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INDUCTION OF LAURATE ω-HYDROXYLASE BY DI(2-ETHYLHEXYL)PHTHALATE IN RAT LIVER MICROSOMES

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Summary - Liver microsomes, prepared from rats fed a diet containing di(2-ethylhexyl)phthalate, were observed to hydroxylate lauric acid at carbon 12 at a specific activity 6 times greater than control rats. There was no increase in the specific activity of the laurate 11-hydroxylase. The specific activity of hepatic microsomal NADPH-cytochrome c reductase was increased 2-fold by phthalate feeding, but no effect was observed in the specific contents of cytochromes b_5 or P-450. These results indicate that di(2-ethylhexyl)phthalate is similar to the hypolipidemic agent and peroxisomal proliferator, clofibrate, which was previously reported to be a novel inducer of the cytochrome P-450 mediated ω-hydroxylation of fatty acids.

Hypolipidemic and peroxisomal proliferating agents, such as clofibrate or di(2-ethylhexyl)phthalate (DEHP), have significant effects on pathways involving fatty acids, e.g., mitochondrial (1) and peroxisomal (2,3) mediated β-oxidation and microsomal mediated desaturation reactions (4). DEHP is a chemical additive that is commonly used in the manufacture of plastic products, and recently was reported to cause hepatic peroxisomal proliferation (5) and hepatic tumors (6) in rats fed DEHP. These effects also are characteristic of clofibrate (7,8). Clofibrate was reported recently to stimulate the ω-hydroxylation of fatty acids in liver microsomes (9,10) and hepatocytes (11).

Parker and Orton (9) have demonstrated that the cytochrome P-450 mediated ω- or 12-hydroxylase of lauric acid was stimulated 28-fold in rats fed clofibrate, while the penultimate (ω-1) or 11-hydroxylase of laurate was stimulated only slightly. This was the first report that the laurate 12-hydroxylation reaction could be induced. Previous studies had demonstrated that phenobarbital induced the laurate 11-hydroxylase 2-3 fold, but had no effect on the 12-hydroxylase (12). Gibson et al. (13) have isolated several

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cytochromes P-450 from liver microsomes of clofibrate-treated rats which supported laurate hydroxylation in reconstituted systems and one cytochrome P-450 form, with a molecular weight of 52,000, demonstrated a high turnover number and specificity for the 12-hydroxylation reaction. Due to the reported similarities between DEHP and clofibrate, DEHP was examined in the present study for its effect on the rat liver microsomal laurate hydroxylation activities.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200-300 gm) were fed a commercially prepared diet containing 2% (w/w) DEHP (Ralston Purina, Richmond, IN) for 14 days. The rats were starved for 14-18 hours prior to sacrificing. Control rats were given normal rat chow (Ralston Purina, Richmond, IN). Liver microsomes were prepared by a modification of the procedure of Remmer et al. (14). The specific activity of NADPH-cytochrome c reductase was determined by the procedure of Masters et al. (15) and the specific contents of cytochromes P-450 and b_5 were determined by the procedure of Estabrook and Werringloer (16) using a Varian 2290 UV-Vis spectrophotometer. Laurate binding spectra were performed in an Aminco-DW2a spectrophotometer at room temperature as described by Jefcoate (17). Benzphetamine (a gift from Upjohn) N-demethylation activity was determined by the procedure of Werringloer (18). The incubation system for the lauric acid hydroxylation system was performed by a modification of the procedure of Okita and Masters (19) using a concentration of 100 μM sodium laurate (Sigma, St. Louis, MO) containing 0.1 μCi[1-^{14}C]lauric acid (Amersham, Arlington Heights, IL) and 0.3 mg microsomal protein in a total volume of 1 ml. The reaction was terminated after 3 min by the addition of 0.2 ml of a 2.5% H_2SO_4 solution and the reaction mixture extracted with ethyl acetate. The hydroxylated derivatives of lauric acid were separated and quantitated by a modification of the HPLC procedure of Parker and Orton (9). Lauric acid and its hydroxylated derivatives were separated as the free acids on a Bio-Sil ODS-5S 150 x 4 mm column (BioRad, Richmond, CA) using an isocratic solvent system of 62% methanol/37.8% water/0.2% acetic acid at a flow rate of 0.7 ml/min to elute the 11- and 12-hydroxylated derivatives and 100% methanol to elute the unmetabolized lauric acid. The chromatographic separations were performed on a Varian 5020 HPLC (Varian Instruments, Palo Alto, CA). The elution pattern of the radioactive metabolites and unmetabolized substrate was monitored by an on-line Radiomatic Flo-One Model HP detector (Radiomatic, Tampa Bay, FL) or by collecting column aliquots in 0.2 min intervals and counting the vials in a LKB Rackbeta 1217 scintillation counter (LKB Instruments, Gathersburg, MA). RPI 3a70B scintillation cocktail (RPI, Mount Prospect, IL) was used in both counting techniques. The retention times for the 11- and 12-hydroxylated derivatives of laurate were 13.0 and 14.5 min, respectively, and were identified by co-chromatography of previously identified standards.

Protein determinations were performed by the method of Lowry et al. (20). using human serum albumin as standard.

RESULTS AND DISCUSSION

DEHP that was administered for two weeks in a commercially prepared diet to male Sprague Dawley rats at a concentration of 2% (w/w) was observed to increase the mean liver weight 61% versus control livers (Table I). The liver

TABLE I. Effect of DEHP feeding on body and liver weights

Diet	Body Weight	Liver Weight	% of Body Weight
Control (n=4)	289±10	10.8±1.2	3.7±0.4
DEHP (n=6)	278±30	17.4±1.7	6.3±0.5

Values represent means ±S.D.

weight in DEHP-treated rats represented 6.3% of the total body weight, whereas in control rats, the liver represented 3.8% (Table I). This increase in liver size is in agreement with the results reported by Ganning et al. (21) who examined the effects of DEHP on liver enlargement.

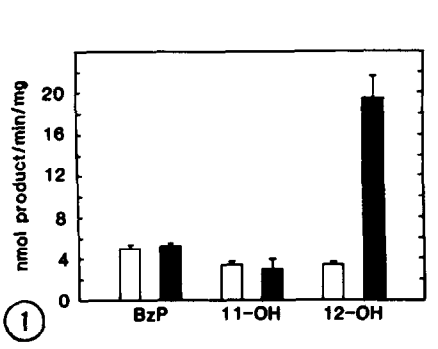
The administration of DEHP had no effect on the specific contents of liver microsomal cytochromes b_5 or P-450 (Table II), but NADPH-cytochrome P-450 reductase was stimulated 2-fold as determined by the increase in cytochrome c reductase activity (Table II). Studies by Agarwal et al. (22) and Ganning et al. (21) have reported a 50-60% increase in cytochrome P-450 content in rats given DEHP by oral administration for one and two weeks, respectively, but no increase in cytochrome P-450 content was observed in this study.

Previous studies have reported that DEHP treatment had minimal stimulatory effects on liver microsomal cytochrome P-450-mediated metabolism of xenobiotics (10,22,23). In agreement with these studies, benzphetamine N-demethylation activity was found not to increase in DEHP-fed versus control rats (Fig. 1). As shown in Fig. 1, the laurate 11-hydroxylation activities were equivalent in DEHP-fed and control rats, but a 6-fold induction in laurate 12-hydroxylase was observed in liver microsomes prepared from DEHP-fed

TABLE II. Effect of DEHP feeding on microsomal electron transport activities in rat liver microsomes.

Diet	NADPH-cytochrome c reductase[a]	cytochrome b_5[b]	cytochrome P-450[b]
Control (n=3)	102.1±22.6	0.6±0.2	1.2±0.4
DEHP (n=4)	212.2±30.9	0.5±0.2	1.2±0.2

[a] nmoles cytochrome c reduced per min per mg microsomal protein
[b] nmoles/mg microsomal protein



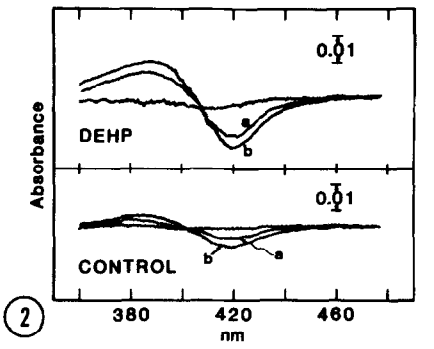


Fig. 1 - The effect of DEHP on benzphetamine (BzP) or laurate 11-(11-OH) and 12-(12-OH)hydroxylation specific activities. The open bars represent values obtained from control rats and the black bars represent DEHP-fed rats. Values are the mean of 3 experiments performed in triplicate ± S.D.

Fig. 2 - The binding spectra of laurate in microsomes prepared from DEHP-fed and control rats. Curves a and b represent binding spectra performed with 8 and 16 μM sodium laurate, respectively. The concentration of cytochrome P-450 in microsomes from control and DEHP-fed rats was 2.8 μM.

rats. The specific activity of the 12-hydroxylase increased from 3.5 to 19.5 nmol/min/mg protein in rats fed DEHP for 2 weeks and the ratio of 12-hydroxy- to 11-hydroxy-laurate increased from 1.0 to 5.6.

Although the specific content of cytochrome P-450 remained unchanged, an enhanced type I laurate-cytochrome P-450 binding spectrum was observed in the rat liver microsomes prepared from DEHP fed rats (Fig. 2). At a concentration of 8 μM lauric acid, the absorbance change at 388-420nm was 3 times greater in liver microsomes from DEHP-fed rats than from control rats. Although no increase in cytochrome P-450 concentration was observed in DEHP-treated versus control rats, the increased type I binding spectra suggests the induction of a specific, but quantitatively minor, form of cytochrome P-450 for the laurate 12-hydroxylase. A cytochrome P-450 with a molecular weight of 52,000 was reported by Gibson et al. (13) as the major cytochrome P-450 form responsible for the 12-hydroxylation of lauric acid in clofibrate treated rats.

This study demonstrates that the phthalate ester, DEHP, is similar to another peroxisomal inducer, clofibrate, in being a potent inducer of the cytochrome P-450 mediated fatty acid ω-hydroxylation reaction (9,10). Aspirin, another inducer of peroxisomal enzyme activities, was recently

observed to induce the laurate 12-hydroxylase 3-fold (unpublished observations).

Orton and Parker (10) reported a 28-fold induction in laurate 12-hydroxylation activity after clofibrate feeding as opposed to a 6-fold induction for DEHP in the present study. This difference in the extent of induction is due to the low specific activity for the 12-hydroxylase in control rats reported in the Orton and Parker study (10) which was 7-fold less than the control activity in the present study. This difference could be due to the different strains of rats used in the two studies.

The ω-hydroxylation reaction is the first step in the oxidation of fatty acids to the dicarboxylic acid derivative and is normally a minor route for fatty acid oxidation in healthy individuals. It, however, may play a more significant role in individuals with diabetic ketosis, mitochondrial defects, or Reye Syndrome (25-27).

These effects on the ω-hydroxylation pathway of fatty acids require further examination to determine if these chemicals may alter fatty acid oxidation *in vivo* or if the products of this pathway may account for the toxic reactions reported for peroxisomal proliferating agents (28).

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