mg protein (to 8% of control). These concentrations of PFDA had no effect on the mitochondrial oxidation of octanoate (data not shown). Long-chain ACS activity was virtually eliminated by incubating the mitochondria with 0.6 mg PFDA/mg protein, resulting in a decrease in palmitoyl-CoA formation to 5% of control. CPT activity remained unaltered or slightly elevated at the 0.06 and 0.6 mg PFDA/mg protein concentrations and was decreased significantly at the highest PFDA concentration. The addition of 1% BSA to the mitochondrial incubations had a protective effect, with the 0.6 mg PFDA/mg protein concentration no longer depressing ACS activity (data not shown).

To further characterize the inhibition of mitochondrial ACS activity by PFDA, the activity of the enzyme was measured at increasing substrate concentrations in mitochondria exposed to vehicle (control) or 0.11 mM PFDA. Figure 2 shows that formation of palmitoyl-CoA increased with increasing concentrations of palmitic acid (left panel), ATP (center panel) or CoA (right panel) in the vehicle and PFDA-treated mitochondria. The kinetics of the ACS reaction are depicted in double-reciprocal plots (Fig. 2, inserts). The K_m for palmitic acid was identical in the vehicle- (0.054 mM) and PFDA-treated (0.057 mM) mitochondria, whereas the V_{max} was reduced by PFDA. Thus, the inhibition of ACS by PFDA is noncompetitive for the palmitic acid binding site. PFDA inhibits ACS in a predominantly competitive fashion for the ATP binding site as indicated by the dissimilar K_m values (0.58 mM control vs 1.13 mM PFDA) and comparable V_{max} values. Similar to that seen for palmitic acid, the K_m for CoA was not affected by incubating the mitochondria with PFDA (0.017 mM control vs 0.018 mM PFDA), whereas the V_{max} was reduced, indicating that PFDA noncompetitively inhibits ACS at the CoA binding site. Thus, at this concentration of PFDA (0.11 mM) the inhibition of ACS activity appears to be via competition for the ATP binding site of the enzyme.

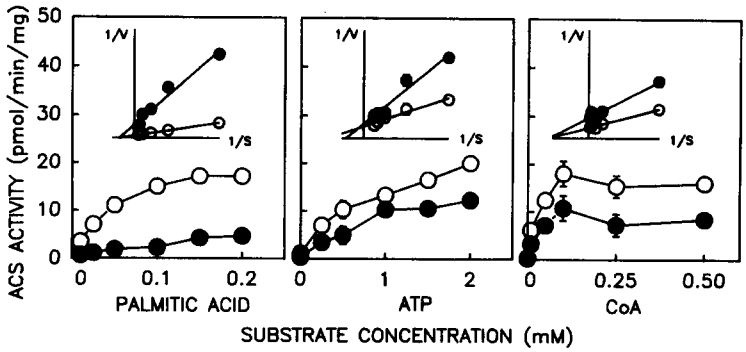

Fig. 2. Inhibition of mitochondrial long-chain acyl-CoA synthetase by PFDA. Mitochondria were incubated with vehicle (○) or 0.11 mM PFDA (●), and the activity of ACS with increasing substrate concentrations was determined. Results are means ± SEM (N = 4). Inserts show double-reciprocal plots ($1/v$ vs $1/s$).

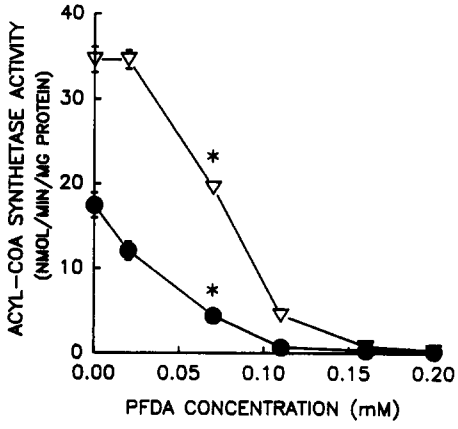

Fig. 3. Effects of increasing PFDA concentrations on microsomal (▽) and mitochondrial (●) long-chain acyl-CoA synthetase activity. Palmitate (0.20 mM) was used as the substrate. Results are means ± SEM (N = 4). An asterisk indicates the lowest PFDA concentration where enzyme activity was significantly different from control ($P < 0.01$).

The effects of PFDA on mitochondrial and microsomal ACS activity were examined to determine if either preparation is more susceptible than the other to the *in vitro* effects of this perfluorinated acid (Fig. 3). As expected, in the absence of PFDA, the activity of long-chain ACS was much higher in microsomal (34.6 nmol/min/mg) than mitochondrial (17.4 nmol/min/mg) preparations. At 0.02 mM PFDA mitochondrial ACS activity was decreased by 25% while microsomal ACS activity remained the same. Mitochondrial and microsomal activity was decreased 75 and 44%, respectively, at 0.07 mM PFDA and 96 and 87%, respectively, at 0.11 mM PFDA. Thus, it appears that mitochondria (IC_{50} 0.03 mM) may be slightly more susceptible than microsomes (IC_{50} 0.08 mM) to the inhibitory effect of PFDA on ACS activity.

The effects of increasing perfluorinated acid chain length on mitochondrial ACS activity were examined (Table 4). 2-Bromo fatty acids, which are known ACS inhibitors [22], were also examined. The short-chain perfluoropropionic acid (three carbon, C3) and perfluorobutyric acid (C4) did not inhibit ACS activity at concentrations up to 2 mM. On the other hand, the medium-chain length perfluorinated fatty acids (C8–C10) were found to be potent inhibitors of ACS ($IC_{50} < 0.1$ mM). The relative potency of the perfluorinated acids for ACS inhibition was: C10 > C9 > C8 >>> C3 = C4. 2-Bromooctanoate (IC_{50} approx. 0.8 mM) was found to be approximately ten times less potent than perfluorooctanoate (IC_{50} approx. 0.08 mM). The most potent inhibitor of mitochondrial ACS examined was 2-bromopalmitic acid which had an IC_{50} value of less than 0.02 mM. The inhibition of ACS by these fatty acid analogues appears to be noncompetitive or uncompetitive due to the fact that the IC_{50} values were similar with either 0.05 or 0.15 mM palmitate used as the substrate for ACS.

DISCUSSION

Inhibition of fatty acid oxidation and peroxisome proliferation. Several peroxisome proliferators have been shown to inhibit the mitochondrial oxidation of fatty acids. A metabolite of di(2-ethylhexyl)phthalate inhibits the oxidation of medium chain fatty acids [23], while POCA [8], 4-THA [7] and bezafibrate [9] inhibit long-chain fatty acid oxidation. The anticonvulsant peroxisome proliferating drug valproic acid inhibits mitochondrial oxidation of branch-chain and long-chain fatty acids [24]. Riboflavin deficiency, a situation which also induces peroxisomal enzyme activity, decreases mitochondrial oxidation of fatty acids of various chain lengths due to a decrease in FAD-dependent acyl-CoA dehydrogenases [25]. Due to the ability of the peroxisome to metabolize fatty acids, the peroxisome proliferative response may be an adaptation to this altered mitochondrial fatty acid oxidation [5].

The peroxisome proliferator PFDA has now been shown to be an inhibitor of long-chain fatty acid oxidation. Apparently, this medium-chained

Table 4. Effects of halogenated fatty acid structure on the ability to inhibit long-chain acyl-CoA synthetase activity

Halogenated fatty acid	IC_{50} (mM)	
	0.05 mM*	0.15 mM*
Perfluoropoprionic acid $CF_3CF_2COO^-$	>2.0	>2.0
Perfluorobutyric acid $CF_3(CF_2)_2COO^-$	>2.0	>2.0
Perfluorooctanoic acid $CF_3(CF_2)_6COO^-$	0.089 (0.077–0.103)	0.144 (0.100–0.187)
Perfluoroananoic acid $CF_3(CF_2)_7COO^-$	0.076 (0.071–0.081)	0.069 (0.063–0.076)
Perfluorodecanoic acid $CF_3(CF_2)_8COO^-$	0.064 (0.058–0.071)	0.059 (0.052–0.066)
2-Bromooctanoic acid $CH_3(CH_2)_5CHBrCOO^-$	0.825 (0.652–1.043)	0.874 (0.685–1.117)
2-Bromopalmitic acid $CH_3(CH_2)_{13}CHBrCOO^-$	<0.02	<0.02

Mitochondria were incubated with increasing concentrations of halogenated fatty acids, and ACS was measured using palmitate as substrate. Values in parentheses are the 95% confidence intervals for the IC_{50} values.

* Concentration of palmitic acid used as substrate for ACS.

perfluorinated acid (10 carbon) is inhibiting long-chain fatty acid oxidation and esterification by inhibiting the activation of palmitate to palmitoyl-CoA. The fact that CPT activity was unaffected at concentrations where ACS activity was reduced significantly suggests that the ACS inhibition is not due to direct damage to the outer mitochondrial membrane. PFDA is not competing with palmitate for ACS, although PFDA does appear to compete for the ATP binding site. The observation that the inhibition by PFDA of mitochondrial ACS was reversed when BSA was added to the medium suggests that protein binding may modulate the effects of PFDA. A possible intracellular binding protein that may also be affected by PFDA is fatty acid-binding protein (FABP), which is important for the uptake and utilization of long-chain fatty acids. Involvement of FABP in modulating the effects of PFDA on hepatic fatty acid metabolism needs to be addressed.

Inhibition of ACS. Activation of fatty acids to acyl-CoA derivatives is an essential step in the oxidation, elongation and esterification of fatty acids. Long-chain ACS from microsomes, mitochondria and peroxisomes of rat liver appear to be identical in kinetic properties, molecular weight (76 kD) and amino acid sequences [26]. Recently it was shown that the mRNA for long-chain fatty acyl-CoA synthetase was increased 7- to 8-fold in rat liver following feeding a high fat or carbohydrate diet [27]. Due to the importance of ACS in fatty acid metabolism, inhibition of this enzyme may have important biological consequences.

To date several ACS inhibitors have been reported. 2-Bromo fatty acids have been shown to be inhibitors of triacylglycerol synthesis in rat hepatocyte cultures, with compounds of chain lengths of 8 or greater being inhibitors of microsomal ACS activity [22]. The effect of perfluorinated fatty acids on ACS follows a similar pattern with short-chain lengths having no ACS inhibition activity while medium-chain lengths are potent inhibitors of mitochondrial ACS. However, chain length is not the only important factor involved in the ability of fatty acid analogs to inhibit this enzyme. The fact that 2-bromo fatty acids are inhibitors suggests that halogenation at the second carbon is important for ACS inhibition. In addition, perfluorinated octanoic acid was much more potent than its 2-bromo counterpart. This suggests that the hydrophobicity of the carbon chain and/or the acidity of the carboxyl terminal may influence inhibition of ACS.

Besides halogenated fatty acids, several other ACS inhibitors have been reported. Triacsins, compounds isolated from the cultured broth of *Streptomyces* strain SK-1894 [28], and phytanic acid [29] also inhibit fatty acid metabolism at this key enzyme. Recently, a dicarboxylic metabolite of tiadenol has been shown to inhibit ACS *in vitro* in the mitochondrial, microsomal and peroxisomal enriched subcellular fractions of rat liver [30]. This metabolite [bis(carboxymethylthio)-1.10 decane, BCMTD], similar to PFDA, is a peroxisome proliferator in rodents [30]. The fact that many peroxisome proliferators are converted to CoA esters by acyl-CoA synthetase [31], and PFDA, PFOA and BCMTD are inhibitors of ACS, suggests that the interaction of peroxisome proliferating compounds with ACS may play a role in the proliferative response.

In vitro *effects on lipid metabolism compared to* in vivo *effects.* PFDA has been shown to increase the levels of triacylglycerols and cholesteryl esters 7 days following administration to male rats [3]. This large increase in neutral lipids led to the hypothesis that PFDA was causing a diversion of fatty acids from catabolic pathways such as oxidation, towards esterification into acylglycerols. The *in vitro* data

presented here support the premise that PFDA is inhibiting catabolism of long-chain fatty acids. However, *in vitro* PFDA is equally effective in inhibiting the synthesis of acylglycerols. Therefore, the discrepancy between *in vivo* and *in vitro* effects of PFDA on lipid metabolism needs to be addressed. We hypothesize that inhibition of ACS may represent a primary response of hepatocytes to PFDA, followed by several secondary responses, including peroxisome proliferation. In primary rat liver cell cultures, exposure to 0.1 mM PFDA for 72 hr caused an induction of peroxisomal β-oxidation as well as ACS activity (Vanden Heuvel JP and Peterson RE, unpublished results). Similar results have been obtained with the ACS inhibitor BCMTD where ACS activity was induced following *in vivo* administration [30]. The secondary induction of ACS activity may explain the increase in hepatic triacylglycerols in rats following PFDA treatment *in vivo* [3] and may represent an adaptive response to the decrease in fatty acid utilization that occurs immediately following PFDA administration.

Acknowledgements—We acknowledge the excellent technical assistance of Dorothy Nesbit, Becky Lettner and John W. Davis II. Portions of this research were presented at the 1991 Annual Meeting of the Society of Toxicology (*Toxicologist* **11**: 185, 1991). This work was supported by NIH Grant GM41131. Contribution 235, Environmental Toxicology Center, University of Wisconsin, Madison WI, 53706. J. P. Vanden Heuvel was supported by NIEHS Training Grant ESO7015 awarded to the Environmental Toxicology Center, University of Wisconsin, Madison, WI.

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