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IDENTIFICATION OF THE PROXIMATE PEROXISOME PROLIFERATOR(S) DERIVED FROM DI (2-ETHYLHEXYL) ADIPATE AND SPECIES DIFFERENCES IN RESPONSE

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Abstract—Identification of the proximate peroxisome proliferator(s) derived from di (2-ethylhexyl) adipate (DEHA) has been achieved using primary hepatocyte cultures derived from different species and cyanide-insensitive fatty acyl CoA oxidase (PCO) as a marker enzyme for peroxisome proliferation. In rat and mouse hepatocytes, the parent compound (DEHA) had no effect on peroxisomal β-oxidation, but primary metabolites of DEHA, mono (2-ethylhexyl) adipate (MEHA) and 2-ethylhexanol (EH), were approximately equipotent in PCO induction (5-fold at 0.5 mM final concentration). The secondary metabolite of DEHA, 2-ethylhexanoic acid (EHA), was in both species the most potent peroxisome proliferator (25- and 9-fold induction in mice and rats, respectively, at 1 mM final concentration). At 2 mM final concentration a tertiary metabolite of DEHA, 2-ethyl-5-hydroxyhexan-1-oic acid, was less effective in mouse and rat hepatocytes at inducing PCO (15- and 5-fold, respectively). 2-Ethyl-5-oxohexan-1-oic acid and 2-ethylhexan-1,6-dioic acid had little effect (2–3-fold in both rat and mouse hepatocytes). Thus, EHA was identified as the proximate peroxisome proliferator of DEHA and mouse hepatocytes were approximately twice as sensitive as rat hepatocytes to peroxisome proliferation due to MEHA, EH and EHA. We investigated further species differences in response to peroxisome proliferators by using guinea pig and marmoset primary hepatocyte culture. None of the chemicals studied stimulated peroxisomal β-oxidation in these species up to a final concentration of 2 mM. Higher concentrations lead to cytotoxicity. This lack of sensitivity of guinea pig and marmoset hepatocytes is in agreement with previous studies with di (2-ethylhexyl) phthalate metabolites, suggesting the absence of a threat of hepatocarcinogenic damage to these species and confirming that primary hepatocytes cultures are useful models for investigating the phenomenon of peroxisome proliferation.

Di (2-ethylhexyl) adipate (DEHA‡) is a plasticizer used to impart "stretch" and "cling" to polyvinylchloride plastics. In some instances, the plasticizer may comprise up to 25% of the finished product. Furthermore, under some conditions the plasticizer may migrate from the plastic into lipophilic surroundings. Hence, the potential exists for human exposure [1].

DEHA is non-genotoxic [2–7] but has been shown to increase the incidence of hepatocellular tumours in mice [8]. DEHA is a hepatic peroxisome proliferator in rodents [9] and the mechanism for its apparent carcinogenicity is postulated to be via this phenomenon [5, 10].

In rats, orally administered DEHA is hydrolysed rapidly to adipic acid and 2-ethylhexanol [11]. The latter compound is metabolized extensively to 2-ethylhexanoic acid (EHA) [12] which is, in turn, oxidized [13]. This metabolic pathway is illustrated in Fig. 1.

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‡ Abbreviations: DEHA, di (2-ethylhexyl) adipate; DiEHA, 2-ethylhexan-1,6-dioic acid; EH, 2-ethylhexanol; EHA, 2-ethylhexanoic acid; 5-OH-EHA, 2-ethyl-5-hydroxyhexan-1-oic acid; 5-keto-EHA, 2-ethyl-5-oxohexan-1-oic acid; MEHA, mono (2-ethylhexyl) adipate; PCO, cyanide-insensitive fatty acyl CoA oxidase.

We have demonstrated previously that the peroxisome proliferation observed with di (2-ethylhexyl) phthalate (a plasticizer related to DEHA) is due largely to monoester metabolites [e.g. mono (2-ethyl-5-oxo-hexyl) phthalate] and that marked species differences in response to these proximate peroxisome proliferators exist [14, 15]. Hence, the objectives of the present studies were, firstly, to identify the proximate peroxisome proliferator(s) derived from DEHA and, secondly, to examine possible species differences in response to these agents.

MATERIALS AND METHODS

Chemicals and culture medium

Leibowitz L15 culture medium, foetal bovine serum, tryptose phosphate broth and collagenase were obtained from Flow Laboratories (Irvine, U.K.). DEHA was obtained from ICI Chemicals and Polymers (Wilton, U.K.) and adipic acid, 2-ethylhexanol and 2-ethylhexanoic acid were purchased from the Sigma Chemical Co. (Poole, U.K.) and the British Drug House (Liverpool, U.K.).

Synthesis of metabolites of DEHA

2-Ethyl-5-oxohexan-1-oic acid (5-keto-EHA). Diethyl ethylmalonate (47 g) was added drop-wise to a suspension of sodium hydride (3 g) in dry boiling

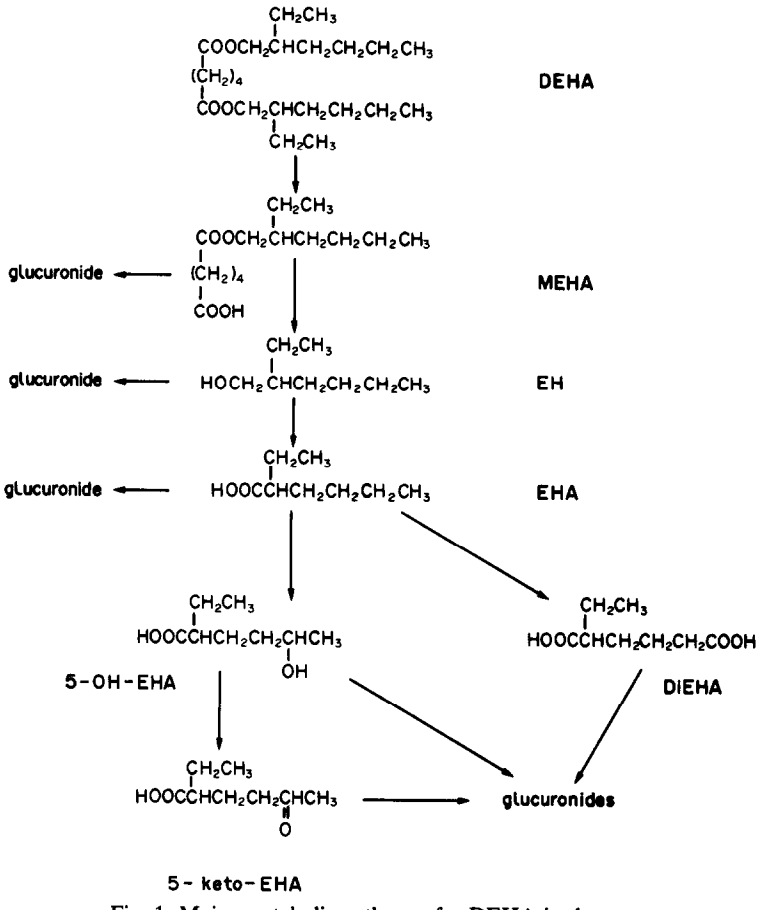

Fig. 1. Major metabolic pathways for DEHA in the rat.

tetrahydrofuran. Methyl vinyl ketone (26.3 g) was added drop-wise to the stirred solution which was then allowed to cool. The cold reaction mixture was poured into water and extracted with ether to give an oil which was distilled (126–130°, 4 Torr) to yield diethyl ethyl-3′-oxobutylmalonate (19 g). This malonate was de-esterified with aqueous potassium hydroxide and de-carboxylated with boiling 6 M sulphuric acid to give a good yield of 5-keto-EHA.

2-Ethyl-5-hydroxyhexan-1-oic acid (5-OH-EHA). 5-Keto-EHA (0.558 g) was dissolved in ethanol (10 mL) and sodium borohydride (0.114 g) was added in three equal portions over 1.5 hr. The mixture was stirred for a further 3 hr and then evaporated to dryness. The residue was dissolved in chloroform (50 mL) and shaken vigorously with 1 M hydrochloric acid (20 mL). The chloroform solution was separated from the aqueous layer, evaporated and the residue purified by silica column chromatography (eluting with hexane/ethyl acetate 3/1) to give 5-OH-EHA as its lactone.

2-Ethylhexan-1,6-dioic acid (DiEHA). The method of Reed and Thornley [16] was used to prepare DiEHA (m.p. 48–49°; lit. m.p. 48°).

Mono (2-ethylhexyl) adipate (MEHA). The method of Komarov and Kotlyarevskii [17] was used to prepare mono (2-ethylhexyl) adipate. The crude compound was purified by acid/base extraction and then by silica column chromatography (eluting with chloroform/methanol 95/5), and, finally, was distilled at reduced pressure.

All compounds gave satisfactory mass spectra and proton nuclear magnetic resonance spectra and were found to be > 97% pure by GLC analysis (after derivatization with diazomethane).

Animals

Experiments were performed on male Alderley Park mice (Swiss, 25–30 g), male Alderley Park rats (Wistar-derived, 180–220 g), male Alderley Park guinea pigs (Dunkin Hartley, 400–500 g) and male marmosets (*Callithrix jacchus*, 350–500 g). All animals were obtained from the Animal Breeding Unit, Imperial Chemical Industries, Pharmaceuticals Division (Alderley Park, Cheshire, U.K.). Rats and guinea pigs were allowed food *ad lib.*, while marmosets were fed once daily. All animals had free access to water. A 12 hr light/dark cycle (6–18 hr) was operated. Prior to isolation of hepatocytes, rats and guinea pigs were killed by inhalation of excessive diethyl ether and marmosets by injection of a lethal dose of pentobarbital.

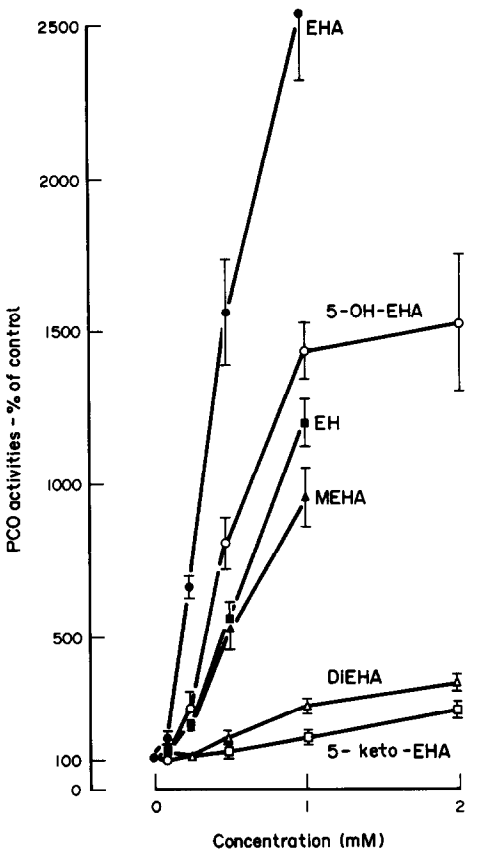

Fig. 2. Effect of DEHA metabolites [(□) 5-keto-EHA; (●) EHA; (▼) DiEHA; (▲) MEHA; (■) EH; (○)5-OH-EHA] on peroxisomal β-oxidation in mouse hepatocyte cultures. Values are expressed as means ± SD of three different cells preparations. Control value = 1.13 ± 0.5 nmol NAD^+ reduced/min/mg protein.

Hepatocyte isolation and culture

Hepatocytes were isolated from male mice, rats, guinea pigs and marmosets, by a two step *in situ* perfusion technique as described previously by Mitchell *et al.* [18] and Elcombe and Mitchell [15]. Strains and weights of animals were also as described in these articles. Aseptic technique were used throughout the procedure. Viability of hepatocyte preparations (> 90%) was determined by Trypan blue dye exclusion.

Falcon tissue culture flasks (25 cm^2) were seeded with 2×10^6 viable hepatocytes in 14 mL of CL15 medium (Leibowitz L15 containing 8.3% foetal bovine serum, 8.3% tryptose phosphate broth, 42.3 IU/mL penicillin G, 8.2 μg/mL glutamine, 10^{-6} M insulin and hydrocortisone). Vitamin C (50 mg/L) was included in the guinea pig and marmoset cultures. Cultures were maintained at 37° in air, and 4, 24, 48, 72 hr after seeding, the spent medium and any detached cells were aspirated and fresh medium applied. Test compounds dissolved in dimethylformamide were added to the monolayers at each 24 hr medium change. Dimethylformamide (10 μL/flask) produced no cytotoxic effect. The monolayer cultures were harvested 96 hr after seeding. The medium was discarded and the monolayer washed twice in 2 mL of SET buffer (0.25 M sucrose/5 mM EDTA/20 mM Tris–HCl, pH 7.4, 4°). The cells were removed from the flask by scraping into 1 mL of SET buffer, disrupted by sonication and stored at −70°.

Enzyme assay

Cyamide-insensitive fatty acyl CoA oxidase (PCO) is a marker enzyme for peroxisomes [19]. This was measured in cell sonicates as the palmitoyl-CoA-dependent reduction of NAD^+, in the presence of cyanide, by the method described by Bronfman *et al.* [20] with some modifications [18].

Protein determination

Protein content of the samples was determined following the method of Lowry *et al.* [21] using bovine serum albumin as standard.

RESULTS

Cultured mouse hepatocytes

Marked differences in potency for the stimulation of peroxisomal β-oxidation (PCO) were observed following the exposure of mouse hepatocytes to various metabolites of DEHA. The primary metabolites of DEHA, MEHA and EH, were approximately equipotent in inducing PCO (Fig. 2). The secondary metabolite EHA was the most potent peroxisome proliferator examined, stimulating PCO by 25-fold at a concentration of 1 mM. The hydroxylated metabolite of EHA, 5-OH-EHA, was considerably less potent than EHA. The other tertiary metabolites of DEHA, 5-keto-EHA and DiEHA, had little effect upon PCO causing only a 2–3-fold induction at a concentration of 2 mM.

EHA, EH and MEHA at concentrations above 1 mM resulted in cytotoxicity which was characterized by "blebbing", rounding of the cells and detachment from the cultured flasks.

Cultured rat hepatocytes

DEHA had no effect upon peroxisomal β-oxidation in rat hepatocyte cultures, even at concentrations of up to 5 mM (Fig. 3). MEHA and EH were again approximately equipotent, inducing PCO by 4–5-fold at 0.5 mM (Fig. 3). Higher concentrations of these agents resulted in cytotoxicity. EHA and 5-OH-EHA (Fig. 3) stimulated PCO by approximately 10- and 5-fold, respectively, at a concentration of 2 mM. 5-Keto-EHA and DiEHA (Fig. 3) had little effect upon PCO activity.

Cultured guinea pig and marmoset hepatocytes

None of the chemicals studied stimulated peroxisomal β-oxidation in guinea pig or marmoset hepatocytes up to concentrations of 2 mM (Fig. 3). Higher concentrations could not be used because of concomittant cytotoxicity.

DISCUSSION

Several laboratories have demonstrated that

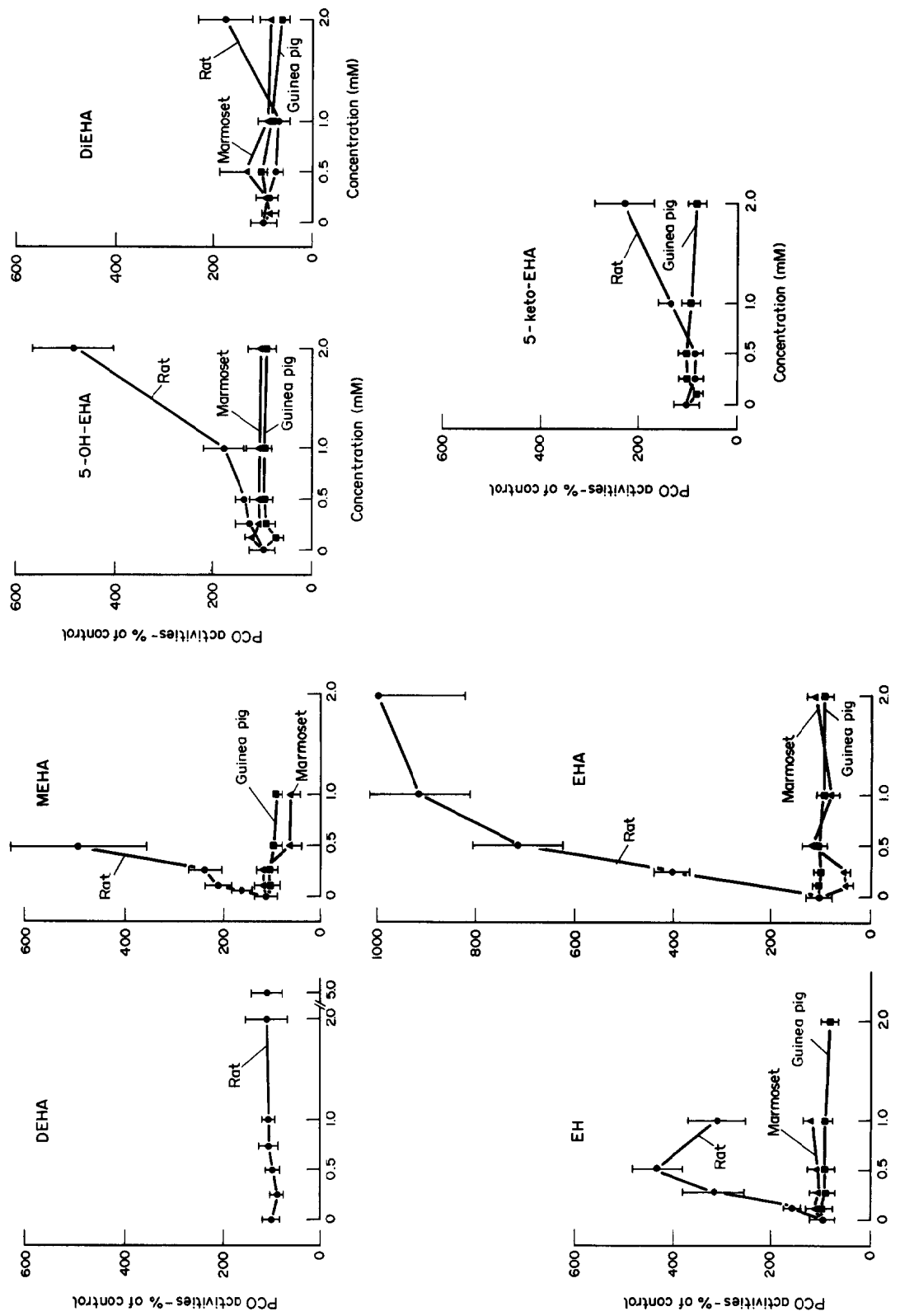

Fig. 3. Effect of DEHA, MEHA, EH, EHA, 5-OH-EHA, DiEHA and 5-keto-EHA on peroxisomal β-oxidation in rat, guinea pig and marmoset hepatocyte cultures. Values are expressed as means ± SD of three different experiments. Control value = 2.02 ± 0.7 nmol NAD^+ reduced/min/mg protein.

primary hepatocyte cultures are useful models for the investigation of peroxisome proliferation [18, 22–24]. Hepatocyte cultures, even allowing for their deficiencies, are useful for the identification of proximate peroxisome proliferators and the study of species differences in response because confounding factors such as absorption and metabolism can be eliminated or controlled [14, 15, 25]. Hence, these culture systems have the ability to define intrinsic cellular differences in response to peroxisome proliferators.

The present study demonstrates the lack of peroxisome proliferation following the exposure of rat and mouse hepatocytes to DEHA. This observation is in conflict with previous data generated *in vivo* [9, 26]. This apparently anomalous result may be explained by the inability of hepatocytes to hydrolyse DEHA to adipic acid and 2-ethylhexanol (Cornu MC, unpublished data).

The two primary metabolites of DEHA, MEHA and EH, were equipotent peroxisome proliferators in cultured rat and mouse hepatocytes. However, the further product of metabolism, EHA, was clearly the most potent metabolite of DEHA examined. This suggests that EHA is the proximate peroxisome proliferator derived from DEHA. Further metabolism of EHA to 5-OH-EHA, 5-keto-EHA or DiEHA markedly decreased its potency as a peroxisome proliferator.

These data have confirmed the initial observations of Moody and Reddy [9] who showed EH and EHA to be peroxisome proliferators in rats. However, from such *in vivo* data it is not possible to define categorically intrinsic potencies of peroxisome proliferators due to uncertainties of administered dose, bioavailability and further metabolism.

It is noteworthy that DEHA has increased the incidence of hepatic tumours in mice, but not in rats [8]. One might speculate that this species difference could be due to quantitative differences in peroxisome proliferation due to DEHA metabolites. Indeed, the present studies illustrate that mouse hepatocytes are approximately twice as sensitive as rat hepatocytes to peroxisome proliferation due to EHA, EH and MEHA. This suggestion should be viewed cautiously since different strains of animals were used in the studies.

Our studies indicate the lack of sensitivity of guinea pig and marmoset hepatocytes to DEHA metabolites, including the proximate proliferator EHA. These data are similar to those obtained previously using di (2-ethylhexyl) phthalate metabolites where little, if any, peroxisome proliferation was observed in guinea pig, marmoset or human hepatocytes [15]. This suggests that human hepatocytes would also not respond to EHA or other DEHA metabolites. Hence, it may be possible that the lack of peroxisome proliferation in guinea pig, marmoset (and human) hepatocytes indicates the absence of a threat of hepatocarcinogenic damage to these species.

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