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IN VITRO INHIBITION OF CARNITINE ACYLTRANSFERASE ACTIVITY IN MITOCHONDRIA FROM RAT AND MOUSE LIVER BY A DIETHYLHEXYLPHTHALATE METABOLITE

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Abstract—The effects of mono(2-ethyl-5-oxohexyl)phthalate [ME(O)HP], a di(2-ethylhexyl)phthalate (DEHP) metabolite and a potent peroxisomal inducer, on the mitochondrial β-oxidation were investigated. In isolated rat hepatocytes, ME(O)HP inhibited long chain fatty acid oxidation and had no effect on the ketogenesis of short chain fatty acids, suggesting that the inhibition occurred at the site of carnitine-dependent transport across the mitochondrial inner membrane. In rat liver mitochondria, ME(O)HP inhibited carnitine acyltransferase I (CAT I; EC 2.3.1.21) competitively with the substrates palmitoyl-CoA and octanoyl-CoA. An analogous treatment of mouse mitochondria produced a similar competitive inhibition of palmitoyl-CoA transport whereas ME(O)HP exposure with guinea pig and human liver mitochondria revealed little or no effect. The addition of clofibric acid, nafenopin or methylclofenopate revealed no direct effects upon CAT I activity. Inhibition of transferase activity by ME(O)HP was reversed in mitochondria which had been solubilized with octyl glucoside to expose the latent form of carnitine acyltransferase (CAT II), suggesting that the inhibition was specific for CAT I. Our results demonstrate that *in vitro* ME(O)HP inhibits fatty acid oxidation in rat liver at the site of transport across the mitochondrial inner membrane with a marked species difference and support the idea that induction of peroxisome proliferation could be due to an initial biochemical lesion of the fatty acid metabolism.

A variety of structurally diverse chemicals elicit the proliferation of hepatic peroxisomes when administered to rats and mice. Examples of such chemicals are phthalate ester plasticizers, hypolipidaemic drugs and organic solvents [1, 2]. Although these compounds are in general non-mutagenic, several of them have been shown to cause an increased incidence of hepatocellular carcinoma upon chronic administration to rats and mice [3]. A marked species difference has been reported with rats and mice being exquisitely sensitive, in terms of peroxisome proliferation and hepatomegaly, and guinea pigs, monkeys and humans exhibiting little or no response [4–6]. Such species differences lead to difficulties when attempting a human hazard assessment for peroxisome proliferators.

The exact mechanisms of peroxisome proliferation and the basis for the species differences are as yet unclear. Two major hypotheses have been proposed, firstly an activation of specific genes by the chemical either directly or mediated by a cytosolic ligand-receptor [7, 8]. Secondly, a substrate overload perturbation of lipid metabolism has been suggested with a close relationship between lipid accumulation, microsomal cytochrome P450 IV induction and peroxisome proliferation [1, 9].

A number of observations are consistent with the second hypothesis: (1) high fat diets and starvation elicit lipid influx and peroxisome proliferation in rat liver [10]. (2) Several enzyme activities involved in fatty acid metabolism were shown to be specifically altered by peroxisome proliferators [1, 2]. (3) *In vitro* addition of long chain fatty acids to rat hepatocyte primary cultures increase the peroxisomal fatty acid oxidase activity [11].

Molecular biology studies have shown that the transcription of cytochrome P450 IVA1 and peroxisomal fatty acid oxidase were activated by hypolipidaemic drugs in 2–6 and 24 hr, respectively, suggesting that the key events of induction occur very early [12, 13]. Elcombe and Mitchell [4] reported that mono(2-ethyl-5-oxohexyl)phthalate (ME(O)HP†), a di(2-ethylhexyl)phthalate (DEHP) metabolite, inhibited palmitic acid oxidation in isolated rat hepatocytes after only 30 min exposure and subsequently produced lipid accumulation and peroxisome proliferation. Lipid accumulation occurring early in the development of peroxisome proliferation, it was interesting to examine the effects of ME(O)HP on hepatic fatty acid metabolism. Our objectives were to understand the mechanism of the inhibition of fatty acid oxidation by ME(O)HP in isolated rat hepatocytes in comparison with other peroxisome proliferators and other species.

MATERIALS AND METHODS

Materials. ME(O)HP (Fig. 1) was synthesized by

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† Abbreviations: CAT, carnitine acyltransferase; DEHP, di(2-ethylhexyl)phthalate; ME(O)HP, mono(2-ethyl-5-oxohexyl)phthalate; POCA, ethyl-2-(5-(4-chlorophenyl)pentyl)oxiran-2-carboxylate.

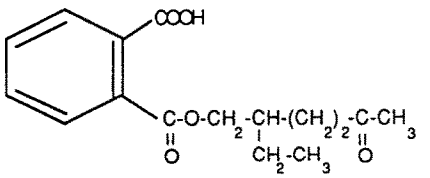

Fig. 1. Structure of ME(O)HP.

Dr R. Moore (Imperial Chemical Industries p.l.c., Macclesfield, U.K.). Methylclofenopate was supplied by Lancaster Synthesis. Clofibric acid was purchased from the Sigma Chemical Co. (Poole, U.K.) and nafenopine was a kind gift of Dr P. Bentley (Ciba-Geigy, Basel, Switzerland). Collagenase and Leibovitz culture media were obtained from Flow Laboratories (Irvine, U.K.). L-[*methyl*-^{3}H]Carnitine hydrochloride (60 Ci/mmol) was purchased from Amersham (Amersham, U.K.). All other chemicals of the highest available purity were obtained from Sigma.

Animals. Experiments were performed on male Alpk:APsSD rats (Wistar-derived rats) weighing 180–220 g, male Alderley Park Guinea pigs (Dunkin and Hartley, 400–460 g) and Alpk:ApsCD-I mice (Swiss). All animals were fed *ad lib.* A 12 hr light/dark cycle (6 a.m.–6 p.m.) was used. Prior to isolation of hepatocytes or mitochondria, animals were killed by excessive diethylether inhalation or cervical dislocation. Normal human liver (available after transplantations) was obtained after compliance with ethical and legal requirements.

Hepatocyte isolation. Hepatocytes were isolated by a two step *in situ* perfusion technique as described before [14]. The hepatocytes were finally resuspended in a Krebs–Ringer hydrogen carbonate buffer (KHB), pH 7.4, at 10×10^6 cells/mL. The cell viability (>85%) was determined by trypan blue exclusion.

Fatty acid oxidation by isolated rat hepatocytes. The production of β-hydroxybutyrate and acetoacetate was determined in the post-incubation supernatant following $HClO_4$ precipitation by the method of Kientsch-Engel and Siess [15]. Cells (3.5×10^6) were preincubated for 20 min with L-carnitine (4 mM) and then incubated under constant shaking for 30 min in KHB buffer containing Hepes (25 mM), fatty acid free albumin (2%) at 37° (final volume = 2 mL). The concentration of the different fatty acid substrates used in the first experiment was chosen in order to yield the same number of acetyl-CoA molecules as palmitate.

Isolation of mitochondria. Livers were initially homogenized in cold 0.25 mM sucrose. In order to minimize the contamination by peroxisomes and lysosomes, mitochondria were isolated from the low-speed nuclear pellet of the initial homogenate. Tissue homogenates were centrifuged at 1000 *g* for 15 min. The supernatant was discarded and replaced by an equivalent volume of 0.25 mM sucrose. The pellet was rehomogenized and centrifuged at 600 *g* for 10 min. Further centrifugation of the resulting supernatant at 15,000 *g* for 15 min yielded a pellet rich in mitochondria with less than 5–10% contamination, as reported by McGarry *et al.* [16]. After successive washes in 0.25 mM sucrose and 0.15 M KCl, the final pellet was resuspended in 0.15 M KCl. Solubilization of mitochondria with octyl glucoside was performed as previously described [17].

Assay of carnitine acyltransferase (CAT) activity. Formation of acylcarnitine from acyl-CoA plus carnitine was studied. The standard incubation mixture contained in a volume of 0.9 mL, 105 μmol Tris–HCl (pH 7.4), 0.25 μmol reduced glutathione, 4 μmol ATP, 4 μmol $MgCl_2$, 15 μmol KCl, 2 μmol KCN, 40 μg rotenone, 10 mg defatted albumin, 5–160 nmol acyl-CoA, 200 nmol L-carnitine, 1 μCi and L-[^{3}H]carnitine. Reactions were initiated by the addition of 100 μL of the mitochondrial suspension (0.1 mg protein), incubated 5 min at 30° and then stopped with $HClO_4$. The labelled palmitoylcarnitine and octanoylcarnitine were extracted by the methods of Bremer *et al.* [18] and Solberg [19] respectively, and quantified by liquid scintillation counting.

Protein assay. Proteins were determined by the method of Lowry *et al.* [20] using bovine serum albumin as a standard.

Statistics. Statistical analysis was performed using Student's *t*-test, relative to concurrent controls. Kinetic constants (K_m, V_{max}) were calculated by weighted linear regressions.

RESULTS

Isolated rat hepatocytes

Ketogenesis, expressed as β-hydroxybutyrate plus acetoacetate production, was inhibited by 0.5 mM

Table 1. Effect of ME(O)HP on ketogenesis in cultured rat hepatocytes

	Ketogenesis (μmol of total ketone bodies/30 min/10^9 cells)				
Substrate added (mM)	Hexanoate (1.04)	Octanoate (0.78)	Laurate (0.51)	Myristate (0.44)	Palmitate (0.39)
Control	150 ± 16	139 ± 20	57 ± 7	85 ± 11	60 ± 6
0.5 mM ME(O)HP	147 ± 11	134 ± 14	53 ± 3	67 ± 4*	47 ± 3*

Values are means ± SD, N = 3 experiments with 3 replicates per concentration.
* $P < 0.05$ compared to control.

Table 2. Effect of ME(O)HP on ketogenesis from palmitate and octanoate in cultured rat hepatocytes

	Ketogenesis (μmol of total ketone bodies/30 min/10^9 cells)					
	Palmitate			Octanoate		
Substrate (mM)	0.5	0.25	0.1	0.5	0.25	0.1
Control	75 ± 7	39 ± 4	16 ± 2	123 ± 13	55 ± 6	25 ± 2
0.5 mM ME(O)HP	61 ± 4*	29 ± 3†	10 ± 5†	125 ± 6	57 ± 3	22 ± 3

Results are presented as means ± SD for three different preparations of hepatocytes with 3 replicates per point.
* $P < 0.05$.
† $P < 0.01$ compared to control.

ME(O)HP as a function of the chain length of the fatty acid substrates (Table 1). Hence, oxidation of palmitic acid (0.39 mM) and myristic acid (0.44 mM) was reduced by 21–22% by ME(O)HP ($P < 0.05$) whereas metabolism of shorter chains was not modified even at low fatty acid concentrations (0.1–0.5 mM) (Table 2). In treated hepatocytes, the inhibition of the ketogenesis rate from palmitate was shown to be dependent on the initial substrate concentration (Table 2). Hence, the 18% inhibition observed for 0.5 mM palmitate ($P < 0.05$) rose to 25% then to 37% for 0.25 and 0.1 mM palmitate, respectively ($P < 0.01$).

Mitochondria

The specific inhibition of long chain fatty acid oxidation suggested a potential action of ME(O)HP on the key enzyme involved in their transfer from cytosol to the mitochondrial matrix, i.e. CAT. As shown in Fig. 2, incubation of 1 mM ME(O)HP with intact mitochondria from rat liver in the presence of various amounts of palmitoyl-CoA and octanoyl-CoAs (5–160 μM) resulted in a lower CAT I activity. The suspected mode of inhibition was confirmed by kinetic studies in which double-reciprocal plots were generated. They indicated a competitive inhibition of the two substrates by ME(O)HP. The apparent K_m raised from 19.1 (control) to 45.2 μM for palmitoyl-CoA and from 65.3 (control) to 137.6 μM for the octanoyl-CoA. The apparent V_{max} values were not affected by ME(O)HP.

The effect of various types of peroxisome proliferators on the rat liver CAT I activity with palmitoyl-CoA as a substrate was examined and the results shown in Table 3. Ethyl-2-(5-(4-chlorophenyl)pentyl)oxiran-2-carboxylate (POCA; 0.1 mM) was used as a positive control and asserted as a competitive inhibitor increasing the apparent K_m value from 20.4 to 75.7 μM without any significant change in the V_{max} value (6 nmol/min/mg protein). Opposite to ME(O)HP, the hypolipidaemic agents did not inhibit CAT I in our experimental conditions, but nafenopin decreased the V_{max} value slightly.

The effect of ME(O)HP was assayed with fresh liver mitochondria from another animal responsive to peroxisome proliferation, the mouse, and two non-responsive species the guinea pig and humans. Human mitochondria were isolated from frozen

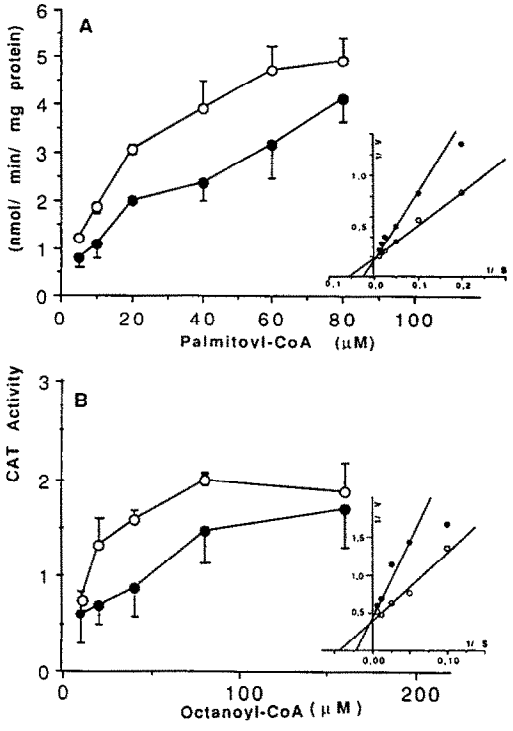

Fig. 2. Inhibition of the CAT I activity in rat liver mitochondria by ME(O)HP 1 mM as a function of palmitoyl-CoA (A) or octanoyl-CoA (B) concentration. Mitochondria were preincubated with 1 mM ME(O)HP in the presence of 5–160 μM palmitoyl-CoA or octanoyl-CoA for 5 min before initiation of the raction. Values represent means ± SD for 3 different experiments with triplicate measurements. Inset: Lineweaver–Burk plot in which v is nmol/min/mg of protein and s is μM acyl CoA in the absence (○) and the presence (●) of ME(O)HP.

liver, and hence direct comparisons with fresh rat mitochondria were difficult. Incubation of 1 mM ME(O)HP with mouse mitochondria resulted in a competitive inhibition of CAT I with respect to palmitoyl-CoA. The apparent K_m value increased from 26.2 to 57.4 μM (+119%) while the V_{max} remained essentially unchanged (Table 4). Guinea

Table 3. Kinetic values of CAT I from rat liver mitochondria treated with different peroxisome proliferators, with palmitoyl-CoA (5–80 μM) as substrate

Compounds	K_m (μM)	V_{max} (nmol/min/mg protein)
Control	19.1	5.75
1 mM ME(O)HP	45.2	5.31
0.1 mM POCA	75.7	6.01
0.5 mM Clofibrate	14.6	5.97
0.2 mM Nafenopin	12.6	5.21
0.2 mM Methylclofenopate	16.1	5.78

Kinetic values were calculated from 5 concentration points, 2 different experiment and triplicate measurements.

pig and human mitochondrial CAT I appeared less sensitive to ME(O)HP than rats and mice. The inhibitory effect of ME(O)HP was also investigated in rat liver mitochondria solubilized with octyl glucoside to expose the latent carnitine acyltransferase II (CAT II). Inhibitions [−33% for palmitoyl-CoA (C16-CoA) and −62% for octanoyl-CoA (C8-CoA)] by 1 mM ME(O)HP were suppressed in solubilized mitochondria suggesting that they were specific for CAT I for both octanoyl- and palmitoyl-CoA (Table 5).

DISCUSSION

Two major hypotheses were suggested to explain the initial mechanism of peroxisome proliferation. One involves an activation of a nuclear receptor protein related to the superfamily of nuclear steroid hormone receptors, by chemical or specific cytosolic receptors [7, 8]. It is supported by the correlation between the rank order of peroxisome proliferators for the receptor activation and, the inducibility of peroxisomal β-oxidation and changes of protein composition in the liver. However, Milton *et al.* [21] failed to demonstrate a cytosolic complex for ciprofibrate and nafenopin. Furthermore, this mechanism does not account for the peroxisome proliferation produced by high fat diets in rat liver and the structural diversity of peroxisome proliferators. An alternative hypothesis involves a lipid substrate overload leading to lipid metabolism alterations, the changes in the fatty acid profile of the cell being responsible for the co-induction of microsomal ω-oxidation and peroxisomal β-oxidation [4, 9]. Elcombe and Mitchell [4] reported that 0.5 mM ME(O)HP produce a transient neutral lipid accumulation in cultured rat hepatocytes which disappeared presumably as a result of the subsequent increased peroxisomal and mitochondrial β-oxidation. Suggesting that this lipid accumulation could trigger peroxisome proliferation, they studied the possible mechanism of lipid metabolism alteration and reported that ME(O)HP inhibited palmitic acid oxidation in isolated rat hepatocytes and produced a selective inhibition of octanoylcarnitine, but not palmitoylcarnitine oxidation in mitochondria from rat liver. However, the fatty acid oxidation was recorded by measurements of oxygen consumption and thus it was not possible to designate the site of action of ME(O)HP.

Table 4. Effect of ME(O)HP on the kinetic values of CAT I from rat, mouse, guinea pig and human liver mitochondria with palmitoyl-CoA as substrate

	K_m (μM)	V_{max} (nmol/min/mg protein)
Rat		
Control	19.1	5.75
1 mM ME(O)HP	45.2	5.31
Mouse		
Control	21.2	8.69
1 mM ME(O)HP	47.4	9.32
Human		
Control	32.4	6.25
1 mM ME(O)HP	30.8	5.75
Guinea pig		
Control	28.7	2.51
1 mM ME(O)HP	30.6	2.23

Kinetic values were calculated from 5 concentration points with triplicate measurements.

In the present study, ME(O)HP was confirmed to decrease ketogenesis in cultured rat hepatocytes according to long chain fatty acid concentration, suggesting a competitive inhibition. Moreover, oxidations from short- and medium-chain substrates which were independent of the formation of carnitine ester [22] were not modified by ME(O)HP. All these data are consistent with a direct effect of ME(O)HP on the specific long chain fatty acid transport across the mitochondrial inner membrane, at the outer located enzyme (CAT I) catalysing the formation of acylcarnitine esters or at the inner located enzyme (CAT II), catalysing the reverse reaction.

Incubation of ME(O)HP with intact and fresh mitochondria from rat liver led to a concentration-dependent inhibition of acylcarnitine formation from palmitoyl-CoA and octanoyl-CoA. Partial overcoming of the inhibition by high concentrations of acyl-CoA and the kinetic data generated with double-reciprocal plots ($1/v$ vs 1/acyl-CoA) revealed that the CAT I inhibition by ME(O)HP was competitive with long and medium chain acyl-CoA esters. Interestingly, the detergent-solubilized preparations of CAT II from rat liver did not show any sensitivity to ME(OH)HP and the activity was even slightly increased. This appeared to be similar to the effects exerted by malonyl-CoA [17] in terms of specificity for CAT I rather than for CAT II. This is an apparent discrepancy with the inhibited octanoylcarnitine oxidation reported by Elcombe and Mitchell [4] in treated rat liver mitochondria. However, the present studies measured the forward reaction (i.e. acylcarnitine formation) with the solubilized CAT II while *in vivo* CAT II is bound to the inner membrane and catalyses acyl-CoA formation from the corresponding acylcarnitine

Table 5. Effect of ME(O)HP on the activities of CAT I and II from rat liver mitochondria

	Activity (nmol/min/mg protein)			
	CAT I		CAT II	
Substrate 40 μM	C16-CoA	C8-CoA	C16-CoA	C8-CoA
Control	3.87 ± 0.41	1.68 ± 0.09	1.20 ± 0.02	1.09 ± 0.06
1 mM ME(O)HP	2.37 ± 0.33*	0.63 ± 0.03*	1.47 ± 0.09*	1.16 ± 0.04

Values are means ± SD for 3 different preparations of mitochondria.
* $P < 0.01$ compared to control.

esters [23]. Thus, this activity with respect to octanoylcarnitine could be regulated differently in the membrane.

ME(O)HP is not the first compound shown to produce both peroxisome proliferation and early mitochondrial CAT I inhibition. Thus, valproic acid [24], tetradecylglycidate [17], POCA [25], 2-hydroxy-3-propyl-4-(6-tetrazol-5-yl)hexyloxy)acetophenone [26] and the leukotriene D4 antagonist LY171883 [27] have been reported to produce the same effects in rat liver mitochondria. The first three of the above chemicals contain a carboxylic function which has to be converted into a CoA ester to produce the active inhibitor. It is plausible that many peroxisome proliferators could inhibit CAT I and sequestrate essential CoA by the chemicals *per se* or by another mechanism leading to a unifying initial lesion. However, conversion of 2-hydroxy-3-propyl-4-(6-tetrazol-5-yl)hexyloxy)acetophenone and ME(O)HP into CoA derivatives has not yet been demonstrated. CAT has been postulated to be a complex oligomeric protein, which can be regulated by a positive cooperative inhibition [23]. Thus, it is possible that ME(O)HP being not further metabolized in the hepatocytes [28] interacts directly with one or more CAT subunits thus decreasing the affinity for acyl-CoA. In our study, administration of nafenopin and clofibrate which are activated in CoA esters in the microsomal fraction [29] failed to inhibit CAT I activity at the concentration which elicits peroxisomal induction in cultured rat hepatocytes. However, in our assay conditions, no CoA chemical esters could be formed as there were no microsomes and no added free CoA. Hence, it may be concluded that free clofibrate and nafenopin do not inhibit CAT I activity, but, it cannot be excluded that these compounds can be converted into CoA esters and thereafter inhibit the fatty acid oxidation in the intact cell. This hypothesis would need further investigation. Our results support the hypothesis that mitochondrial β-oxidation could be a primary lesion causing further lipid accumulation and peroxisome proliferation in ME(O)HP-treated rat hepatocytes.

Marked species differences in response to peroxisome proliferators have been reported in terms of peroxisome proliferation, hepatomegaly and lipid accumulation in the liver. The rat and mouse are highly sensitive, while guinea pigs and humans are essentially non-responsive. Interestingly, we observed a competitive inhibition of CAT I activity in mice and rat mitochondria by 1 mM ME(O)HP and no effect with guinea pig and human mitochondria. These results could be related to the accumulation of neutral lipids in cultured rat hepatocytes [4] and suggest that this enzymatic alteration could be a key event of peroxisomal proliferation. However, Mitchell [30] showed an inhibition of mitochondrial β-oxidation and lipid accumulation in cultured guinea pig hepatocytes treated with a high concentration of ME(O)HP (2 mM). In this case the mitochondrial lesion did not lead to peroxisome proliferation. Thus, CAT from guinea pig mitochondria seemed to be less sensitive to the inhibitory effect of ME(O)HP than rat and mouse mitochondria. Moreover, the lack of peroxisomal induction in response to lipid accumulation suggested a possible species difference in the metabolism of hepatic lipids or the fact that the inhibition of β-oxidation and the lipid overload were not essential for peroxisome proliferation.

In conclusion, our data confirm that ME(O)HP inhibits specifically CAT I activity in a competitive manner possibly leading to lipid accumulation in rats, mice and to a lesser extent in guinea pig liver supporting in part the hypothesis of lipid substrate overload [2, 4]. However, the species differences should still be investigated. The relationship between CAT inhibition and peroxisome proliferation seems very complex since Hertz and Bar-Tana [31] reported that POCA decreased the induction of peroxisomal β-oxidation by bezafibrate. Moreover, peroxisome proliferators have been shown to induce different mitochondrial CAT activities several days after exposure [32, 33]. All these results suggest that the receptor hypotheses and the lipid accumulation model are not exclusive. A general scheme summarizing these hypothesis is given in the review of Lock *et al.* [1].

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