Biochemical Pharmacology, Vol. 43, No. 12, pp. 2621–2629, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

MICROSOMAL LAURIC ACID HYDROXYLASE ACTIVITIES AFTER TREATMENT OF RATS WITH THREE CLASSICAL CYTOCHROME P450 INDUCERS AND PEROXISOME PROLIFERATING COMPOUNDS

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(*Received* 11 *September* 1991; *accepted* 9 *March* 1992)

Abstract—In order to investigate a proposed relationship between induction of hepatic microsomal lauric acid hydroxylase activity and peroxisome proliferation in the liver, male Wistar rats were treated with peroxisome proliferating compounds, and the lauric acid hydroxylase activity, the immunochemical detectable levels of cytochrome P450 4A1 and the activities of peroxisomal enzymes were determined. In addition, the levels of cytochrome P450 4A1 and lauric acid hydroxylase activities were studied after treatment of rats with three cytochrome P450 inducers. After treatment with aroclor-1254, phenobarbital or 3-methylcholanthrene total cytochrome P450 was 1.7–2.7 times induced. However, no induction of lauric acid ω-hydroxylase activities or P450 4A1 levels were found. After treatment of rats with di(2-ethylhexyl)phthalate (DEHP) a dose-dependent induction of lauric acid ω-hydroxylase activities, levels of cytochrome P450 4A1 and peroxisomal fatty acid β-oxidation was found. Even at a dose-level of 100 mg DEPH/kg body weight per day a significant induction of these activities was observed. The main metabolites of DEHP, mono(2-ethylhexyl)phthalate and 2-ethyl-1-hexanol, also caused an induction of levels of P450 4A1, lauric acid ω-hydroxylase activities and the activity of peroxisomal palmitoyl-CoA oxidase. 2-Ethyl-1-hexanoic acid did not influence lauric acid ω-hydroxylase activities, but did induce levels of P450 4A1 and palmitoyl-CoA oxidase activities. Three other compounds (perfluoro-octanoic acid, valproate and nafenopin) induced both lauric acid ω-hydroxylase activity and peroxisomal palmitoyl-CoA oxidase activity. The plasticizer, di(2-ethylhexyl)adipate, did not induce levels of P450 4A1, lauric acid ω-hydroxylase activities or palmitoyl-CoA oxidase activities. With the compounds tested a close association between the induction of lauric acid ω-hydroxylase activities and peroxisomal palmitoyl-CoA oxidase activity was found. These data support the theory that peroxisome proliferating compounds do induce lauric acid ω-hydroxylase activities and that there might be a mechanistic inter-relationship between peroxisome proliferation and induction of lauric acid ω-hydroxylase activities.

A large number of chemicals that induce hepatic peroxisome proliferation cause the emergence of liver cell carcinoma in lifetime animal assays. This group includes highly used hypolipidaemic drugs, like clofibrate and nafenopin, chlorophenoxyacid herbicides and industrial plasticizers, like di(2-ethylhexyl)phthalate (DEHP†). Chemically, these compounds seem to be a heterogenous group, however many of them can be metabolized to a hydrophobic backbone with a carboxylic function. Administration of these compounds to rodents results in an increase in the number and volume of peroxisomes in hepatocytes and also in an increase in the number of cells undergoing DNA synthesis (for review: [1, 2]). Peroxisome proliferators are called non-genotoxic hepatocarcinogens since they cause liver cell carcinoma but fail to cause DNA damage directly [3]. The exact mechanistic relationship of peroxisome proliferation and tumour formation remains unknown.

Proliferation of peroxisomes is proposed to be mediated by an increased formation of ω-hydroxy fatty acids due to cytochrome P450 4A1 activity, which are converted to dicarboxylic acids in the cytoplasm of hepatocytes [4]. Dicarboxylic acids are mainly metabolized by the peroxisomal β-oxidation system [5, 6]. In the metabolism of fatty acids by the peroxisomal β-oxidation system hydrogen peroxide is produced. It has been proposed that the imbalance between hydrogen peroxide production and degradation, results in an oxidative stress and ultimately in DNA damage. Propagation of this lesion by the well-documented hyperplastic response to peroxisome proliferators might result in liver cell carcinoma [7].

After treatment of rats *in vivo* or hepatocytes of rats *in vitro* with peroxisome proliferators it was shown that RNA encoding cytochrome P450 4A1 appears prior to RNA encoding peroxisomal enzymes [8–10]. Bell *et al.* [9] showed by immunohistochemical staining that cytochrome P450 4A and peroxisomal acyl-CoA oxidase were induced in the same region

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† Abbreviations: DEHP, di(2-ethylhexyl)phthalate; MEHP, mono-(2-ethylhexyl)phthalate; DEHA, di(2-ethylhexyl)adipate; Br-mdmc, 4-(bromo-methyl)-6,7-dimethoxycoumarin.

of the liver by the peroxisome proliferator methylclofenapate. Cytochrome P450 4A1, formerly termed cytochrome P452, is a fatty acid hydroxylating enzyme (for review: [11]). Medium chain-length (C6–C12) fatty acids are oxidized by either of two microsomal pathways, ω- or (ω-1)-hydroxylation. Tamburini *et al.* [12] reported that purified cytochrome P450 4A1 in a reconstituted enzyme system exhibits a lauric acid ω- and (ω-1)-hydroxylase activity in a 6:1 ratio. However, Thomas *et al.* [13] found that purified cytochrome P450 4A1 in a reconstituted system only catalyses the ω-hydroxylase activity, while an equimolar mixture of 4A2 and 4A3 catalyse both ω- and (ω-1)-hydroxylase activity in a 3:1 ratio. Tanaka *et al.* [14] found that multiple purified cytochrome P450 enzymes have lauric acid (ω-1)-hydroxylase activities but not ω-hydroxylase activities, so lauric acid (ω-1) hydroxylase activity is not specific for cytochrome P450 4A1.

It was determined that ω-hydroxylase activity towards lauric acid and cytochrome P450 4A1 can be induced by xenobiotics like hypolipidaemic agents [4], DEHP [15], chlorophenoxyacid herbicides [16] and perfluorinated compounds [17]. DEHP is widely used as plasticizer in PVC plastics and is an important environmental pollutant [18]. In the body, DEHP is rapidly hydrolysed to mono-(2-ethylhexyl)phthalate (MEHP) and 2-ethyl-1-hexanol. 2-Ethyl-1-hexanol can be further oxidized to 2-ethyl-1-hexanoic acid [19]. 2-Ethylhexanoic acid is also used as a wood preservative agent. 2-Ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, the plasticizer di(2-ethylhexyl)adipate (DEHA), the epileptic drug valproate and perfluorooctanoic acid enhance peroxisomal β-oxidation enzymes but the effects on cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity have not been reported yet [20–23]. Studies on the effects of other cytochrome P450 inducers, on lauric acid hydroxylase activities have been limited to phenobarbital, 3-methylcholanthrene and aroclor 1254. Since different effects were found we have repeated these studies and in addition we have quantified the levels of immunochemical detectable P450 4A1.

In this paper we further studied the relationship between induction of microsomal lauric acid ω-hydroxylase activity and peroxisomal palmitoyl-CoA oxidase activity by testing a number of chemicals, known to induce peroxisomal β-oxidation, on their ability to induce lauric acid ω-hydroxylase activities and cytochrome P450 4A1.

MATERIALS AND METHODS

Chemicals and reagents. Nafenopin was a gift of Ciba Geigy (Basle, Switzerland). Valproate (2-propylpentanoic acid), 3-methylcholanthrene, lauric acid, ω-hydroxylauric acid, NADPH, palmitoyl-CoA, acetyl-CoA and aminotriazole were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2,7-Dichlorofluorescin diacetate was purchased from the Eastman Kodak Co. (Rochester, NY, U.S.A.). DEHP, DEHA and 2-ethyl-1-hexanol were obtained from Janssen Chimica (Beerse, Belgium). 2-Ethyl-1-hexanoic acid, 18-crown-6 ether and 4-(bromo-methyl)-6,7-dimethoxycoumarin (Br-

Table 1. The effect of aroclor-1254, phenobarbital and 3-methylcholanthrene on cytochrome P450 content, P450 4A1 content and lauric acid hydroxylase activities

Treatment	Total cytochrome P450 (nmol/mg protein)	Specific cytochrome P450 4A1 (nmol/mg protein)	Specific cytochrome P450 4A1 (% of total cytochrome P450)	Lauric acid hydroxylase activity: (ω-1)-Hydroxylase (nmol/min.nmol P450)	Lauric acid hydroxylase activity: (ω-1)-Hydroxylase (nmol/min.mg protein)	Lauric acid hydroxylase activity: ω-Hydroxylase (nmol/min.nmol P450)	Lauric acid hydroxylase activity: ω-Hydroxylase (nmol/min.mg protein)
Aroclor-1254	2.25 ± 0.3*	0.017 ± 0.01	0.74 ± 0.3†	0.54 ± 0.1	1.21 ± 0.3*	0.18 ± 0.05*	0.40 ± 0.1
Olive oil control	0.82 ± 0.1	0.015 ± 0.004	1.78 ± 0.4	0.67 ± 0.07	0.55 ± 0.1	0.72 ± 0.1	0.60 ± 0.2
Phenobarbital	1.93 ± 0.3*	0.010 ± 0.005	0.58 ± 0.3†	0.64 ± 0.05	1.21 ± 0.1*	0.18 ± 0.08*	0.36 ± 0.1
Saline control	0.75 ± 0.03	0.014 ± 0.004	1.93 ± 0.5	0.69 ± 0.1	0.52 ± 0.1	0.80 ± 0.3	0.60 ± 0.3
3-Methylcholanthrene	1.71 ± 0.08*	0.015 ± 0.008	0.87 ± 0.4†	0.69 ± 0.2*	1.18 ± 0.3	0.47 ± 0.1*	0.81 ± 0.2
Olive oil control	0.98 ± 0.09	0.021 ± 0.008	2.2 ± 0.9	1.1 ± 0.2	1.07 ± 0.1	1.21 ± 0.4	1.18 ± 0.4

Values are means ± SD of four rats.
* Statistically different from control $P \leq 0.05$ [Student's *t*-test for unpaired samples (with correction for unsufficient homogeneity of variance)].
† Statistically different from control $P \leq 0.05$ (Mann–Whitney U test).

mdmc) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Perfluoro-octanoic acid was from Lancaster Synthesis Ltd (Morecambe, U.K.). Aroclor-1254 was from Alltech Associated, Inc. (Deerfield, IL, U.S.A.) and phenobarbital was obtained from OPG (Utrecht, The Netherlands).

MEHP was synthesized by refluxing phthalic anhydride (14.8 g) and 2-ethyl-1-hexanol (13 g) in 20 mL toluene for 2 hr in the presence of 15 mg 4-dimethylaminopyridine. The toluene was distilled off and the product was dissolved in diethylether and extracted into 0.5 M K_2CO_3. The product was released for extraction into diethylether by acidification with 2 M $KHSO_4$. The product was 99% pure and the structure was confirmed by NMR and mass spectrometry.

Antibodies against cytochrome P450 4A1 were a generous gift of Dr G. Gordon Gibson, University of Surrey, Guildford, U.K. All other chemicals were of the highest purity obtainable.

Animals and treatment. Male random-bred Wistar rats (Cpb:WU (SPF), body wt 175–225 g) were maintained at 23–25° with an alternating 12-hr light and dark cycle and with free access to RMH food pellets (Hope Farms, Woerden, The Netherlands) and water. For the treatment with 3-methyl-cholanthrene male random bred Wistar rats (Bor:WIS (SPF), body wt 350 g) were used.

Rats were treated i.p with aroclor-1254 in olive oil (500 mg/kg body wt) or with olive oil (2 mL/kg body wt). Five days after the aroclor-1254 treatment the rats were killed by decapitation. Rats were treated i.p. for 4 days with phenobarbital in saline (75 mg/kg body wt) or with saline (1 mL/kg body wt) and with 3-methylcholanthrene in olive oil (25 mg/kg body wt) or with olive oil (0.6 mL/kg body wt). Twenty-four hours after the last dose rats were killed. All the next doses are expressed as mg/kg body wt per day. In brackets is the dose expressed as mmol/kg body wt per day. Rats were pretreated by gastric intubation once a day for 3 days with DEHP (dose levels: 50 (0.13), 100 (0.26), 250 (0.64), 500 (1.28) and 1000 (2.6) mg/kg; MEHP (250 (0.9) mg/kg); DEHA (475 (1.3) mg/kg); 2-ethyl-1-hexanol (500 (3.8) mg/kg); 2-ethyl-1-hexanoic acid (550 (3.8) mg/kg); valproate (550 (3.8) mg/kg); nafenopin (100 (0.3) mg/kg) and perfluoro-octanoic acid (50 (0.1) mg/kg). Five to seven animals were used per group. Olive oil was used as a vehicle for administration. Control animals were given olive oil at 5 mL/kg body wt per day. One group of five rats was pretreated by gastric intubation with saline. Twenty-four hours after the last dose, animals were weighed and then killed by decapitation prior to perfusion of the livers.

Preparation of liver fractions. Livers were perfused with 0.9% NaCl (w/w) for 10 min. Entire livers were removed and weighed. Whole liver homogenates (20%, w/v) were prepared in 0.25 M sucrose, 2 mM EDTA and 10 mM Tris–HCl (pH 7.4) (SETH buffer) using a teflon–glass homogenizer. A part of this homogenate was freezed/thawed three times, followed by centrifugation at 9000 *g* for 5 min. The supernatants were flash frozen in liquid nitrogen and stored at −80° until they were thawed for enzymatic analysis of palmitoyl-CoA oxidase and carnitine acetyltransferase activity.

The remaining part of the whole liver homogenate was centrifuged at 9000 *g* for 20 min and the supernatant was centrifuged at 105,000 *g* for 1 hr. The pellet (microsomal fraction) was homogenized in SETH buffer and aliquots were flash frozen in liquid nitrogen and stored at −80° until they were thawed for determination of cytochrome P450 content, cytochrome P450 4A1 concentration and lauric acid hydroxylase activity.

Other methods. Palmitoyl-CoA oxidase activity was determined according to Reubsaet *et al.* [24]. Carnitine acetyltransferase was determined at 25° according to Gray *et al.* [25]. Lauric acid hydroxylase activities were determined by reverse phase HPLC and fluorimetric detection based on the derivatization of lauric acid and its metabolites with the fluorescent compound Br-mdmc as previously presented elsewhere [26]. Total cytochrome P450 was determined from the CO-difference spectrum by the method of Omura and Sato [27]. Cytochrome P450 4A1 was determined immunochemically by an ELISA method described by Sharma *et al.* [4]. On immunoblots the antibodies react with two protein bands (51.5 and 52 kDa). The 51.5 kDa protein co-migrates with authentic rat cytochrome P450 4A1, the 52 kDa

Table 2. The effect of DEHP on liver size, cytochrome P450 and cytochrome P450 4A1

Dose	Liver/body weight ratio	Total cytochrome P450 (nmol/mg protein)	Specific cytochrome P450 4A1 (nmol/mg protein)	(% of total cytochrome P450)
0	4.8 ± 0.4	0.55 ± 0.08	0.013 ± 0.005	2.4 ± 0.9
50	5.1 ± 0.3	0.64 ± 0.1	0.032 ± 0.01*	4.9 ± 0.9†
100	5.2 ± 0.4	0.64 ± 0.1	0.049 ± 0.01*	7.7 ± 1.4†
250	5.6 ± 0.5†	0.59 ± 0.06	0.069 ± 0.01*	10.9 ± 1.4†
500	5.7 ± 0.4†	0.69 ± 0.1	0.094 ± 0.03*	13.5 ± 2.4†
1000	5.8 ± 0.2†	0.71 ± 0.1	0.109 ± 0.03*	15.4 ± 0.6†

Values are means ± SD of 5–7 rats.
* Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).
† Statistically different from control $P \leq 0.05$ (Mann–Whitney U test with Bonferroni correction).

band is probably 4A2 or 4A3 [28]. Protein concentrations were determined by the method of Bradford [29] using crystalline bovine serum albumin as standard.

Statistical analysis. Data were analysed using SAS-software, version 6. All tests performed are two tailed.

RESULTS

Studies with classical cytochrome P450 inducers

The effects of phenobarbital, 3-methylcholanthrene and aroclor-1254 administration on total cytochrome P450 content, the specific cytochrome P450 4A1 levels and lauric acid (ω-1)- and ω-hydroxylase activities are summarized in Table 1.

All compounds tested increased the total cytochrome P450 content of liver microsomes (1.7–2.7 times). Specific cytochrome P450 4A1 levels in control animals as determined by ELISA were shown to be 1.8–2.2% of total cytochrome P450, and no induction of cytochrome P450 4A1 by phenobarbital, 3-methylcholanthrene or aroclor-1254 was observed. When the levels of P450 4A1 were expressed as a percentage of total cytochrome P450 all three compounds decreased the P450 4A1 content. Furthermore, no induction was found of the ω-hydroxylase activity towards lauric acid, the marker substrate for cytochrome P450 4A1, when this activity was expressed per mg protein. When this activity was expressed per nmol P450 a decrease was found. A 2-fold increase in the (ω-1)-hydroxylase activity was found after aroclor-1254 and phenobarbital treatment when this activity was expressed per mg protein. No increases were found when the (ω-1)-hydroxylase activity was expressed per nmol P450. We prefer this latter expression because it is possible that total cytochrome P450, including P450 4A1 levels and lauric acid hydroxylase activities, is induced by the test compound, which can obscure the specific induction of lauric acid hydroxylase activities.

Our results clearly show that classical enzyme inducers used in toxicological studies such as phenobarbital, 3-methylcholanthrene or aroclor-1254 do not induce lauric acid hydroxylase activities or cytochrome P450 4A1.

Dose–response studies with DEHP

The effects of DEHP administration on liver/body weight ratio, total cytochrome P450 content and specific P450 4A1 isoenzyme levels are shown in Table 2. In rats receiving DEHP doses of more than 250 mg/kg body weight per day a significant hepatomegaly was found. Total cytochrome P450 levels were not altered by the DEHP treatment. Following DEHP treatment immunochemically determined cytochrome P450 4A1 concentrations were strongly increased in a dose-dependent manner. Even at the lowest DEHP dose (50 mg/day per kg body weight) a 2-fold induction of cytochrome P450 4A1 was found. At dose levels of 100 mg DEHP/kg body weight per day or higher, the associated cytochrome P450 4A1-driven lauric acid ω-hydroxylase activity was induced in a dose-dependent manner (Fig. 1a). At dose-levels of 500 or 1000 mg DEHP/kg body weight per day this activity was increased 8–9-fold. No increase in the lauric acid (ω-1)-hydroxylase activity was found. Acyl-CoA oxidase activity is rate-limiting in the peroxisomal β-oxidation and it was shown that palmitoyl-CoA oxidase was the most sensitive marker for peroxisome proliferation in the liver [30]. At dose levels of

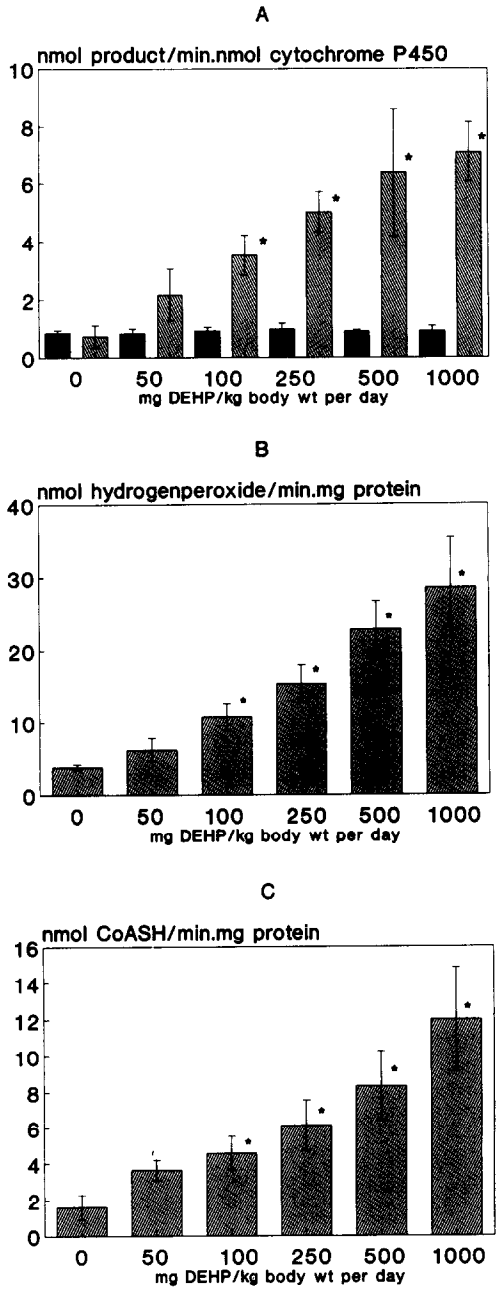

Fig. 1. Effect of DEHP on lauric acid hydroxylase activities (A), peroxisomal palmitoyl-CoA oxidase activities (B) and carnitine acetyltransferase activities (C). Each bar represents the mean ± SD of 5–7 rats. Key: (■) (ω-1)-hydroxylauric acid and (▨) ω-hydroxylauric acid. *Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).

Table 3. The effect of DEHP and metabolites of DEHP on liver size, cytochrome P450 and cytochrome P450 4A1

Treatment	Dose (mmol/kg body weight)	Liver/body weight ratio	Total cytochrome P450 (nmol/mg protein)	Specific cytochrome P450 4A1 (nmol/mg protein)	Specific cytochrome P450 4A1 (% of total cytochrome P450)
Control	—	5.0 ± 0.2	0.69 ± 0.1	0.011 ± 0.005	1.6 ± 0.7
DEHP	1.3	5.9 ± 0.4†	0.96 ± 0.16*	0.129 ± 0.03*	13.5 ± 1.8†
MEHP	0.9	5.6 ± 0.8	0.76 ± 0.05	0.084 ± 0.1*	11.0 ± 1.6†
2-Ethyl-1-hexanol	3.8	5.4 ± 0.3	0.73 ± 0.07	0.037 ± 0.01*	5.1 ± 1.2†
2-Ethyl-1-hexanoic acid	3.8	5.8 ± 0.4	0.77 ± 0.09	0.033 ± 0.005*	4.3 ± 0.7†

Values are means ± SD of 5–7 rats.
* Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).
† Statistically different from control $P \leq 0.05$ (Mann–Whitney U test with Bonferroni correction).

100 mg DEHP/kg body wt per day or higher palmitoyl-CoA oxidase activities were dose-relatedly induced by the DEHP treatment (Fig. 1b). It has been reported that the increase in number and size of peroxisomes in rats treated with peroxisome proliferators correlates well with increased carnitine acetyl-CoA oxidase activity, although it is not a specific peroxisomal enzyme [4]. At dose levels of 100 mg DEHP/kg body wt per day or higher carnitine acetyl transferase activity also exhibits a dose-dependent increase following the administration of DEHP (Fig. 1c). A dose of 100 mg DEHP/kg body wt per day gives an increase in both peroxisomal enzyme activities and of lauric acid ω-hydroxylase activities. This dose-level is 2.5 times lower than in another study [15].

Studies on DEHP metabolites

The effects of the metabolites MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid were compared with the effects of DEHP. Administration of DEHP did result in a liver enlargement and in an increase in the total cytochrome P450 content of the liver microsomes (Table 3). DEHP, MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid increased cytochrome P450 4A1 levels (Table 3). None of the compounds altered lauric acid (ω-1)-hydroxylase activities, while DEHP, MEHP and 2-ethylhexanol resulted in a significant induction of lauric acid ω-hydroxylase activities (Fig. 2a). Palmitoyl-CoA oxidase activity, a marker for peroxisome proliferation was increased by all four compounds tested (Fig. 2b).

Studies on other compounds possible having a peroxisome proliferating action

Effects of administration of valproate, perfluoro-octanoic acid and DEHA to rats were compared with the well known effects of the peroxisome proliferator nafenopin.

Administration of perfluoro-octanoic acid and nafenopin resulted in a hepatomegaly (Table 4). Perfluoro-octanoic acid was also able to increase the total cytochrome P450 level 2.5 times. Perfluoro-

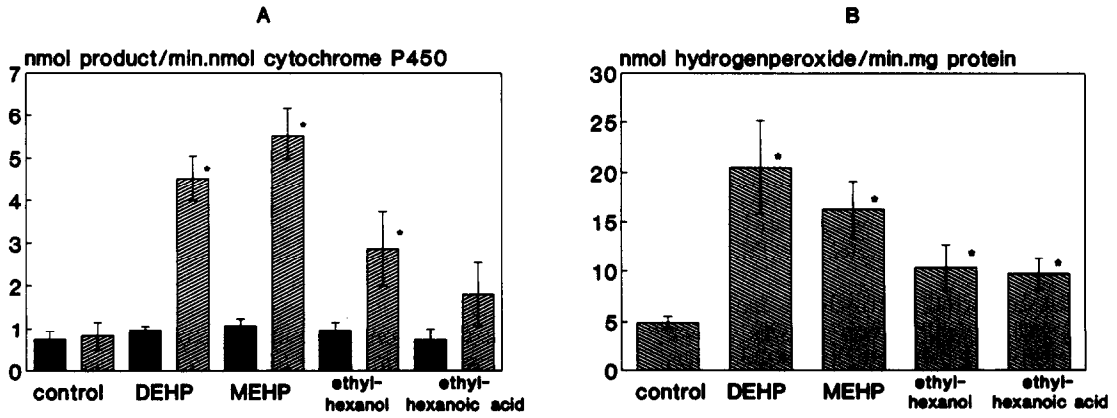

Fig. 2. Effect of DEHP, MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid on lauric acid hydroxylase activities (A) and peroxisomal palmitoyl-CoA oxidase activities (B). Each bar represents the mean ± SD of 5–7 rats. Key: (■) (ω-1)-hydroxylauric acid and (□) ω-hydroxylauric acid. *Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).

Table 4. The effect of olive oil, saline, DEHA, valproate, perfluoro-octanoic acid and nafenopin on liver size, cytochrome P450 and cytochrome 4A1

Treatment	Dose (mmol/kg body weight)	Liver/body weight ratio	Total cytochrome P450 (nmol/mg protein)	Specific cytochrome P450 4A1 (nmol/mg protein)	(% of total cytochrome P450)
Olive oil	—	5.0 ± 0.2	0.69 ± 0.1	0.011 ± 0.005	1.63 ± 0.7
Saline	—	4.9 ± 0.3	0.74 ± 0.1	0.009 ± 0.002	1.21 ± 0.4
DEHA	1.3	5.0 ± 0.4	0.67 ± 0.1	0.014 ± 0.003	2.14 ± 0.4
Valproate	3.8	5.5 ± 0.5	0.74 ± 0.1	0.039 ± 0.007*	5.29 ± 0.4†
Perfluoro-octanoic acid	0.1	7.1 ± 0.5†	1.69 ± 0.3*	0.199 ± 0.03*	11.86 ± 1.7†
Nafenopin	0.3	6.8 ± 0.8†	0.71 ± 0.07	0.133 ± 0.02*	18.76 ± 1.6†

Values are means ± SD of 5–7 rats.
* Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).
† Statistically different from control $P \leq 0.05$ (Mann–Whitney U test with Bonferroni correction).

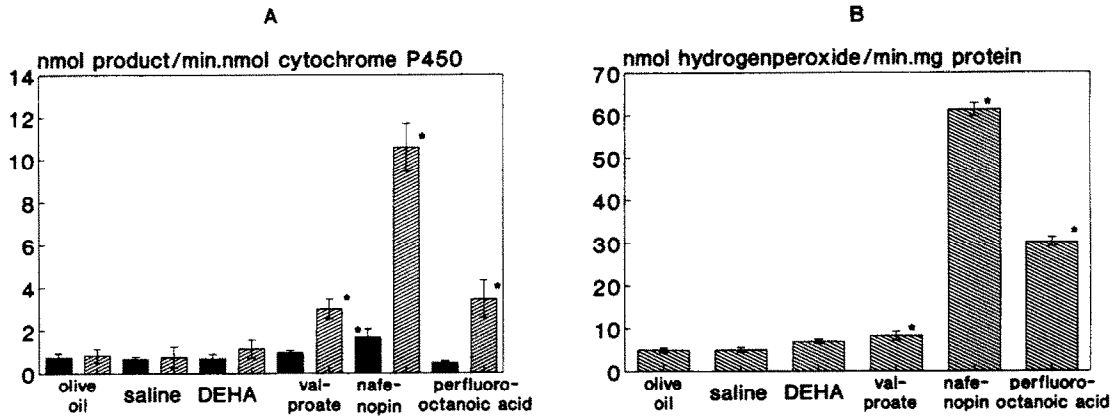

Fig. 3. Effect of olive oil, saline, DEHA, valproate, perfluoro-octanoic acid and nafenopin on lauric acid hydroxylase activities (a) and peroxisomal palmitoyl-CoA oxidase activities (b). Each bar represents the mean of 5–7 rats. Key: (■) (ω-1)-hydroxylauric acid and (▨) ω-hydroxylauric acid. *Statistically different from control $P \leq 0.05$ (ANOVA and Dunnett's test).

octanoic acid, valproate and nafenopin induced levels of cytochrome P450 4A1 (3–11-fold). These three compounds also increased the ω-hydroxylase activity towards lauric acid 3–11-fold, but only nafenopin increased the lauric acid (ω-1)-hydroxylase activity (Fig. 3a). Palmitoyl-CoA oxidase activities were induced in the liver of rats treated with these compounds. No (significant) increase in cytochrome P450 4A1 levels, in the ω-hydroxylase activity towards lauric acid or palmitoyl-CoA oxidase activities were found after the DEHA treatment (Fig. 3b).

It was reported that high fat diets also produce lauric acid ω-hydroxylase activity induction [31]. Therefore, we studied a possible bias in our experiments of olive oil, which was the vehicle in most of our experiments. A group of rats treated once a day by gastric intubation for 3 days with olive oil was compared with a group of rats treated with saline. No differences were found in total cytochrome P450 concentrations, cytochrome P450 4A1 levels (Table 4), or in hydroxylase activities towards lauric acid and palmitoyl-CoA oxidase activities (Fig. 3a and b) between the olive oil- or the saline-treated group. The vehicle used in our experiments appeared to have no effect on the parameters studied.

DISCUSSION

None of the classical P450 inducers tested increased cytochrome P450 4A1 levels or the ω-hydroxylase activity towards lauric acid. The results obtained with aroclor-1254 are in contrast with the results of Borlakoglu *et al.* [32] who reported a 5-fold increase in the formation of ω-hydroxylauric acid, expressed per mg protein, in the liver of female rats treated with aroclor-1254. Since we have used male rats in our experiments, the observed differences in inducibility of the ω-hydroxylase activity towards lauric acid after arochlor-1254 treatment might be due to sex differences.

Comparisons of our data with the data of others

Table 5. Matrix of Pearson correlation coefficients of several morphological and biochemical effects on rat liver after administration of peroxisome proliferating compounds*

	Total cytochrome P450 (nmol/mg protein)	P450 4A1 (nmol/mg protein)	Lauric acid (ω-1)-hydroxylase activity (nmol/min.mg protein)	Lauric acid ω-hydroxylase activity (nmol/min.mg protein)	Palmitoyl-CoA oxidase activity
Liver/body weight	0.539‡	0.745‡	0.450†	0.779‡	0.765‡
Total cytochrome P450		0.776‡	0.274	0.448†	0.282
P450 4A1			0.633‡	0.852‡	0.727‡
(ω-1)-Hydroxylase activity				0.808‡	0.665‡
ω-Hydroxylase activity					0.895‡

* Data for the following compounds were used: DEHP (1.3 mmol/kg body wt), MEHP, 2-ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, DEHA, valproate, perfluoro-octanoic acid, nafenopin and olive oil (N = 46).
Statistically significant effects are indicated by † $P < 0.005$ and ‡ $P < 0.001$.

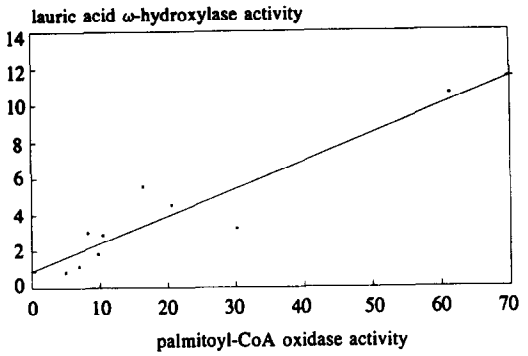

Fig. 4. Association between peroxisomal palmitoyl-CoA oxidase activities and microsomal lauric acid ω-hydroxylase activities after treatment of rats with DEHP (500 mg/kg per day), MEHP, 2-ethyl-1-hexanol, 2-ethyl-1-hexanoic acid, nafenopin, valproate, perfluoro-octanoic acid, DEHA and olive oil. Of each group mean values were used. ($y = 0.88 + 0.15x$, $r = 0.91$, N = 9).

is somewhat complicated because lauric acid hydroxylase activities are by some authors expressed per mg protein and by others per nmol cytochrome P450. We found a 2-fold increase of (ω-1)-hydroxylase activity in the livers of rats treated with aroclor-1254 and phenobarbital, when the activity is presented per mg protein, but no increase was found when the activity was expressed per nmol cytochrome P450. Similar increases in lauric acid (ω-1)-hydroxylase activities (when expressed per mg protein) after phenobarbital treatment were also found by Okita and Masters [33] and Cresteil *et al.* [34]. Tanaka *et al.* [14] found that both activities are decreased after phenobarbital treatment when these activities were expressed per nmol P450. After 3-methylcholanthrene treatment we found a decrease in both ω- and (ω-1)-hydroxylase activities when this activity was expressed per nmol P450. Similar findings were reported by Tanaka *et al.* [14]. We found no differences between the two activities when the activity was expressed per mg protein, but Cresteil *et al.* [34] found a decrease. Taken collectively our data and the data of others [14, 33, 34] make clear that phenobarbital and 3-methylcholanthrene do not induce lauric acid ω-hydroxylase activities or levels of immunochemical detectable P450 4A1. These findings are supported by the recent finding that levels of RNA levels encoding two other members of the cytochrome P450 4A family (cytochrome P450 4A2 and 4A3) were increased in rats after treatment with clofibrate, but not after treatment with the P450 inducers phenobarbital, 3-methylcholanthrene and dexamethasone [35].

Recent studies with hypolipidaemic peroxisome proliferators, with chlorinated phenoxy acid herbicides and with perfluorinated compounds have demonstrated an excellent correlation between microsomal cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity on the one hand, and peroxisomal volume and peroxisomal β-oxidation activity on the other in the liver of rats

[4, 16, 17]. After treatment of rats with different doses of DEHP we found a dose-related increase in cytochrome P450 4A1 levels and associated lauric acid ω-hydroxylase activity, as well as peroxisomal enzymes like palmitoyl-CoA oxidase and carnitine acetyl transferase. This is in close agreement with data presented by Sharma *et al.* [15], although we have used 2.5 times lower dose levels. In most studies DEHP is administered to rats as part of the diet. The lowest dose of DEHP used in our study which induced both lauric acid ω-hydroxylase- and palmitoyl-CoA activity is equal to a diet containing 0.1% DEHP (calculated on the basis of previously published data [36]) and this dose is one of the lowest doses which has been reported to induce palmitoyl-CoA oxidase activities and lauric acid ω-hydroxylase activities.

When the DEHP metabolites MEHP, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid were administered orally to rats, an induction of the levels of P450 4A1 was found. MEHP and 2-ethyl-1-hexanol, but not 2-ethyl-1-hexanoic acid increased the lauric acid ω-hydroxylase activity. All three compounds induced peroxisomal palmitoyl-CoA oxidase.

Another plasticizer, DEHA, was also reported to enhance peroxisomal β-oxidation activity [20]. With the dose level used in our study, DEHA gave no (significant) induction of palmitoyl-CoA oxidase activity or lauric acid ω-hydroxylase activity.

Our results also indicate that the anti-epileptic drug valproic acid, a chemical analogue of the DEHP metabolite 2-ethylhexanoic acid, and perfluoro-octanoic acid are inducers of cytochrome P450 4A1 and lauric acid ω-hydroxylase activities. The strong inducibility of peroxisome proliferation by perfluorinated fatty acids is unexpected because the structures of these chemicals are dissimilar to any known peroxisome proliferating compounds. Owing to their metabolically inert properties these compounds may be proximate inducers themselves. The data obtained in the experiments with DEHP (500 mg/kg per day), 2-ethyl-1-hexanoic acid, 2-ethyl-1-hexanol, MEHP, DEHA, nafenopin, valproate and perfluoro-ocatanoic acid were used to study the correlation of biological effects of peroxisome proliferating compounds on the liver. A correlation matrix is shown in Table 5. Total cytochrome P450 did not correlate well with palmitoyl-CoA oxidase activities. A high correlation was found between P450 4A1 levels, and its associated lauric acid ω-hydroxylase activities and peroxisomal palmitoyl-CoA oxidase activities. The high degree of association between palmitoyl-CoA oxidase activity and lauric acid ω-hydroxylase activity is also shown in Fig. 4. Although cause and effect cannot be separated by a correlation analysis, our data and data from others [4, 17] are suggestive of a mechanistic interrelationship between induction of lauric acid ω-hydroxylase activity by P450 4A1 and peroxisome proliferation.

In conclusion, the induction of cytochrome P450 4A1 and its associated lauric acid ω-hydroxylase activity seems to be specific for peroxisome proliferating compounds. Determination of levels of P450 4A1 and lauric acid ω-hydroxylase activities in identifying peroxisome proliferators is very useful since these parameters are very sensitive. The demonstration of the ability of several peroxisome proliferating compounds to induce cytochrome P450 4A1 further support the hypothesis that there might be a mechanistic interrelationship between peroxisome proliferation and induction of lauric acid ω-hydroxylase activities.

Acknowledgements—The authors wish to thank Nicole Palmen (University of Limburg, The Netherlands) for the treatment of the rats with 3-methylcholanthrene. We also thank Prof. P. Th. Henderson (University of Limburg, The Netherlands) for critical reading of the manuscript.

REFERENCES

1. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* **18**: 441–515, 1987.
2. Moody DE, Reddy JK, Lake BG, Popp JA and Reese DH, Peroxisome proliferation and nongenotoxic carcinogens: commentary on a symposium. *Fundam Appl Toxicol* **16**: 223–248, 1991.
3. Conway JG, Cattley RC, Popp JA and Butterworth BE, Possible mechanisms in hepatocarcinogenesis by the peroxisome proliferator di(2-ethylhexyl)phthalate. *Drug Metab Rev* **21**: 65–102, 1989.
4. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. A mechanistic inter-relationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
5. Poosch MS and Yamazaki RK, The oxidation of dicarboxylic acid CoA esters via peroxisomal fatty acyl-CoA oxidase. *Biochim Biophys Acta* **1006**: 291–298, 1989.
6. Suzuki H, Yamada J, Watanabe T and Suga T, Compartimentation of dicarboxylic acid β-oxidation in rat liver: importance of peroxisomes in the metabolism of dicarboxylic acids. *Biochim Biophys Acta* **990**: 25–30, 1989.
7. Rao MS and Reddy JK, The relevance of peroxisome proliferation and cell proliferation in peroxisome proliferator-induced hepatocarcinogenesis. *Drug Metab Rev* **21**: 103–110, 1989.
8. Milton MN, Elcombe CR and Gibson GG, On the mechanism of microsomal cytochrome P450IVA1 and peroxisome proliferation in rat liver by clofibrate. *Biochem Pharmacol* **40**: 2727–2732, 1990.
9. Bell DR, Bars RG, Gibson GG and Elcombe CR, Localization and differential induction of cytochrome P450IVA and acyl-CoA oxidase in rat liver. *Biochem J* **275**: 247–252, 1991.
10. Bieri F, Meier V, Stäubli W, Muakkassah-Kelley SF, Waechter F, Sagelsdorff P and Bentley P, Studies on the mechanism of induction of microsomal cytochrome P452 and peroxisomal bifunctional enzyme mRNA by nafenopin in primary cultures of adult rat hepatocytes. *Biochem Pharmacol* **41**: 310–312, 1991.
11. Gibson GG, Comparative aspects of the mammalian cytochrome P-450 IV gene family. *Xenobiotica* **19**: 1123–1148, 1989.
12. Tamburini PP, Masson HA, Bains SK, Makowski RJ, Morris B and Gibson GG, Multiple forms of hepatic cytochrome P-450, purification, characterisation and comparison of a novel clofibrate-induced isoenzyme with other major forms of cytochrome P-450. *Eur J Biochem* **139**: 235–246, 1984.
13. Thomas H, Molitor E, Kuster H, Savoy C, Wolf CR and Waechter F, Hydroxylation of lauric acid and

testesterone by rat liver cytochrome P450 IVA proteins in reconstituted systems. In: *Proceedings of the Third International ISSX Meeting, Amsterdam, 24–25 June*, p. 303 (1991).
14. Tanaka S, Imaoka S, Kusunose E, Kusunose M, Maekawa M and Funae Y, ω- and (ω-1)-hydroxylation of arachidnoic acid, lauric acid and prostaglandin A1 by multiple forms of cytochrome P-450 purified from rat hepatic microsomes. *Biochim Biophys Acta* **1043**: 177–181, 1990.
15. Sharma R, Lake BG and Gibson GG, Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid β-oxidation pathway in the rat by clofibrate and di-(2-ethyl-hexyl)phthalate. Dose–response studies. *Biochem Pharmacol* **37**: 1203–1206, 1988.
16. Bacher MA and Gibson GG, Chlorophenoxyacid herbicides induce microsomal cytochrome P-450 IVA1 (P452) in rat liver. *Chem Biol Interact* **65**: 145–156, 1988.
17. Kozuka H, Watanabe T, Horie S, Yamada J, Suga T and Ikeda T, Characteristics of peroxisome proliferation: co-induction of peroxisomal fatty acid oxidation-related enzymes with microsomal laurate hydroxylase. *Chem Pharm Bull* **39**: 1267–1271, 1991.
18. Wams TJ, Diethylhexylphthalate as an environmental contaminant—a review. *Sci Total Environ* **66**: 1–16, 1987.
19. Albro PW, The biochemical toxicology of di-(2-ethylhexyl) and related phthalates: testicular atrophy and hepatocarcinogenesis. *Rev Biochem Toxicol* **8**: 73–119, 1986.
20. Moody DE and Reddy JK, Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. *Toxicol Appl Pharmacol* **45**: 497–504, 1978.
21. Katoh H, Nakajima S, Kawashima Y, Kozuka H and Uchiyama M, Induction of rat hepatic long-chain acyl-CoA hydrolases by various peroxisome proliferators. *Biochem Pharmacol* **33**: 1081–1085, 1984.
22. Horie S and Suga T, Enhancement of peroxisomal β-oxidation in the liver of rats treated with valproic acid. *Biochem Pharmacol* **34**: 1357–1362, 1985.
23. Kawashima Y, Uy-yu N and Kozuka H, Sex-related difference in the induction by perfluoro-octanoic acid of peroxisomal β-oxidation, microsomal 1-acylglycerophosphocholine acyltransferase and cytosolic long-chain acyl-CoA hydrolase in rat liver. *Biochem J* **261**: 595–600, 1989.
24. Reubsaet FAG, Veerkamp JH, Bukkens SGF, Trijbels JMF and Monnens LAH, Acyl-CoA oxidase activity and peroxisomal fatty acid oxidation in rat tissues. *Biochim Biophys Acta* **985**: 434–442, 1988.
25. Gray TJB, Beamand JA, Lake BG, Foster JR and Gangolli SD, Peroxisome proliferation in cultured rat hepatocytes produced by clofibrate and phthalate ester metabolites. *Toxicol Lett* **10**: 273–279, 1982.
26. Dirven HAAM, de Bruijn AAGM, Sessink PJM and Jongeneelen FJ, Determination of the cytochrome P-450 IV marker, ω-hydroxylauric acid, by high-performance liquid chromatography and fluorimetric detection. *J Chromatogr* **564**: 266–271, 1991.
27. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* **239**: 2379–2385, 1964.
28. Dirven HAAM, Peters JGP, Gibson GG, Peters WHM and Jongeneelen FJ, Lauric acid hydroxylase activity and cytochrome P450 IV family proteins in human liver microsomes. *Biochem Pharmacol* **42**: 1841–1844, 1991.
29. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **131**: 248–254, 1976.
30. Tomaszewski KE, Derks MC and Melnick RL, Acyl CoA oxidase is the most suitable marker for hepatic peroxisomal changes caused by treatment of F344 rats with di-(2-ethylhexyl)phthalate. *Toxicol Lett* **37**: 203–212, 1987.
31. Nilsson A, Arey H, Pedersen JI and Christiansen EN, The effect of high-fat diets on microsomal lauric acid hydroxylation in rat liver. *Biochim Biophys Acta* **879**: 209–214, 1986.
32. Borlakoglu JT, Edwards-Webb JD, Dils RR, Wilkins JGP and Robertson LW, Evidence for the induction of cytochrome P-452 in rat liver by aroclor 1254, a commercial mixture of polychlorinated biphenyls. *FEBS Lett* **247**: 327–329, 1989.
33. Okita RT and Masters BSS, Effect of phenobarbital treatment and cytochrome P-450 inhibitors on the laurate ω- and (ω-1)-hydroxylase activities of rat liver microsomes. *Drug Metab Dispos* **8**: 147–151, 1980.
34. Cresteil T, Beaune P, Celier C, Leroux JP and Guengerich FP, Cytochrome P-450 isoenzyme content and monooxygenase activities in rat liver: effect of ontogenesis and pretreatment by phenobarbital and 3-methylcholanthrene. *J Pharmacol Exp Ther* **236**: 269–276, 1986.
35. Kimura S, Hardwick JP, Lozak CA and Gonzalez FJ, The rat clofibrate-inducible CYP4A subfamily II. cDNA sequence of IVA3, mapping of the Cyp4a locus to mouse chromosome 4, and coordinate and tissue-specific regulation of the CYP4A genes. *DNA* **8**: 517–525, 1989.
36. Dirven HAAM, van den Broek PHH and Jongeneelen FJ, Effect of di-(2-ethyl-hexyl)phthalate on enzyme activity levels in liver and serum of rats. *Toxicology* **65**: 199–207, 1990.