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EFFECTS OF DIETARY TREATMENT WITH 11 DICARBOXYLIC ACIDS, DIETHYLCARBOXYLIC ESTERS AND FATTY ACIDS ON PEROXISOMAL FATTY ACID β-OXIDATION, EPOXIDE HYDROLASES AND LAURIC ACID ω-HYDROXYLATION IN MOUSE LIVER

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Abstract—C57B1/6 male mice were exposed through their diet to 11 dicarboxylic acids, carboxylic acids and diethyldicarboxylesters for 10 days. For the diacids and diethylesters this treatment resulted in a chain length-dependent induction of lauryl-CoA oxidase and cyanide-insensitive palmitoyl-CoA oxidation activities. A chain length of 12 carbon atoms or more seemed to be necessary for induction of these two activities. In addition, the same chain length dependence was observed for induction of lauric acid ω + ω−1 hydroxylase activity and increase in the protein content of the mitochondrial fraction. Treatment with two "natural" fatty acids, i.e. lauric and palmitic acid gave no effect at all on these various parameters. In no case was induction of cytosolic and mitochondrial epoxide hydrolase activities observed. Instead, a slight decrease in these activities was observed after administration of diacids with a chain length of 4–8 carbon atoms, whereas microsomal epoxide hydrolase activity was concurrently induced.

During the past decade it has become clear that exposure to a large number of structurally different xenobiotics can increase the number and size of peroxisomes in the liver of rodents [1, 2]. Substances presently known to cause peroxisome proliferation include industrial chemicals such as phthalates and trichloroacetic acid, perfluoro fatty acids and di(2-ethylhexyl)phosphate, as well as various medical drugs, e.g. acetylsalicylic acid and hypolipidemic drugs such as clofibrate (for a review see Refs 3–5).

Exposure to such xenobiotics causes not only an increase in the number and volume of peroxisomes, but also an enlargment of the liver, a decrease in mitochondrial size and a dramatic increase in the peroxisomal capacity for fatty acid oxidation in rat and mouse (for a review see Ref. 6). These substances can also cause liver cancer in rodents after chronic administration, although the genotoxic mechanism involved is still unclear [7].

Of relevance to this question is the observation that the fatty acid β-oxidation system in mammalian peroxisomes, which Lazarow and DeDuve [8] were the first to demonstrate and which differs from the mitochondrial β-oxidation system in several ways, is induced many-fold after treatment with peroxisome proliferators [8]. Since peroxisomal β-oxidation involves production of hydrogen peroxide and the level of catalase is relatively unchanged [9], peroxisome proliferation is thought to lead to an increase in reactive oxygen species and, therefore, an increased intracellular oxidative stress [10, 11].

Exposure to peroxisome proliferators also affects several xenobiotic-metabolizing enzymes in rodent liver. Microsomal cytochrome P450 IVA1 (P452), which demonstrates a high ω + ω − 1-hydroxylation activity with lauric acid, is induced several-fold [12]. Cytosolic and microsomal epoxide hydrolases are also induced [13]. In contrast, the activity of glutathione transferase (measured with 1-chloro-2,4-dinitrobenzene as the second substrate) is often decreased [14, 15].

In several studies we have investigated the structural requirements for peroxisome proliferation and for effects on mitochondria in mouse liver [16, 17]. In the present study we have tested the ability of several different dicaboxylic acids and dicarboxylic ethyl esters to induce peroxisomal fatty acid β-oxidation, in order to investigate the hypothesis that dicarboxylic acids are the direct signal for peroxisome proliferation.

MATERIALS AND METHODS

Chemicals

Succinic acid, suberic acid, 1,10-decanedicarboxylic acid, hexadecanedioccarboxylic acid, docosanedioccarboxylic acid (Aldrich-Chimie, Steinheim, Germany), lauric acid, NAD^+, palmitic acid, diethylsuccinate, diethylsuberate, diethyltetradecanedioate and diethyldecanedioate (E.

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Merck, Darmstadt, Germany), *S*-acetyl-CoA, palmitoyl-CoA, lauroyl-CoA, cytochrome *c* (Sigma Chemical Co., St Louis, MO, U.S.A.), *trans*-stilbene oxide (EGA-Chemie, Albruch, Germany) and horseradish peroxidase (Boehringer-Mannheim, Bromma, Sweden) were all purchased from the sources indicated.

trans- and *cis*-[8-^{3}H]Stilbene oxide were synthesized by Dr Åke Pilotti and Winnie Birberg of the Department of Organic Chemistry, University of Stockholm, according to the method of Gill *et al.* [18] and purified by TLC using *n*-hexane:ethyl acetate (95:5 v/v) with two developments. The bands containing pure *trans*- and *cis*-stilbene oxide were removed from the plate and eluted twice with 2 mL ethanol, followed by filtration through glass-wool. The final purities were estimated to be 99.9%. Unlabelled *cis*-stilbene oxide was synthesized by oxidation of *cis*-stilbene with *m*-chloroperoxybenzoic acid and purified in the same manner before use.

All lipids and dicarboxylic acids used were ≥98% pure. All other chemicals were of analytical grade and obtained from common commercial sources.

Animals and treatment

Male C57B1/6 mice (ALAB, Sollentuna, Sweden) weighing 20–25 g and approximately 8 weeks old were used throughout this investigation. The animals were housed in plastic cages with steel bottoms in groups of three with a 12 hr light–dark cycle at 25°. They were given free access to commercial powdered food R3 containing 5% fat, 24% protein and 49% carbohydrate (Astra Ewos AB, Södertälje, Sweden) before commencement of the experiment. All animals were acclimatized to these conditions for at least 2 weeks before use.

The experimental diets were prepared by grinding the solid substances to a fine powder and mixing them thoroughly with powdered R3 food to give a dose of 10% (w/w), except in the case of diethylsuberate and palmitic acid, where a 5% (w/w) dose was used in order to avoid acute toxicity. Liquid substances were dissolved in acetone (pa) and this solution poured over the powdered chow, which was subsequently mixed and dried in a ventilated hood [13]. These diets were stored at −20° prior to use and mice were fed them for 10 days. Both control and exposed mice consumed about 3 g food per day.

Preparation of subcellular fractions

At the end of the treatment period, the animals were killed by cervical dislocation. The livers were dissected out, the gallbladders removed and the tissue placed in ice-cold 0.25 M sucrose and weighed.

The liver was subsequently homogenized in 2 vol. ice-cold 0.25 M sucrose using four up-and-down strokes of a Potter–Elvehjem homogenizer at 440 rpm. After dilution to 1 g liver/5 mL suspension, this homogenate was centrifuged at 600 g_{av} for 10 min and the resulting supernatant was then centrifuged at 10,000 g_{av} for 10 min. This second pellet, the mitochondrial pellet, was resuspended, washed twice in sucrose by centrifugation, and finally resuspended in 0.25 M sucrose to give a final volume of 2.0 mL.

The 10,000 g_{av} supernatant was centrifuged at 133,000 g_{av} for 60 min and the resulting microsomal pellet washed once in 0.15 M Tris–Cl, pH 8.0, and thereafter resuspended in 0.25 M sucrose to give a final volume of 2.0 mL. The volume of the 133,000 g_{av} supernatant fraction was also measured (about 3 mL).

The subfractions obtained by this procedure have been thoroughly characterized in an earlier report [19].

Enzyme assays

All enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein, as determined using fractions from both control and induced animals. Cytosolic and microsomal epoxide hydrolase activities and microsomal cytochrome P450 content were assayed using freshly prepared subfractions. Other measurements were performed with subfractions flushed with

Table 1. Effects of the different treatments on liver weight, liver somatic index and protein content of the hepatic mitochondrial fraction

Dietary additive	Liver weight (g)	Liver weight/body weight	Mitochondrial protein (mg/mL)
None (control; N = 16)	1.18 ± 0.19	0.052 ± 0.009	4.09 ± 1.18
Dicarboxylic acids (chain length)			
Succinic acid (4)	0.82 ± 0.30	0.048 ± 0.012	3.45 ± 0.23
Suberic acid (8)	0.72 ± 0.18*	0.043 ± 0.007*	3.18 ± 0.14
Decandioc acid (12)	1.35 ± 0.30	0.052 ± 0.003	7.54 ± 0.81†
Hexadecandioc acid (16)	1.22 ± 0.28	0.052 ± 0.004	5.76 ± 0.91
Docosandioc acid (22)	1.12 ± 0.11	0.060 ± 0.007	4.02 ± 0.49
Fatty acids (chain length)			
Lauric acid (12)	0.81 ± 0.17*	0.045 ± 0.006	4.18 ± 0.77
Palmitic acid (16)	1.26 ± 0.16	0.060 ± 0.005	3.80 ± 0.41
Diethylesters of dicarboxylic acids			
Diethylsuccinate	1.20 ± 0.14	0.052 ± 0.007	5.65 ± 2.41
Diethylsuberate	1.07 ± 0.03	0.061 ± 0.003	5.40 ± 0.17
Diethyldecanedioate	1.15 ± 0.01	0.050 ± 0.001	4.97 ± 0.68
Diethyltetradecanedioate	1.33 ± 0.16	0.058 ± 0.004	8.18 ± 1.64*

* $P < 0.05$, † $P < 0.01$ compared to the control values in the same experiment.

nitrogen and stored at −80°, a procedure which did not affect the other enzyme activities investigated here.

Cytosolic and mitochondrial epoxide hydrolase. Activities were measured by a published radiometric procedure [18] with 50 μM [^{3}H]*trans*-stilbene oxide as substrate in 0.1 M potassium phosphate, pH 7.0. Essentially the same procedure was used to assay *microsomal epoxide hydrolase*, except that in this case the substrate was *cis*-stilbene oxide and the medium 0.1 M sodium glycine, pH 9.0. In both cases product and remaining substrate were separated by an extraction procedure prior to scintillation counting.

Cytochrome P450. Cytochrome P450 content was quantified in the microsomal fraction according to Omura and Sato [20] from the difference spectrum between the reduced cytochrome and its carbon monooxide complex.

Catalase. Catalase was assayed in the mitochondrial fraction by monitoring the disappearance of hydrogen peroxide at 240 nm [21].

Palmitoyl-CoA oxidation. Palmitoyl-CoA oxidation by the mitochondrial fraction was measured as the reduction of NAD^+ at 340 nm in the presence of KCN as an inhibitor of mitochondrial β-oxidation [8, 22]. *Lauroyl-CoA oxidase activity* was monitored in this same fraction by assaying hydrogen peroxide production with a fluorimetric procedure based on the peroxidase-coupled oxidation of 4-hydroxyphenylacetic acid [23].

Lauric acid hydroxylation. Lauric acid hydroxylation was measured using [^{14}C]lauric acid essentially as described by Parker and Orton [24]. The incubation medium was applied to Merck silica gel TLC plates equipped with a pre-absorbing zone and the plate was developed and then scanned and the radioactivity quantified using a Rita radioactivity TLC scanner. The results presented are the combined ω and ω−1 hydroxylations of lauric acid, since the TLC method used does not separate these two metabolites.

Protein. Protein was determined according to Lowry *et al.* [25] with bovine serum albumin as standard.

SDS–PAGE. SDS–PAGE was performed according to Laemmli [26]. Phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000) ovalbumin (M_r 43,000), carboanhydrase (M_r 30,000) and soybean trypsin inhibitor (M_r 20,100) (Pharmacia, Uppsala, Sweden) were employed as standard molecular weight markers.

Statistical analysis. All experimental groups contained three or four animals. Data are given as means ± SD and, where appropriate, statistical significance has been tested for using Student's *t*-test.

RESULTS

Effect on peroxisomes

Table 1 shows the effects of the substances tested on liver weight, liver somatic index (liver weight/body weight) and protein content in the mitochondrial fraction. No significant increases in liver weight were observed, in contrast to the effects of virtually all known potent peroxisome proliferators. Indeed, two substances, i.e. suberic acid and palmitic acid, caused a slight, but significant ($P < 0.05$) decrease in this parameter. However, the protein content of the mitochondrial fraction was significantly increased (about 2-fold) by decandioc acid and diethyltetradecanedioate. No changes were observed in the protein contents of the microsomal or cytosolic fractions (data not shown).

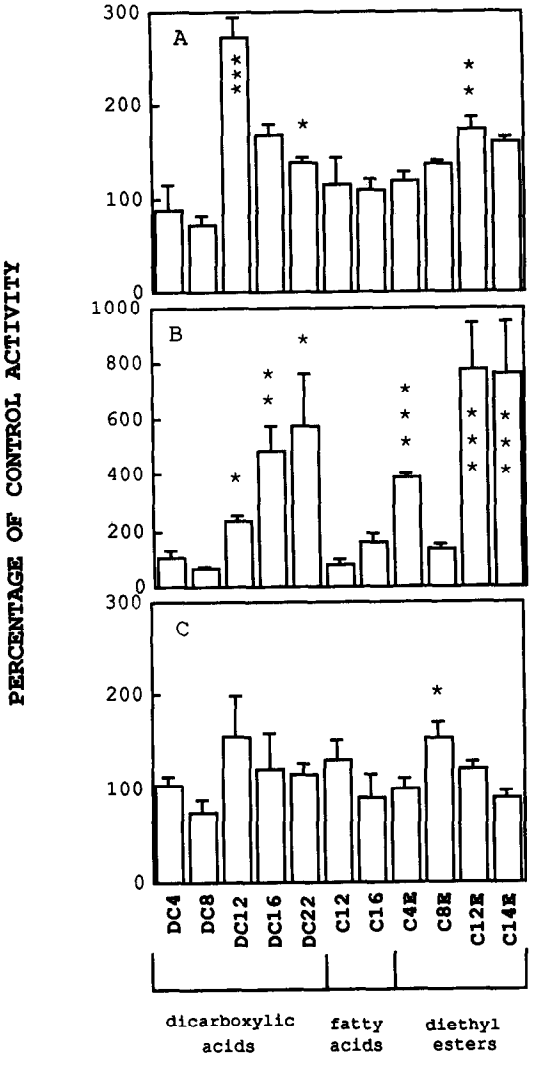

Fig. 1. Hepatic levels of three peroxisomal activities—(A) cyanide-insensitive palmitoyl-CoA oxidation. (B) lauroyl-CoA oxidase and (C) catalase—after dietary treatment of mice with dicarboxylic acids and related substances. Abbreviations: succinic acid (DC4), suberic acid (DC8), decandioc acid (DC12), hexadecandioc acid (DC22), lauric acid (C12), palmitic acid (C16), diethylsuccinate (C4E), diethylsuberate (C8E), diethyldecandioate (C12E) and diethyltetradecabdioate (C14E). Control values were as follows: palmitoyl-CoA oxidation = 18.6 ± 4.31 nmol/min mg mitochondrial protein; lauryl-CoA oxidase = 12.5 ± 4.89 nmol/min mg mitochondrial protein; and catalase = 2110 ± 378 nmol/min mg mitochondrial protein. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control values in the same experiment.

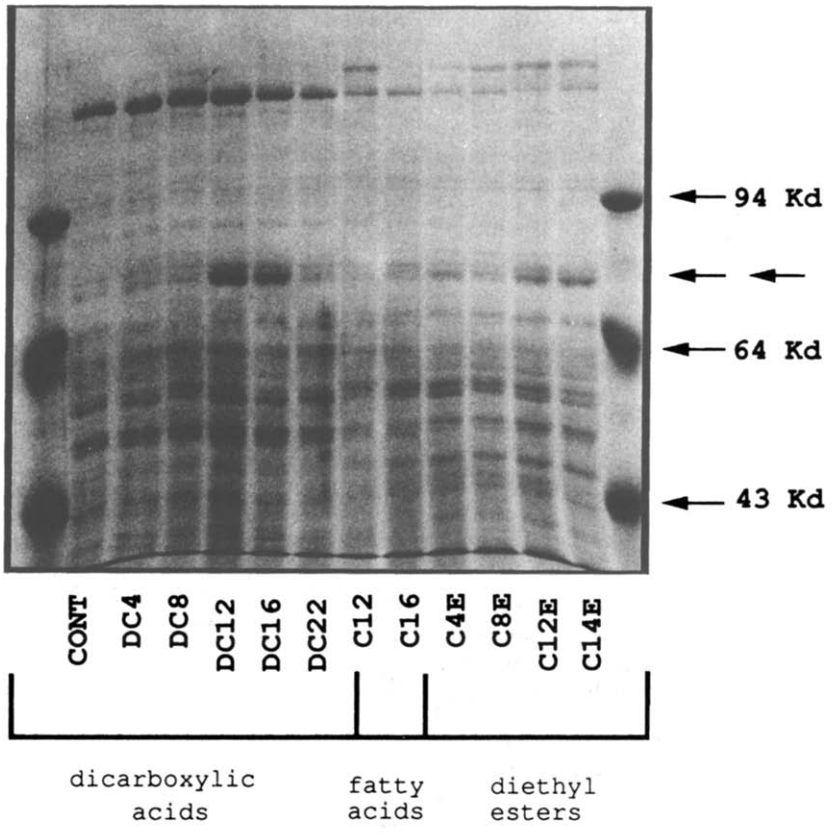

Fig. 2. SDS–PAGE of the liver mitochondrial fraction from control and treated animals. Electrophoresis was performed as described in Material and Methods using a 7.5% separating gel and 10 μg mitochondrial protein per slot. CONT = control. For the other abbreviations used, see the legend to Fig. 1. The double arrow indicates the position of PPA-80.

Table 2. Hepatic activities of cytosolic, mitochondrial and microsomal epoxide hydrolases after dietary treatment of mice with dicarboxylic acids and related substances

Dietary additive	Epoxide hydrolase activity		
	Cytosolic	Mitochondrial (% of control)	Microsomal
Dicarboxylic acids (chain length)			
Succinic acid (4)	33 ± 15†	67 ± 15	252 ± 25†
Suberic acid (8)	42 ± 14†	63 ± 10*	160 ± 33*
Decandioc acid (12)	78 ± 20	55 ± 50*	242 ± 13‡
Hexandecandioc acid (16)	83 ± 50	87 ± 25	117 ± 44
Docosandioc acid (22)	73 ± 23	147 ± 20	111 ± 70
Fatty acids (chain length)			
Lauric acid (12)	47 ± 20†	85 ± 90	104 ± 18
Palmitic acid (16)	115 ± 11	109 ± 13	122 ± 18
Diethylesters of dicarboxylic acids			
Diethylsuccinate	132 ± 12	103 ± 15	268 ± 61*
Diethylsuberate	73 ± 15	155 ± 18	141 ± 21
Diethyldecanedioate	105 ± 60	156 ± 50	151 ± 37
Diethyltetradecanedioate	84 ± 40	119 ± 15	75 ± 28

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ compared to control values from the same experiment. Control values for cytosolic, mitochondrial and microsomal activities were 5.69 ± 1.46, 3.84 ± 0.79 and 2.06 ± 0.34 nmol product formed/min mg protein, respectively. Each experimental group contained three or four animals.

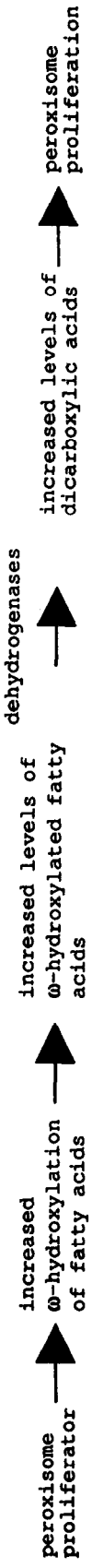

Fig. 3. Hypothesis for the mechanism of peroxisome proliferation.

Figure 1 illustrates the effects of the dietary treatments on three peroxisomal activities, i.e. cyanide-insensitive palmitoyl-CoA oxidation, lauroyl-CoA oxidase activity and catalase. In the case of total peroxisomal capacity for β-oxidation, as judged by palmitoyl-CoA oxidation (Fig. 1A), there were only three compounds which caused a significant induction ($P > 0.05$), i.e. decandioc acid, docosandioc acid and diethyldecandioate. The increase observed for these substances was about 1.5–3-fold.

Figure 1B shows peroxisomal oxidase activity measured with lauroyl-CoA as substrate. For this enzyme, which is the first enzyme in the peroxisomal β-oxidation pathway, several substances were found to be effective inducers. The most significant increases in activity were found with the longer diacids and diethyldiesters. The corresponding fatty acids caused no or smaller changes, i.e. about 1.5-fold compared to 4–6-fold increases with the diacids and diethylesters.

Figure 1C documents the catalase activity in the mitochondrial fraction after dietary treatment. This activity was not significantly ($P < 0.01$) affected by any of the substances employed.

SDS–PAGE

Figure 2 illustrates the results of SDS–PAGE of the mitochondrial fractions. An increased level of a protein located in the 80,000 Da region could be observed with several of the substances administered, indicating an induction of the peroxisomal so-called PPA-80 protein [27]. This band is increased visibly with the diacids decandioc acid and hexadecandioc acid and the corresponding diesters, i.e. diethyldecanedioate and diethyltetradecanedioate. The fatty acids used, i.e. lauric acid and palmitic acid caused no visible increase in this protein.

Effects on xenobiotic-metabolizing enzymes

Table 2 illustrates the effects of dicarboxylic acids, dicarboxylic diethylesters and fatty acids on cytosolic, mitochondrial and microsomal epoxide hydrolase activities. For cytosolic or mitochondrial epoxide hydrolase no significant induction was observed with the dietary additions used. Indeed, there was a slight, but significant depression of these activities with several of the acids administered. Microsomal epoxide hydrolase was induced about two-fold with certain diacids, as well as the corresponding diesters, i.e. those with a chain-length of four to eight carbon atoms.

In Table 3, the effects of the dietary treatments on microsomal cytochrome P450 content and cytochrome P452-catalysed $\omega + \omega-1$-hydroxylation are documented. Total microsomal cytochrome P450 content showed no increases. However, suberic acid caused a slight, but noticeable decrease in the content of this enzyme. The $\omega + \omega-1$-hydroxylation activity showed only a slight, but significant increase of about 2-fold with several of the acids tested.

DISCUSSION

The goal of this investigation was to test the hypothesis that dicarboxylic acids could act as the

immediate proliferators of peroxisomes (see Fig. 3). Since the proliferation of peroxisomes is invariably accompanied by the induction of ω-hydroxylation [28–30], it is possible that dicarboxylic acids could be formed from ω-hydroxylation of fatty acids and further oxidation catalysed by alcohol and aldehyde dehydrogenase activities [31]. Such dicarboxylic acids can then form acyl-coenzyme A esters, which can be used as substrate by the peroxisomal oxidase [32] and are, indeed, preferentially β-oxidized in peroxisomes [33].

In agreement with this hypothesis no induction of microsomal cytochrome P450 content was observed here. Furthermore, the induction of cytochrome P452-catalysed ω-hydroxylation activity by some of the substances employed here is very modest compared to that observed with other peroxisome proliferators [4, 34].

Dietary treatment with the compounds employed caused little or no effects on several parameters which are usually affected during peroxisome proliferation, e.g. increase in liver weight and protein content of the mitochondrial protein fraction. However, it is clear that the diacids with 12 carbon atoms and corresponding esters cause other changes normally associated with such proliferation (as judged from increases in peroxisomal fatty acid β-oxidation activities and SDS–PAGE patterns). On the other hand, the changes in peroxisomal enzymes observed here are considerably smaller than those caused by the plasticizer di(2-ethylhexyl)phthalate or the hypolipidemic drug clofibrate. It must, of course, be remembered that the occurrence of peroxisome proliferation has not been confirmed using electron microscopy in the present study (since the low potency of the effects observed would necessitate a very large morphometric study to definitely demonstrate changes in the size and number of hepatic peroxisomes). Nonetheless, the changes observed here may reflect a more "natural" cellular response than that observed with the more potent peroxisome proliferators.

The lack of effect of the administered fatty acids on these same parameters clearly indicates that the corresponding diacids behave differently metabolically and, furthermore, that the observed enzymatic changes cannot be a consequence of an increased caloric content of the diet. Lauric and palmitic acids were also found to cause acute toxic responses when administered to mice at a dose of 10% in their diet (w/w), which may explain the decrease in liver weight observed with these substrates.

Peroxisomal lauryl-CoA oxidase activity was increased by several of the substances employed, e.g. decanedioc acid, hexadecanedioc acid, docosanedioc acid, diethyldecanedioate and diethyltetradecanedioate. These increases did not correlate with the changes in peroxisomal palmitoyl-CoA oxidation, a correlation which is usually observed [35]. This finding suggests independent regulation and a possible imbalance in the components of peroxisomal β-oxidation. If this is indeed the case, a minor increase in peroxisomal β-oxidation capacity (as judged by cyanide-insensitive palmitoyl-CoA oxidation) could be accompanied by a much larger induction in the first oxidase step. Thus, even a small peroxisome proliferation might result in elevated hydrogen peroxide levels which could be dangerous to the cell. However, it may be that the level of catalase is sufficient to reduce even the increased amounts of hydrogen peroxide which might be formed by the induced oxidase. This question demands more attention in the future.

In a recent investigation of partially purified rat liver peroxisomal oxidase, the existence of at least three different peroxisomal fatty-acyl CoA oxidases was demonstrated [36]. These three forms differ in

Table 3. Hepatic microsmal cytochrome P450 content and lauric acid ω + ω−1-hydroxylase activity after dietary treatment of mice with dicarboxylic acids and related substances

Dietary additive	Cytochrome P450 content	Lauric acid ω-hydroxylase activity
	(% of control activity)	
Dicarboxylic acids (chain length)		
Succinic acid (4)	81 ± 14	120 ± 32
Suberic acid (8)	68 ± 15*	103 ± 14
Decandioc acid (12)	82 ± 18	366 ± 24†
Hexadecandioc acid (16)	85 ± 20	278 ± 22*
Docosandioc acid (22)	60 ± 18*	148 ± 31
Fatty acids (chain length)		
Lauric acid (12)	121 ± 30	178 ± 41*
Palmitic acid (16)	119 ± 25	179 ± 70†
Diethylesters of dicarboxylic acids		
Diethylsuccinate	125 ± 28	212 ± 14†
Diethylsuberate	102 ± 13	138 ± 58
Diethyldecanedioate	119 ± 25	156 ± 16*
Diethyltetradecanedioate	94 ± 6	110 ± 10

* $P < 0.05$, † $P < 0.001$ compared to the control values for the same experiment.

The control value for cytochrome P450 content was 0.620 ± 0.050 nmol/mg microsomal protein. The control value for lauric acid ω + ω−1-hydroxylation activity was 0.88 ± 0.09 nmol/min mg microsomal protein.

Each group contained three or four animals.

their inducibility by the hypolipidemic drug clofibrate, i.e. they include an inducible fatty acyl-CoA oxidase, a non-inducible fatty-acyl CoA oxidase and a non-inducible trihydroxycoprostanoyl-CoA oxidase. It will be of great interest to determine the possible differential effects of other peroxisomal proliferators on these different oxidases.

The lack of induction of cytosolic epoxide hydrolase here is unusual, since in earlier investigations we have shown that the proliferation of peroxisomes and the induction of this enzyme seem to be coupled [13]. This has led us to believe that this enzyme is at least partially localized in peroxisomes. One possible explanation for the lack of correlation observed here is that the induction of peroxisomes caused by the substances used is not large enough to also increase the hydrolase which is usually induced only 2-fold.

In conclusion, it seems likely from our findings that longer diacids and corresponding diesters can act as peroxisome proliferators. However, the low degree of induction of peroxisomal fatty acid β-oxidation and lauryl-CoA oxidase obtained here compared to other peroxisome proliferators such as di(2-ethylhexyl)phthalate or clofibrate argues against the hypothesis that dicarboxylic acids are involved in the biological effects of these other substances.

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