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Comparison of the effects of selected monocarboxylic, dicarboxylic and perfluorinated fatty acids on peroxisome proliferation in primary cultured rat hepatocytes

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Administration of hypolipidemic agents and phthalate ester plasticizers to rodents increases hepatic peroxisome numbers and peroxisome-associated enzyme activities [1]. High fat diets [2] also cause peroxisome proliferation with an induction of the peroxisomal fatty acid β-oxidation system, although the extent of the induction compared to xenobiotics is low. In contrast, metabolically stable perfluorinated carboxylic acids [3] and non-β-oxidizable sulfur-containing fatty acid analogues [4] are potent peroxisome proliferators *in vivo*. Hepatic peroxisomal responses to xenobiotics may be mediated by interaction with a specific receptor(s) leading to increased synthesis of enzymes of the peroxisomal fatty acid β-oxidation system [1, 5] or through a perturbation in lipid metabolism which initiates a cellular adaptive response leading to a proliferation of peroxisomes [6, 7]. Microsomal cytochrome P450IVA1 which mediates ω- and (ω-1)-hydroxylation of lauric acid (laurate hydroxylase, LH) is co-induced along with peroxisomal fatty acyl-CoA oxidase (FACO) by agents which cause peroxisome proliferation [8–10]. Dicarboxylic acids, products of the microsomal LH system, may be the proximal stimulus for hepatic peroxisome proliferation [6, 7].

To further our understanding of structural requirements for fatty acids in mediating peroxisome proliferation, we have evaluated a series of monocarboxylic, dicarboxylic and metabolically stable perfluorinated fatty acids of a chain length of 4, 8 and 10 carbons (C_4, C_8 and C_{10}, respectively) for their induction of peroxisome-associated enzymes (LH and FACO) in cultured adult rat hepatocytes. Perfluorinated octanol was also examined to determine whether a carboxylic acid function is required for hepatic peroxisome proliferation. Clofibric acid was included as a positive standard for peroxisome proliferation [9, 11]. Structures and abbreviations of the fatty acid analogues used in this study are given in Table 1. A preliminary report of this work has appeared elsewhere [12].

Materials and Methods

Materials. Biochemicals used were: clofibric acid (CPIB), DB, DO and DD (Sigma Chemical Co., St. Louis, MO), OA, DA, PFBA, PFOA, PFDA and PFOL (Aldrich Chemical Co., Milwaukee, WI), BA (Fischer Scientific, Cincinnati, OH), collagenase type IV (Cooper Biochemical, Malvern, PA), [1-^{14}C]lauric acid (58 mCi/mmol) (Amersham, Arlington Heights, IL), Nu-Serum (Collaborative Research Inc., Lexington, MA), Vitrogen (Collagen Corp., Palo Alto, CA) and Williams Medium E (Gibco, Grand Island, NY). Other biochemicals were obtained from the Sigma Chemical Co. Male Sprague–Dawley rats (225–325 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN).

Primary culture of hepatocytes. Hepatocytes were isolated from adult rats by the collagenase perfusion method as

Table 1. Chemical structures, names and abbreviations of fatty acid analogues

Structures	Names (abbreviations)
Monocarboxylic acids	
$CH_3(CH_2)_2COOH$	Butanoic acid (BA)
$CH_3(CH_2)_6COOH$	Octanoic acid (OA)
$CH_3(CH_2)_8COOH$	Decanoic acid (DA)
Dicarboxylic acids	
$HOOC(CH_2)_2COOH$	Butanedioic or succinic acid (DB)
$HOOC(CH_2)_6COOH$	Octanedioic or suberic acid (DO)
$HOOC(CH_2)_8COOH$	Decanedioic or sebacic acid (DD)
Perfluorinated fatty acid analogues	
$CF_3(CF_2)_2COOH$	Perfluorobutanoic acid (PFBA)
$CF_3(CF_2)_6COOH$	Perfluorooctanoic acid (PFOA)
$CF_3(CF_2)_8COOH$	Perfluorodecanoic acid (PFDA)
$CF_3(CF_2)_6CH_2OH$	Perfluorinated octanol (PFOL)

reported previously [11]. Cell viability, determined by trypan blue exclusion, was in the range of 75–95%. Compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium (0.4% DMSO). Aliquots of hepatocyte homogenates were taken for the assay of protein and enzyme activities after 72 hr. The cytotoxicity of the compounds was assessed by measuring lactic acid dehydrogenase (LDH) activities in cell homogenates and medium [11]. Treatment with PFBA (≥1000 μM), PFOA (≥300 μM), PFOL (≥300 μM) and PFDA (≥100 μM) gave percent viabilities that were significantly ($P < 0.05$) less than control, whereas CPIB and mono- and dicarboxylic acids at concentrations up to 1000 μM did not produce any significant differences in cell viabilities. Consistent with the LDH measurement, morphological observations confirmed that high concentrations of PFBA, PFOA, PFOL and PFDA produced disruption of the hepatocyte monolayer and reduced cell number.

Assays. Protein concentrations of cell homogenates were determined by the method of Lowry *et al.* [13]. LDH activities were determined by following the pyruvate-dependent oxidation of NADH at 340 nm. FACO and LH activities were measured by previously reported methods [14, 15]. Statistical analysis of the data was performed using Dunnett's test.

Results and Discussion

PFBA, PFOA, PFDA and CPIB but not PFOL produced concentration-dependent increases in peroxisomal FACO and microsomal LH activities. Whereas PFOA (100 μM) induced a maximal FACO induction response similar to CPIB (1000 μM), responses to PFBA (300 μM) and PFDA (60 μM) were only 46.2 and 33.5%, respectively, when compared to CPIB (Table 2). The inductive responses to PFBA, PFOA and PFDA decreased markedly at higher concentrations, and the effects were correlated to a reduction in cell viability. PFBA, PFOA and PFDA produced concentration-dependent increases in LH activity giving maximal elevations at 1000, 30 and 30 μM which were 90.4, 60.5 and 40.8%, respectively, of that exhibited

Table 2. Relative potencies and maximal effects (E_{max}) for induction of fatty acyl-CoA oxidase (FACO) and laurate hydroxylase (LH) in primary cultured hepatocytes by CPIB and perfluorinated fatty acid analogues

	FACO activity			LH activity		
Compound	Concentration*	Potency†	E_{max}‡	Concentration*	Potency†	E_{max}§
CPIB	111.1 ± 5.1	1.00	9.1 ± 1.0‖	112.8 ± 6.8	1.00	17.4 ± 1.3‖
PFBA	83.0 ± 11.0	1.34	4.7 ± 0.8‖	83.5 ± 31.0	1.35	15.7 ± 2.9‖
PFOA	9.2 ± 2.7	12.07	9.4 ± 0.7‖	3.2 ± 1.4	35.25	10.5 ± 0.8‖
PFDA	21.4 ± 10.9	5.19	3.4 ± 0.2‖	7.6 ± 1.9	14.84	7.1 ± 2.7‖
PFOL	—¶	—¶	1.6 ± 0.4**	—¶	—¶	2.0 ± 0.8**

* Cells were cultured for 72 hr with various concentrations of test compounds, and the concentration (μM) of each compound required for a 2.5-fold induction of each enzyme activity was determined (mean ± SEM, N = 2–3 separate experiments, with three replicates per concentration).

† Relative potencies were calculated as the ratio of concentration of clofibric acid (μM) to concentrations of test compound (μM).

‡ E_{max} values for FACO (nmol H_2O_2/min/mg protein) were determined using 1000, 300, 100 and 60 μM concentrations of CPIB, PFBA, PFOA and PFDA, respectively (control = 1.2 ± 0.2 nmol H_2O_2/min/mg protein; mean ± SEM of N = 7).

§ E_{max} values for LH (nmol/hr/mg protein) were determined using 1000, 1000, 30 and 30 μM concentrations of CPIB, PFBA, PFOA and PFDA, respectively (control = 2.8 ± 0.6 nmol/hr/mg protein; mean ± SEM of N = 7).

‖ E_{max} values were significantly different ($P < 0.05$) from the corresponding control value.

¶ PFOL was not active in the concentration range of 10–1000 μM.

** E_{max} value of PFOL (at 300 μM) was not significantly different ($P > 0.05$) from the corresponding control value.

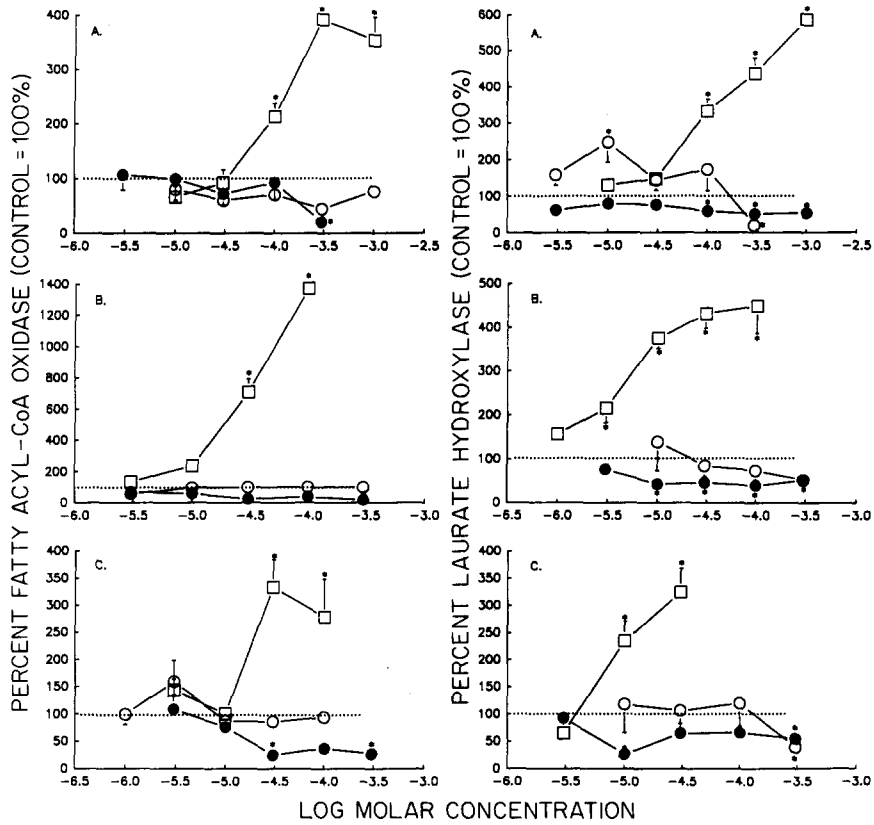

Fig. 1. Comparison of the effects of monocarboxylic acids (○), dicarboxylic acids (●), and perfluorinated fatty acids (□) of the same chain length, C_4 (panel A), C_8 (panel B) and C_{10} (panel C), on the induction of FACO (left) and LH (right). Cultured hepatocytes were incubated for 72 hr in the presence of compound. Activities are expressed as a percent of the control (. . . .). The control activities for FACO and LH were 0.79 ± 0.10 nmol H_2O_2/min/mg protein and 2.35 ± 0.34 nmol/hr/mg protein, respectively. Each point is the mean ± SEM of determinations from 3 to 10 dishes of cells. An asterisk (*) indicates that the value is significantly different ($P < 0.05$) from the control.

by 1000 μM CPIB. The concentrations that produced 2.5-fold inductions and the relative potency for inductions of FACO and LH by these perfluorinated fatty acids are shown in Table 2. The rank order of potency of the compounds for the inductions of FACO and LH was the same, i.e. PFOA > PFDA > PFBA ≥ CPIB.

Perfluorinated fatty acids elevated FACO and LH activities, whereas their corresponding mono- and dicarboxylic acids did not (Fig. 1). Maximum inductions of FACO and LH activities by PFOA, PFDA and PFBA, compared to the normalized control (as 100%), were 1372, 333, and 390% and 450, 324, and 584%, respectively. Enzyme activities in the presence of the monocarboxylic acids (BA, OA, and DA) at various concentrations (30–1000 μM) were not significantly different from control values. Similarly, the corresponding dicarboxylic acids (DB, DO and DD) did not induce FACO or LH activities. Moreover, the dicarboxylic acids appeared to inhibit these enzyme activities, although there were no signs of cytotoxicity shown at the concentrations used. More information is needed to evaluate the mechanism of the inhibitory effects of these dicarboxylic acids.

Our experiments show that perfluorinated fatty acids were equally or more potent than CPIB as peroxisomal proliferators in cultured rat hepatocytes. Using hepatic catalase activity as a marker of *in vivo* peroxisome proliferation, Ikeda *et al.* [3] observed the same pattern of rank order of potency as our studies (PFOA > PFDA > PFBA). As reported for other peroxisome proliferators [8–10], the perfluorinated fatty acids also produced a close concentration-dependent relationship for inductions of peroxisomal FACO and the microsomal cytochrome P450IVA1 enzyme, LH.

Ikeda *et al.* [3] found that a single injection of PFOL to rats causes peroxisome proliferation and suggested that PFOL is metabolized to an active compound, PFOA. In cultured hepatocytes, we found that PFOA but not PFOL was an inducer of FACO and LH activities. Thus, either the oxidation of PFOL occurs to a significant degree only *in vivo* or PFOL may act through an indirect mechanism independent of the liver (e.g. hormone-mediated action). However, our studies strongly suggest that a carboxylic group(s) is an important structural requirement of perfluorinated fatty acid-mediated peroxisome proliferation. The carboxylic acid group of fatty acids may be converted to an acyl-CoA thioester which either directly or indirectly perturbs lipid metabolism leading to peroxisome proliferation [16].

Lock *et al.* [6] reported that long-chain dicarboxylic acids (C_{14}–C_{18}) induce peroxisomal β-oxidation, but not microsomal ω-oxidation of fatty acids in cultured rat hepatocytes. Others [2, 4] have reported that dodecanedioic acid and hexadecanedioic acid possess only marginal effects on peroxisomal activities *in vivo*. However, we found that short- and medium-chain dicarboxylic acids (C_4–C_{10}) did not induce either FACO or LH activities. Mitochondria oxidize short- and medium-chain dicarboxylic acids [17] which may explain the inability of C_4, C_8 and C_{10} dicarboxylic acids to cause peroxisome proliferation in hepatocytes.

Several studies have shown that fatty acids, including those present in marine oil [2], *trans* fatty acids [18] and non-β-oxidizable fatty acids [4, 19], which are poorly oxidized by the mitochondrial β-oxidation system, are inducers of hepatic peroxisomal β-oxidation. Our results also reveal that only non-oxidizable perfluorinated fatty acids induce peroxisome-associated enzyme activities whereas mono- and dicarboxylic acids do not. Thus, the ability of perfluorinated fatty acids to resist mitochondrial β-oxidation may be an important factor which regulates hepatic peroxisome proliferation [4, 20].

In conclusion, the results of this study have demonstrated that only metabolically stable perfluorinated analogues of fatty acids cause hepatic peroxisome proliferation as indicated by the increases in activities of peroxisomal FACO and microsomal LH. Whereas inductions of FACO and LH by the perfluorinated fatty acids (PFOA, PFDA, PFBA) were equally or more potent than those by CPIB, corresponding mono- and dicarboxylic acids were unable to induce peroxisome-associated enzyme activities in primary cultures of rat hepatocytes. PFOL, which lacks a carboxylic group, did not induce peroxisome proliferation in cultured hepatocytes. Thus, our data confirm that important requirements for fatty acids as inducers of peroxisome proliferation include the presence of a carboxylic function linked to a hydrophobic backbone and an ability to resist mitochondrial fatty acid β-oxidation.

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Role of glutathione *S*-transferases in heme transport

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Newly synthesized heme must cross the inner and outer membranes of mitochondria and then diffuse through cytoplasm preceding incorporation into apocytochromes within the endoplasmic reticulum. Senjo *et al.* [1] reported that the reconstitution of apo-b_5* by mitochondrial heme is increased in the presence of GST Yb_2Yb_2 (see [2] for nomenclature). The latter result suggests a dual role for the GSTs, i.e. enhancing efflux of heme from mitochondria and then shuttling it to the apocytochromes. To define whether these transport functions are limited to GST Yb_2Yb_2, we examined the effect of homogenous GSTs on mitochondrial heme efflux and subsequent reconstitution of apo-b_5.

Methods

Materials. All fine chemicals were purchased from Sigma. Heme was prepared fresh daily.

Preparation of rat liver mitochondria and cytosol. Rat livers were perfused *in situ* with ice-cold 0.25 M sucrose and then excised, minced and resuspended in 5 vol. of a 0.25 M sucrose, 10 mM Hepes (pH 7.0) buffer for preparation of cytosol [3] and mitochondria. The mitochondria were prepared according to standard techniques and were washed five times with an EDTA-containing buffer [1]. Mitochondrial coupling was determined on each preparation with succinate, and only preparations with a respiration control ratio of >4.0 were used.

Enzymatic and protein assays and preparation of enzymes. GST activity was determined spectrophotometrically [4]. Protein was determined as described previously with bovine serum albumin as the standard [5]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Maizel [6]. The various GST isozymes were purified using a GSH affinity column and chromatofocusing [3]. GSTs also were purified according to Senjo *et al.* [1].

Preparation of apocytochrome b_5. Detergent-solubilized holocytochrome b_5 was prepared from rabbit liver microsomes [7]. Apo-b_5 was prepared by acid/acetone extraction of the holocytochrome [7, 8].

Measurement of heme transfer. Our experimental approach was similar to that of Senjo and colleagues [1] in which the reconstitution of apo-b_5 is used to quantitate the movement of heme from mitochondria into the aqueous phase. Apo-b_5 (0.02 to 0.04 mg) was mixed in 1.0 mL of buffer (10 mM Tris, pH 7.4) with the putative transfer protein and/or mitochondria (2 to 3.5 mg). The mixture was incubated for 10 min at 37°. The mitochondria were removed by centrifugation in a microfuge, and 0.5 mL of the supernatant was used for measurement in a spectrophotometer of the difference spectra (424–409 nm) of the dithionite reduced minus oxidized cytochrome b_5 [1]. Heme transfer activity is defined as nanomoles of cytochrome b_5 reconstituted per milligram of transfer protein. In experiments without mitochondria, the reconstitution of the apocytochrome also was measured at 410 nm and the concentration of the holocytochrome calculated using an absorption coefficient of 117 mM^{-1} cm^{-1} [9].

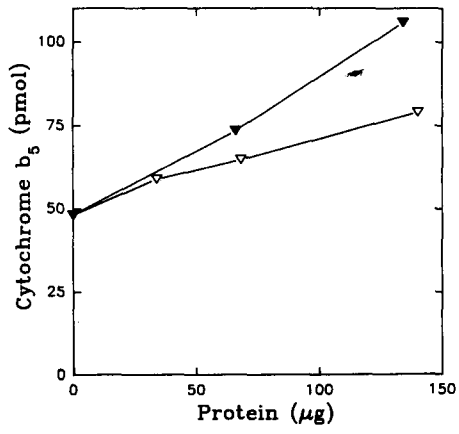

Fig. 1. Reconstitution of apocytochrome b_5 with heme in the presence of GSTs. The transfer proteins [mixture of purified GST (▽) or GST Yb_2Yb_2 (▼) were mixed with 100 pmol heme. Apocytochrome b_5 (1 nmol) was added and the mixture was incubated at 37° for 10 min. Cytochrome b_5 content was measured at 410 nm as described in Methods. Each point is the average of two determinations.

* Abbreviations: apo-b_5, apocytochrome b_5; and GST, glutathione *S*-transferase.