Biochemical Pharmacology, Vol. 45, No. 12, pp. 2425–2434, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EFFECTS OF THE PEROXISOME PROLIFERATOR MONO(2-ETHYLHEXYL)PHTHALATE IN PRIMARY HEPATOCYTE CULTURES DERIVED FROM RAT, GUINEA PIG, RABBIT AND MONKEY

RELATIONSHIP BETWEEN INTERSPECIES DIFFERENCES IN BIOTRANSFORMATION AND PEROXISOME PROLIFERATING POTENCIES

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(*Received* 14 *December* 1992; *accepted* 3 *March* 1993)

Abstract—Primary hepatocyte cultures derived from rat, rabbit, guinea pig and monkey have been treated *in vitro* with metabolites of di(2-ethylhexyl)phthalate, i.e. mono(2-ethylhexyl)phthalate (MEHP), mono(5-carboxy-2-ethylpentyl)phthalate (metabolite V) and mono(2-ethyl-5-oxohexyl)-phthalate (metabolite VI). In rat hepatocyte cultures MEHP and metabolite VI were equally potent in inducing peroxisome proliferation, while metabolite V was much less potent. In rat hepatocytes a 50% increase in both peroxisomal palmitoyl-CoA oxidase activity and microsomal lauric acid ω-hydroxylation activity was found after treatment with 5–15 μM MEHP. In guinea pig, rabbit and monkey hepatocyte cultures, a 50% increase in peroxisomal palmitoyl-CoA oxidase activity was found after treatment with 408–485 μM MEHP. No induction of lauric acid ω-hydroxylation activity was found. These results indicate that peroxisome proliferation can be induced by MEHP in rabbit, guinea pig and monkey hepatocytes, but that these species are at least 30-fold less sensitive to peroxisome proliferation induction than rats. The proposed mechanistic inter-relationship between induction of lauric acid ω-hydroxylation activity and peroxisome proliferation is found in rat hepatocytes, but not in hepatocytes of the other three species. Treatment of guinea pig hepatocyte cultures with MEHP resulted in an increase in triglyceride concentrations in the hepatocytes. In rat and rabbit hepatocyte cultures, triglyceride concentrations were much less altered by MEHP. In monkey hepatocytes a decrease in hepatic triglyceride concentration was found after treatment with MEHP. These effects are in agreement with *in vivo* effects observed before. After treatment of primary hepatocyte cultures with MEHP, high concentrations of ω- and (ω-1)-hydroxylated metabolites of MEHP were found in media from rat, rabbit and guinea pig cultures. The formation of these metabolites did not decline in time. During treatment the metabolite profile in media from rat hepatocyte cultures moved towards ω-hydroxy metabolites of MEHP. In media from monkey hepatocyte cultures the lowest concentrations of hydroxylated metabolites were determined. No major species differences were found in the potency to form oxidized MEHP metabolites, and thus no unique metabolite differences were found, which could explain the species differences in sensitivity for peroxisome proliferation.

The current interest in peroxisome proliferation caused by xenobiotics is the result of the finding that chronic exposure of rats and mice to peroxisome proliferators eventually results in the development of hepatic tumours. The basic mechanism(s) by which peroxisome proliferators induce tumours in rats and mice is unknown. Tumour formation might be due to promotion of preneoplastic cells to cancer and/or might be the result of an increase in the number and volume of peroxisomes [1]. In this latter model it is proposed that hydrogen peroxide, produced by the increased peroxisomal fatty acid β-oxidation system, results in oxidative damage of the genome [2]. Peroxisome proliferation might be the result of a disturbed fatty acid homeostasis in liver cells, probably related to an increased formation of ω-hydroxy fatty acids by cytochrome P450 4A1 [3]. If indeed peroxisome proliferation is an intermediate step in tumour formation, it remains uncertain whether peroxisome proliferating compounds are a health hazard for humans, since humans are considered to be less sensitive for peroxisome proliferation than rats.

Peroxisome proliferation has been demonstrated in most mammalian species. Rats and mice are considered to be very sensitive, hamsters and possible rabbits are intermediately sensitive, while dogs, cats, and monkeys are weak reactors [4–11]. Mechanisms underlying these species differences in sensitivity for peroxisome proliferation are not very well understood. Species differences in the presence of specific receptors [12] and species differences in the

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metabolism or pharmacokinetics of peroxisome proliferating compounds, have been proposed [13, 14].

For the polyvinylchloride plasticizer di(2-ethylhexyl)phthalate (DEHP*), formation of a specific metabolite (e.g. metabolite VI; see Fig. 1) is proposed to be essential for the induction of peroxisome proliferation [14]. DEHP is a relatively weak peroxisome proliferator, but since this compound is used in large quantities and since DEHP is also an important environmental pollutant [15], it is believed that a large number of people are exposed to this compound.

Primary cultures of rat, monkey, human, dog, hamster and guinea pig hepatocytes have been treated *in vitro* with peroxisome proliferating compounds [9, 11, 14–18]. Primary cultures of rabbit hepatocytes have not been tested, yet. Several markers for the response have been determined including peroxisome morphometrics, increases in the activity of peroxisomal enzymes, lauric acid ω-hydroxylation activity and levels of RNA for peroxisomal enzymes and P450 4A1. Other parameters associated with peroxisome proliferation, like hepatic lipid levels (triglyceride and cholesterol concentrations) [7], have not been determined after *in vitro* treatment of hepatocytes with peroxisome proliferators. These parameters are of interest since peroxisome proliferators alter the fatty acid homeostasis in liver cells, indicating that both the fatty acid catabolic reactions (β-oxidation activities), as well as anabolic reactions (triglycerides synthesis) might be altered.

Studies on species differences in peroxisome proliferation should include studies with human hepatocytes. However, practical considerations (i.e. the poor availability of human livers, technical difficulties in establishing good monolayer cultures and the relatively large numbers of cells required for analysis of microsomal enzyme activity) prompted us to use monkey hepatocytes in our experiments.

In the experiments described in this paper, we have compared the effects of metabolites of DEHP (see Fig. 1) in primary hepatocyte cultures of rat, rabbit and guinea pig, and on cynomolgus monkey hepatocytes. Parameters determined (peroxisomal palmitoyl-CoA oxidase activity, lauric acid hydroxylation activities, RNA levels for acyl-CoA oxidase and P450 4A1, triglyceride and cholesterol concentrations) were studied in relation to potency to form metabolites.

MATERIALS AND METHODS

Chemicals. Collagenase B, cholesterol and triglycerides test kits were obtained from Boehringer Mannheim GmbH (Germany). Palmitoyl-CoA, hydrocortisone-21-hemisuccinate, insulin, gentamycin sulphate, bovine serum albumin, aminotriazole, NADPH and lauric acid 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.). 18-Crown-6-ether and 4-(bromomethyl)-6,7-dimethoxycoumarin were from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Triethyloxonium tetrafluoroborate was from Lancaster Synthesis Ltd (Morecambe, U.K.). Newborn calf serum, William's E powder and L-glutamine were obtained from Flow Laboratories (Rickmansworth, U.K.).

A cDNA probe for rat cytochrome P450 4A1 was provided by Dr G. G. Gibson, University of Surrey (U.K.). A cDNA probe for rat acyl-CoA oxidase was provided by Dr Hashimoto, Shinshu University School of Medicine (Japan). Mono(2-ethylhexyl)-phthalate (MEHP) (99% pure) was synthesized as described elsewhere [19]. Monohexylphthalate was synthesized according to the same procedure. Mono-(2-ethyl-5-oxohexyl)phthalate (metabolite VI) (99% pure) and mono(2-ethyl-5-hydroxyhexyl)phthalate (metabolite IX) (91% pure) were synthesized by Drs Nefkens and van Zeist (Department of Organic Chemistry, University of Nijmegen). Mono-(5-carboxy-2-ethyl-pentyl)phthalate (metabolite V) (96% pure) was a kind gift of Dr Sjöberg (Department of Drugs, National Board of Health and Welfare, Uppsala, Sweden). The identity of all metabolites was confirmed with NMR and MS.

Isolation of hepatocytes. Male Wistar rats (Cpb : WU) (*Rattus norvegicus*) (200 g), male Dunkin Hartley guinea pigs (*Cavia porcellus*) (350 g) and male New Zealander rabbits (*Oryctolagus cuniculus*) (2500 g) were purchased from the Central Animal Laboratory, University of Nijmegen.

Cynomolgus monkeys (*Macaca fascicularis*) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). Young monkeys (2–3 years old) served as donors for kidneys, used in the production of poliomyelitis vaccine at that institute.

Rats, rabbits and guinea pigs were anesthetized by i.p. injection of sodium pentobarbital. In rabbits and guinea pigs, 2% lidocaine was injected subcutaneously in the abdominal wall as well. Monkeys were premedicated i.m. with atropine and xylazine and anaesthetized i.m. with ketamine. Monkey livers were perfused with ice-cold saline *in situ* and transported on ice to our laboratory within 60 min after hepatectomy.

Isolation of hepatocytes was based on methods described by Berry and Friend [20] and by Seglen [21]. Monkey liver was perfused with 500 mL of a Ca^{2+}-free HEPES buffer containing 0.25 mM EGTA and 1000 mL of this buffer without EGTA, followed by a recirculating perfusion with a 0.05% (w/v) collagenase containing HEPES buffer for 30 min. Guinea pig and rabbit liver were perfused with 500 mL Ca^{2+}-free HEPES buffer containing 0.25 mM EGTA and 500 mL of this buffer without EGTA, followed by a recirculated perfusion with 0.05% (w/v) collagenase for 10 min. Rat liver was perfused with 500 mL buffer of the Ca^{2+}-free buffer, followed by a recirculating perfusion with 0.05% (w/v) collagenase for 10 min. The perfusion rate was 50 mL/min and the temperature 37°.

The livers were gently torn apart and cells were dispersed in a Ca^{2+}-containing HEPES-TES buffer

* Abbreviations: DEHP, di(2-ethylhexyl)phthalate; MEHP, mono(2-ethylhexyl)phthalate; BNF, β-naphthoflavone; EROD, ethoxyresorufin O-dealkylation.

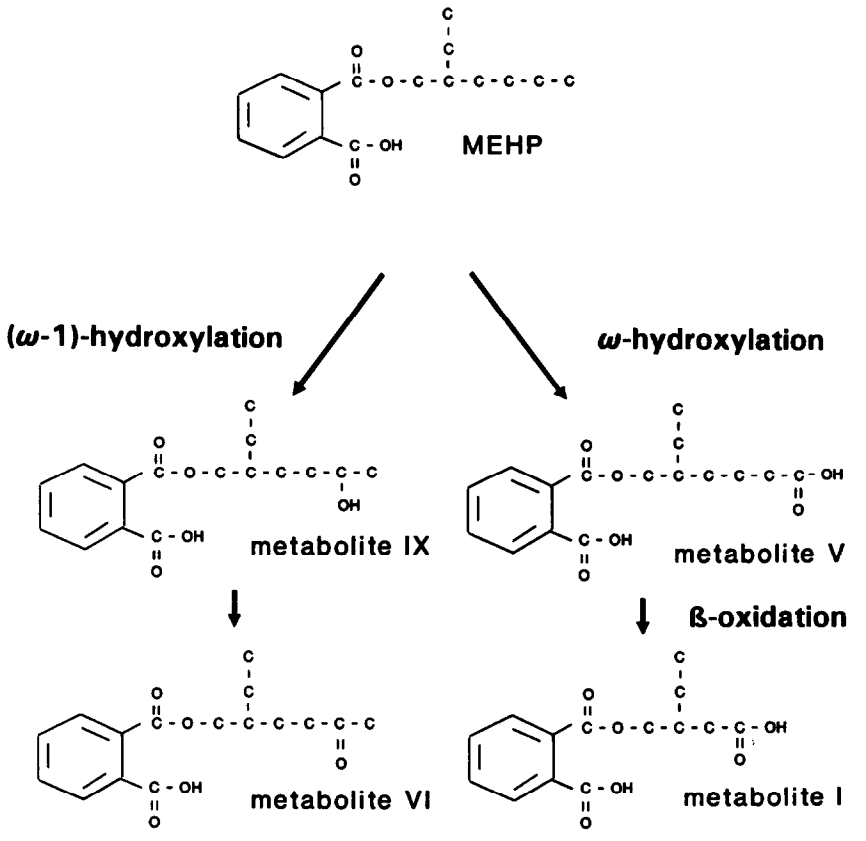

Fig. 1. The metabolism of MEHP. MEHP can undergo ω- and (ω-1)-hydroxylation reactions, with as (final) products metabolite IX, VI, V and I. Numbering of the metabolites is according to Albro *et al.* [38].

with 2% bovine serum albumin. For all four species, hepatocytes were obtained by a similar purification procedure of repeated filtering, washing and centrifugation at 50 *g* (rabbit, rat and guinea pig) and 100 *g* (monkey). Cell yields and viability were assessed using the Trypan blue exclusion test. Cell yield was for rabbits 1280×10^6 cells (80%), monkeys 1100×10^6 cells (89%), guinea pigs 700×10^6 cells (90%) and rats 400×10^6 cells (90%). Numbers in parentheses are the viability of the cells.

Cell culture and treatment. Hepatocytes were plated at a density of 9×10^6/10 mL medium in a 9 cm tissue culture dish (Nunc) in Williams' medium E supplemented with 5% (v/v) newborn calf serum, 2 mM glutamine, 1 μM insulin, 10 μM hydrocortison and gentamycin (50 mg/L). Cells were incubated in a humidified atmosphere in air (95%) and CO_2 (5%) at 37°. During the first 4 hr 4 mM Ca^{2+} and 4 mM Mg^{2+} were added to the medium. After 4 hr the medium was changed.

After 24 hr the medium was replaced by medium containing various concentrations of MEHP, metabolite V or metabolite VI dissolved in dimethylformamide [final concentration of dimethylformamide in medium was 0.1% (v/v)]. The culture media, containing the appropriate concentration of test compounds, were renewed every 24 hr.

The appearance of monolayers derived from all four species was very similar: cells attached and formed confluent monolayers within 24 hr of seeding. Cytotoxicity of the DEHP metabolites tested was studied in parallel experiments. In all four species, there was no decrease observed in the activity of the mitochondrial enzyme succinate-dehydrogenase towards 3-4,5-dimethylthiazpol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (assayed according to Mossman [22] and Denizot and Lang [23]), neither did the concentrations tested influence the lysosomal accumulation of neutral red (assayed as described by Borenfreund and Puerner [24]). Ethoxyresorufin O-dealkylation (EROD) activities were determined after treatment for 72 hr with 50 μM β-naphthoflavone (BNF) according to Wortelboer *et al.* [25].

Cell harvesting and preparation of cell homogenates. After 96 hr cells were harvested by removing the medium, washing the monolayer twice with ice-cold saline and scraping with a rubber policeman in cold sucrose–EDTA–Tris buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris–HCl, pH 7.4). Cells were centrifuged and the pellet was flash frozen in liquid nitrogen and stored at −80°. Cells were sonicated as described by Wortelboer *et al.* [25] using an MSE 100 Watt Ultrasonic Desintegrator. Part of the homogenate was flash frozen in liquid nitrogen and stored at −80° and it was used for determining lauric acid hydroxylation activities, and triglyceride and cholesterol con-

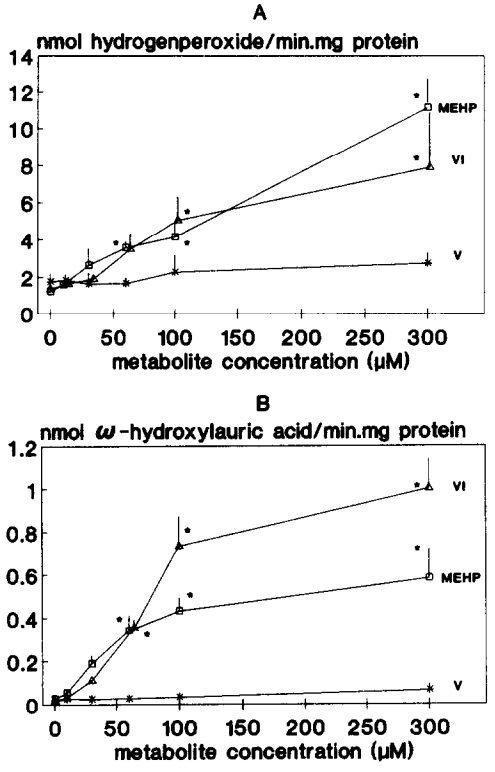

Fig. 2. Effect of MEHP, metabolite VI and metabolite V on palmitoyl-CoA oxidase (A) and lauric acid ω-hydroxylation (B) activities in primary cultures of rat hepatocytes. Hepatocytes were preincubated for 24 hr, followed by 72 hr treatment. Each metabolite was tested in three to four experiments; in each experiment, hepatocytes of different rats were used. Mean values ± SD of these experiments are presented. *Significantly different ($P \leq 0.05$) from 0 μM (ANOVA and Dunnett's test).

centrations. Part of the homogenate was freeze–thawed three times, followed by centrifugation at 9000 *g* for 10 min. Supernatants were quickly frozen in liquid nitrogen and stored at −80° until they were thawed for determination of palmitoyl-CoA oxidase activity.

For the extraction of total RNA, cells were harvested in 4 M guanidium isothiocyanate solution (pH 7), containing 0.1 M 2-mercaptoethanol, 0.01 M Tris (pH 7.4) and 0.5% *N*-lauroyl sarcosine. Total RNA was extracted according to the method of Cathala *et al.* [26]. Typical extractions yielded 80 μg RNA/90 mm dish.

Biochemical determinations. Palmitoyl-CoA oxidase was measured as described by Reubsaet *et al.* [27]. Lauric acid hydroxylation activities were determined as described previously [28]. Protein concentration was determined by the Coomassie brilliant blue method [29] using crystalline bovine serum albumin as standard. Triglyceride and cholesterol concentrations were determined enzymatically using test kits from Boehringer Mannheim.

Northern blotting. Total RNA fractions were submitted to electrophoresis and transferred by capillary blotting to Hybond-N filters (Amersham International, U.K.). Filters were then hybridized to ^{32}P randomly labelled full length cDNA probes. Filters were washed with high stringency (0.5 × SSC, 55°). Filters were autoradiographed for 72 hr.

Analysis of metabolites in culture media. After 48 (24–48) and 96 (72–96) hr medium was collected and stored at −20°. A part of the medium was hydrolysed overnight with β-glucuronidase/arylsulphatase and the other part was left untreated. After the addition of the internal standard monohexylphthalate, samples were acidified and extracted with diethylether. After derivatization with triethyloxonium tetrafluoroborate samples were analysed by GC–MS (Varian Saturn, GC–MS) as described elsewhere [30]. Identification of the metabolites was carried out by the combination of full scan spectra, selected ion fragments (*m/z* 149 and *m/z* 177) and retention times of pure standards.

Quantification was carried out by reference to calibration curves constructed from (media) samples containing MEHP, metabolite IX, metabolite VI and metabolite V (see Fig. 1). Knowing that the slope of the calibration curves of all four metabolites was comparable, metabolite I was quantified using the calibration curve of metabolite V.

RESULTS

Effects of metabolites of DEHP in rat hepatocytes

The effect of treatment of rat hepatocytes with MEHP, metabolite VI and metabolite V on peroxisomal palmitoyl-CoA oxidase activity is shown in Fig. 2A. MEHP and metabolite VI induced this activity dose dependently. At the highest concentration tested (300 μM), this activity was 6–9-fold induced. MEHP and metabolite VI appeared to have the same potency to induce peroxisomal β-oxidation. Metabolite V did not induce palmitoyl-CoA oxidase activity.

Lauric acid ω-hydroxylation activity was dose-dependently induced by both MEHP and metabolite VI (Fig. 2B). At 300 μM this activity was 20-fold increased by MEHP, 55-fold by metabolite VI and 3-fold by metabolite V. Lauric acid (ω-1)-hydroxylation activity was 6–9-fold increased by both MEHP and metabolite VI (results not shown), but this activity is not supposed to be characteristic for P450 4A1 [31].

In Fig. 3 it is shown that after 96 hr in culture, the RNA levels for both cytochrome P450 4A1 and acyl-CoA oxidase are dose-relatedly induced by treatment with MEHP.

The lowest concentration tested which significantly induced both palmitoyl-CoA oxidase activity and lauric acid ω-hydroxylation activity was for MEHP 60 μM and for metabolite VI 100 μM. From this experiment we concluded that metabolite VI and MEHP are equally potent in inducing peroxisome proliferation. In the subsequent experiments we have limited the testing in hepatocytes of several species to only one metabolite, i.e. MEHP.

Effects of MEHP in hepatocytes of rabbits, guinea pig and monkey compared to rat

In parallel experiments it was shown that primary cultures of rat, monkey and rabbit hepatocytes did respond to the known P450 inducer BNF. EROD

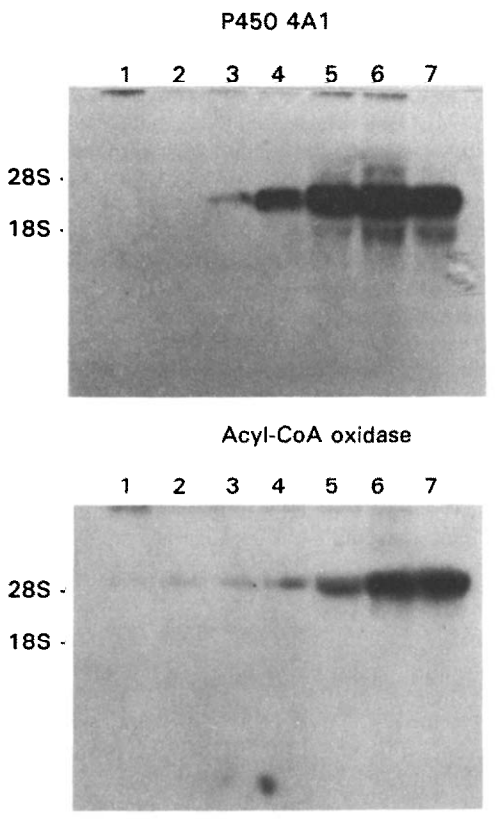

Fig. 3. Northern analysis of RNA isolated from rat hepatocytes treated *in vitro* with MEHP. RNA samples (20 μg) were hybridized with probes for peroxisomal acyl-CoA oxidase and cytochrome P450 4A1. Lane 1: 24 hr after hepatocyte isolation; lane 2–7: 96 hr after hepatocyte isolation and after treatment for 72 hr with 0, 10, 30, 60, 100 and 300 μM MEHP.

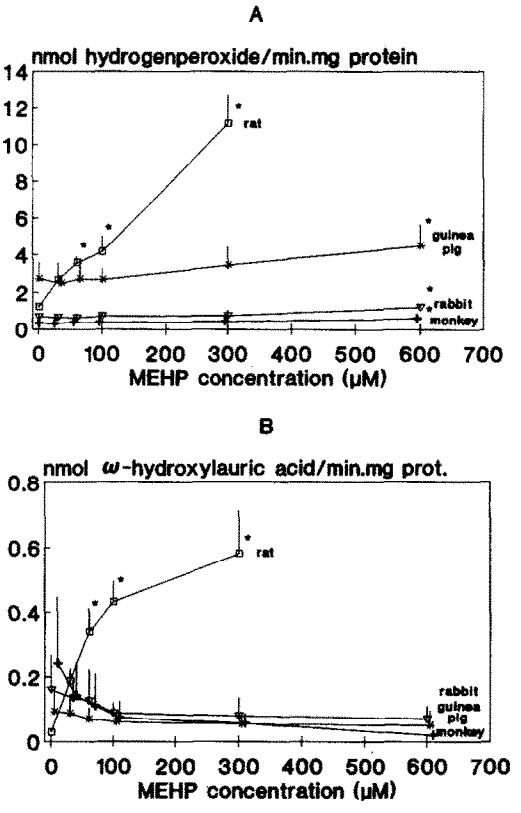

Fig. 4. Effect of MEHP on palmitoyl-CoA oxidase (A) and on lauric acid ω-hydroxylation (B) activities in primary cultures of rat, rabbit, guinea pig and monkey hepatocytes. Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in three to five experiments; in each experiment, hepatocytes of different animals were used. Mean values ± SD of these experiments are presented. *Significantly different ($P \leq 0.05$) from 0 μM (ANOVA and Dunnett's test).

activities from BNF-treated hepatocytes were 8–44-fold increased in rats, 70–140-fold in rabbits and 30–60-fold in monkeys. No induction of EROD activity by BNF was found in guinea pig hepatocytes.

Only at the highest concentration of MEHP tested (600 μM), a 1.8-fold induction of palmitoyl-CoA oxidase activity was found in primary cultures of rabbit, guinea pig and male monkey hepatocytes (Fig. 4A). No induction of lauric acid ω-hydroxylation (Fig. 4B) or (ω-1)-hydroxylation (results not shown) activities was found in these three species. A considerable inter-individual variation was observed in the parameters studied. In hepatocytes of three female monkeys a significant (1.9-fold) induction of palmitoyl-CoA oxidase activities, after treatment with 600 μM MEHP, was determined. No increase in lauric acid ω-hydroxylation activity was observed (results not shown).

With the P450 4A1 and acyl-CoA oxidase cDNA probes used in the rat studies, no signals could be detected after northern blot analysis of RNA samples of hepatocytes of rabbit, guinea pig or monkey treated *in vitro* with MEHP.

After treatment of guinea pig hepatocytes with MEHP, an increase in triglyceride level in the hepatocytes was observed (Fig. 5A), while in (male) monkey hepatocytes the triglyceride level was decreased. In hepatocytes of rabbits, triglyceride levels were slightly decreased after treatment with 60 and 100 μM MEHP, but a small increase was found after treatment with 600 μM MEHP. In rat hepatocytes no significant alteration in triglyceride levels upon treatment with MEHP was found. The cholesterol concentration in the liver cells of rat, guinea pig and monkey was not influenced by treatment with MEHP, while in hepatocytes of rabbits, a small decrease in cholesterol concentration was found after treatment with high concentrations of MEHP (Fig. 5B).

Summarizing, the effects of MEHP in rat hepatocytes are much stronger than in hepatocytes of the other three species.

Biotransformation of DEHP metabolites in primary cultures of rats, rabbits, guinea pigs and monkeys

Rats. Media samples were collected after treatment of hepatocytes with 100 μM MEHP. In the 24–48 hr

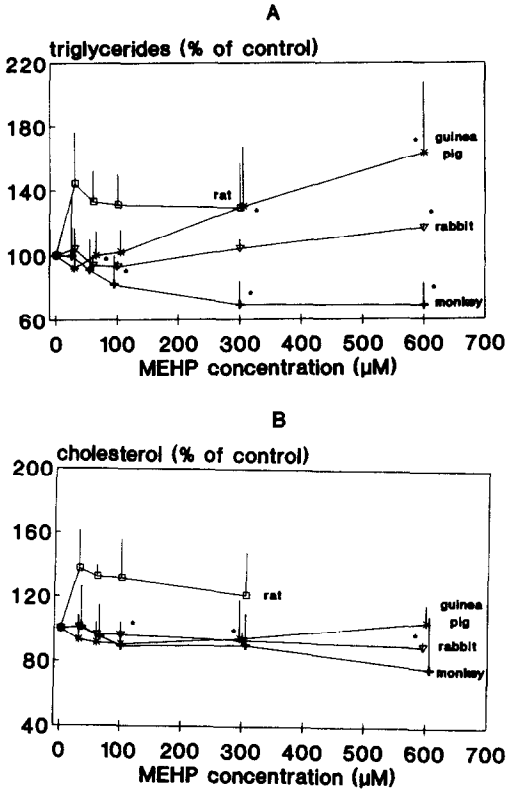

Fig. 5. Effect of MEHP on triglyceride concentration (A) and cholesterol concentration (B) in hepatocytes of rats, rabbits, guinea pigs and monkeys. All values are expressed as percentages of control values. Control triglyceride values are for rat, 135 nmol/mg protein; for guinea pig, 72 nmol/mg protein; for rabbit, 148 nmol/mg protein and for monkey, 172 nmol/mg protein. Control cholesterol values are for rat, 145 nmol/mg protein; for guinea pig, 82 nmol/mg protein; for rabbit, 148 nmol/mg protein and for monkey, 196 nmol/mg protein. Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in three to five experiments; in each experiment, hepatocytes of different animals were used. Means ± SD of these experiments are presented. *Statistically significant ($P \leq 0.05$) from 0 μM (Kruskal–Wallis and Mann–Whitney test).

samples at least four metabolites were identified. Due to the availability of reference compounds for the metabolites VI, IX and V, we could positively identify these metabolites in our samples. The unknown metabolite is probably metabolite I, since the mass spectrum of this metabolite had a characteristic fragment of m/z 143. Almost all metabolites and the parent compound were present in non-conjugated form. In the 72–96 hr samples the same four metabolites were determined, but now metabolite V and metabolite I were the major metabolites formed (Fig. 6). In media of primary cultures of rat hepatocytes treated with metabolite V, 20% was metabolized to metabolite I. Neither metabolite V nor metabolite I was conjugated. After treatment with metabolite VI no other metabolites were detected. A small proportion of metabolite VI was conjugated (results not shown).

Guinea pig, rabbit and monkey. In media of hepatocytes of these three species treated *in vitro* with MEHP, the same four metabolites were identified as with the rat hepatocytes. In media from guinea pig and rabbit hepatocytes the concentration of oxidized metabolites in 24–48 hr and 72–96 hr periods was comparable to the findings in the rat (Fig. 6). In media of primary cultures of monkey hepatocytes, lower concentrations of the oxidized metabolites were detected compared to rabbit and guinea pig. Furthermore, the concentrations of these metabolites in media from monkey hepatocytes declined with time. In guinea pig, rabbit and monkey, a major part of the parent compound MEHP and a part of the metabolites VI and IX was conjugated. This conjugation activity was not lost during culture.

Summarizing, all species showed metabolic capacity towards MEHP with a low oxidizing activity in the monkey and a low conjugating capacity in the rat.

DISCUSSION

A major question in the assessment of carcinogenicity of DEHP and other peroxisome proliferators is what mechanisms underly species differences in sensitivity for peroxisome proliferation. In addition, to assess the risk of these compounds for man it is necessary to quantify these species differences.

In rat hepatocytes peroxisomal palmitoyl-CoA oxidase activity is already significantly increased (2.9-fold) at a concentration of 60 μM MEHP. In rabbit, guinea pig and monkey hepatocytes, a significant induction of this activity (1.8-fold) is observed only after treatment with 600 μM MEHP. These results indicate that MEHP can induce peroxisome proliferation in the monkey, guinea pig and rabbit, but that these latter species are less sensitive for peroxisome proliferation than the rat. Using linear regression analysis, we have calculated at which MEHP concentration the palmitoyl-CoA oxidase activity is 50% increased in the four species tested (Table 1). This value is at least 30-fold higher for rabbits, guinea pigs and monkeys, than for rats.

Our results with primary cultures of monkey and guinea pig hepatocytes are in agreement with observations that these species are less sensitive for peroxisome proliferation than rats and mice after *in vitro* and *in vivo* treatment with peroxisome proliferating compounds [4–11, 18]. Gibson [8] considered rabbits and hamsters to be intermediately sensitive for peroxisome proliferation. This conclusion was made after *in vivo* treatment with the peroxisome proliferating compound ciprofibrate. Our results on *in vitro* treatment of rabbit hepatocytes with MEHP indicate that rabbits are not very sensitive for peroxisome proliferation.

After treatment of rat hepatocytes with MEHP, we have found a close association between induction of lauric acid ω-hydroxylation activity and peroxisomal palmitoyl-CoA oxidase activity, in accordance with earlier published studies [3, 4, 19]. Using linear regression analysis, we have calculated

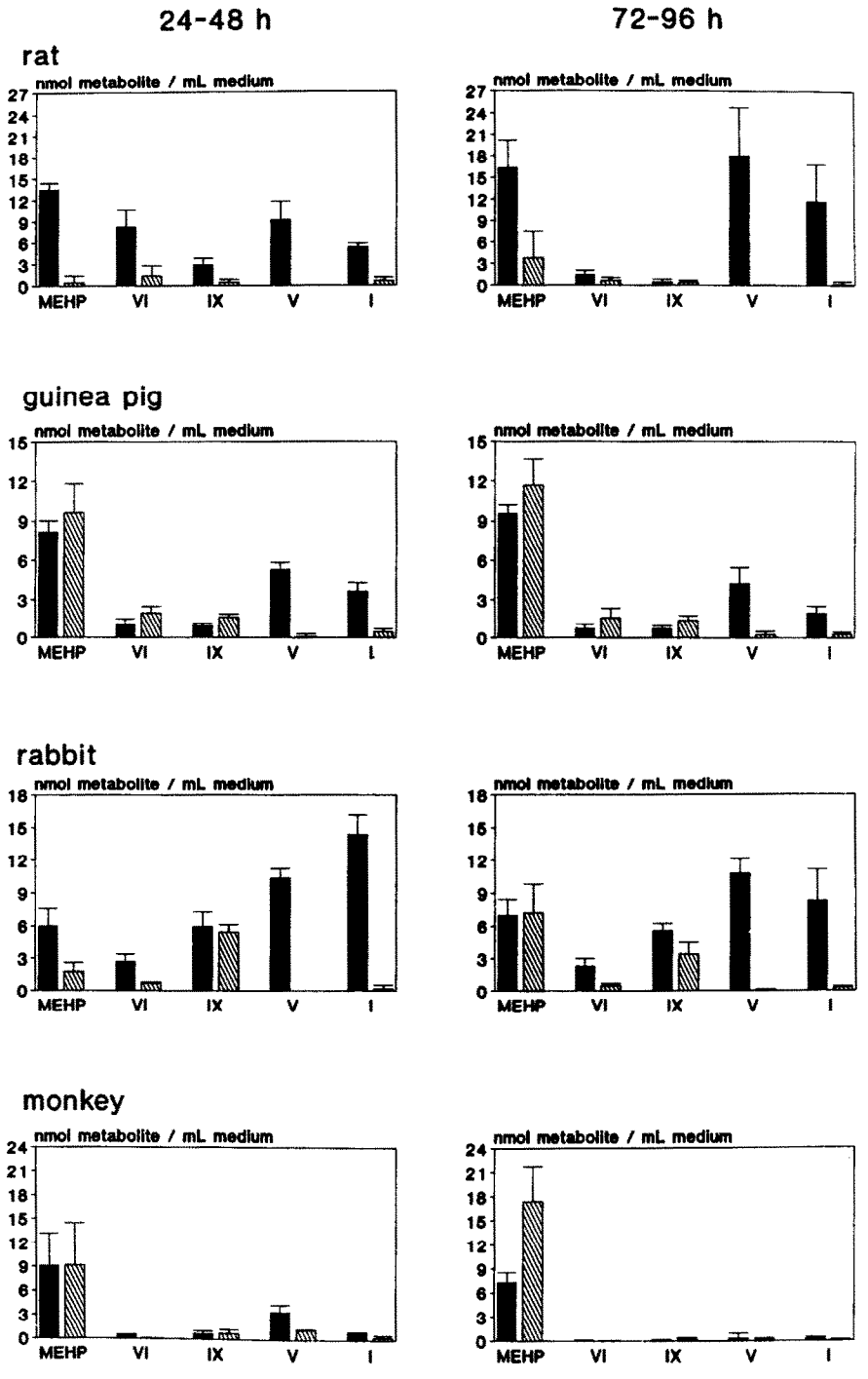

Fig. 6. Concentrations of MEHP and its metabolites in media from primary hepatocyte cultures of rats, guinea pigs, rabbits and (male) monkeys treated *in vitro* with 100 μM MEHP. Results after 48 hr (24–48 hr) and 96 hr (72–96 hr) in culture are shown. Hepatocytes were preincubated for 24 hr, followed by 72 hr of treatment. Each metabolite was tested in three experiments; in each experiment, hepatocytes of different animals were used. Mean values ± SD of these experiments are presented. Key: (▨) glucuronidated metabolites; (■) free metabolites.

Table 1. Estimation of the concentration of DEHP metabolites causing a 50% increase in enzyme activity after *in vitro* treatment of hepatocytes of different species

		Palmitoyl-CoA oxidase activity (μM)	Lauric acid ω-hydroxylation activity (μM)
Rat			
MEHP		14.8	4.5
Metabolite VI		12.1	6.9
Metabolite V		262	78.6
Rabbit	MEHP	440	ND
Guinea pig	MEHP	485	ND
Monkey	MEHP	408	ND

ND, it was not possible to calculate these values since activities decreased with increasing MEHP concentration.

that a 50% increase in lauric acid hydroxylation activities in rat hepatocytes is found at lower MEHP concentrations than a 50% increase in peroxisomal palmitoyl-CoA oxidase activities (Table 1), again demonstrating that induction of lauric acid ω-hydroxylation activities is the more sensitive marker for peroxisome proliferation. The increase in activity of these enzymes is accompanied by increases in mRNA level for these enzymes. This is partly, if not entirely, due to an increase in the transcription of the respective genes [32, 33]. However, the observed increase in palmitoyl-CoA oxidase activities in guinea pig, monkey and rabbit hepatocytes after treatment with MEHP is not accompanied by an increase in lauric acid ω-hydroxylation activity. Similar findings have been reported after *in vivo* treatment of marmoset monkeys with ciprofibrate [10]. The proposed mechanistic inter-relationship between induction of peroxisomal enzymes and induction of lauric acid ω-hydroxylation activities, as proposed by Sharma *et al.* [3], might therefore not be valid in rabbits, guinea pigs and monkeys.

When we studied the triglyceride concentration in the MEHP-treated hepatocytes, we observed different trends in the species tested. In guinea pig hepatocytes, hepatic triglyceride levels were increased by MEHP treatment. In rat and rabbit hepatocytes these levels were much less altered, while in monkey hepatocytes, a decrease in hepatic triglyceride levels was found. Watanabe *et al.* [7] reported that after *in vivo* treatment of guinea pigs with the peroxisome proliferator bezafibrate, an increase in hepatic triglyceride level was observed; in rhesus monkeys a decrease was found, while in rats and rabbits no alteration of liver triglyceride levels was observed. The effects observed in our *in vitro* studies with rats, rabbits, monkeys and guinea pigs, show striking similarities with those observed after *in vivo* exposure. Further studies on species differences in fatty acid metabolism are necessary to explain these results. Kocarek and Feller [34] found a slight increase in triglyceride biosynthesis in rat hepatocytes treated *in vitro* with three peroxisome proliferating compounds.

Mitchell *et al.* [14] have proposed that metabolite VI is the active peroxisome proliferator of DEHP in rats. We doubt if this is true since our studies indicate that both MEHP and metabolite VI have the same potency to induce palmitoyl-CoA oxidase activities and lauric acid ω-hydroxylation. In addition, our biotransformation studies indicate that the concentration of metabolite VI formed from MEHP in rats is low according to the effects found.

Our metabolism studies indicate that in all species the pattern of oxidized metabolites does not differ between the species. Metabolite VI is formed in all four species. In monkeys, the formation of oxidized metabolites decreased in time, indicating a loss of cytochrome P450 activity, since the hydroxylation of MEHP is P450 dependent [35]). In guinea pigs and rabbits the amounts and pattern of oxidized metabolites found between 24–48 hr and 72–96 hr were comparable. In rats, higher concentrations of metabolite V and metabolite I were formed between 72–96 hr compared to 24–48 hr. This indicates that ω-hydroxylation activity towards MEHP is induced after 48 hr. Lhuguenot *et al.* [36] studied the metabolism of MEHP *in vivo* and *in vitro* in the rat and observed that after repeated administration the concentrations of ω-oxidation metabolites of MEHP were increased. It is possible that the ω-hydroxylation of MEHP is mediated by P450 enzymes associated with the ω-hydroxylation of fatty acids [35]. Induction of these enzymes by MEHP results not only in an increased ω-hydroxylation of fatty acids, but might also result in an increased ω-hydroxylation of MEHP.

Metabolite I is supposed to be formed by (peroxisomal) β-oxidation of metabolite V, and Turnbull and Rodricks [37] have proposed that monitoring the formation of metabolite I might be useful for quantifying the target dose. Since guinea pig and rabbit, although less sensitive for peroxisome proliferation, do form high concentrations of metabolite I, we think that formation of metabolite I is not *per se* an indicator of increased peroxisomal β-oxidation activities.

In media of hepatocytes from the rabbit, guinea pig and monkey, glucuronide conjugates of MEHP, metabolite VI and metabolite IX were found. Metabolites V and I were hardly conjugated. The rat hardly formed any conjugated products at all. This finding is in accordance with the observations that rats do not excrete conjugated metabolites of DEHP in urine [38]. A lower conjugating activity towards DEHP metabolites in the rat might mean an increased bioavailability of these metabolites in the hepatocytes. However, we doubt whether there is a causal relationship between induction of peroxisome proliferation and conjugating activity, since mice are very sensitive for peroxisome proliferation and do form conjugated DEHP metabolites [38].

Our studies show that the observed species differences in peroxisome proliferation are not the result of species differences in the formation of active metabolites. We propose that species differences in peroxisome proliferation might be due to differences in fatty acid metabolism, in particular species differences in the partitioning of fatty acids between β-oxidation and esterification. With the recent description of a peroxisome proliferator-

activated receptor as a transcription factor for peroxisomal β-oxidation genes (review: Green [12]), it is also possible that species differences in peroxisome proliferation might be caused by variation in expression of peroxisome proliferator-activated receptor, or in the regulation of the genes that are under the control of this receptor. To assess the toxicity of peroxisome proliferating compounds to humans, further studies on species differences in peroxisome proliferation should focus on these two aspects.

Acknowledgements—The authors wish to thank Dr A. A. J. van Iersel and A. Timmerman (National Institute of Public Health and Environmental Protection (RIVM, Bilthoven) for assistance in obtaining the monkey livers. We wish to thank Dr Baadehuysen from the Clinical–Chemical Laboratory of the Radboud Hospital (University of Nijmegen) for performing the triglycerides and cholesterol determinations. We wish to thank Dr G. G. Gibson (University of Surrey, U.K.) for his hospitality and advice in determining RNA levels in the hepatocytes. H.D. received financial support from the European Science Foundation to work at the laboratory of Dr Gibson.

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