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CO-INDUCTION OF MICROSOMAL CYTOCHROME P-452 AND THE PEROXISOMAL FATTY ACID β-OXIDATION PATHWAY IN THE RAT BY CLOFIBRATE AND DI-(2-ETHYLHEXYL)PHTHALATE

DOSE–RESPONSE STUDIES

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Abstract—Male Wistar rats have been pretreated with either clofibrate or diethylhexylphthalate and the dose-dependency of induction of the microsomal, cytochrome P-452-driven fatty acid hydroxylase and peroxisomal fatty acid β-oxidation system investigated. Both clofibrate and DEHP specifically induced (approximately 10-fold) the 12-hydroxylation of lauric acid in a dose-dependent manner and only marginally increased the associated 11-hydroxylase activity. This dose-dependent increase in fatty acid hydroxylase activity was accompanied by a similar ten-fold increase in the specific content of the cytochrome P-452 isoenzyme responsible for this activity, as assessed by an immunochemical-based ELISA method. Similarly, both clofibrate and DEHP induced the peroxisomal fatty acid β-oxidation pathway in a dose-dependent manner. Furthermore, our results provide evidence that, after oral administration, clofibrate has a higher *in vivo* potency in inducing the above enzymes of fatty acid metabolism than is exhibited by DEHP.

A correlation matrix analysis of the above data indicated a close association between the induction of microsomal cytochrome P-452 (and its associated fatty acid hydroxylase activity) and peroxisomal β-oxidation enzymes, implicating a mechanistic inter-relationship between changes in fatty acid metabolising enzymes in these two hepatic subcellular organelles.

A number of hypolipidaemic agents which produce hepatomegaly and peroxisome proliferation have been shown to induce liver tumours in rats and mice [1–3]. The therapeutic drug clofibrate and the industrial plasticizer di(2-ethylhexyl)phthalate (DEHP)§ are two structurally dissimilar hypolipidaemic agents which have been studied with respect to their induction of microsomal and peroxisomal fatty acid oxidation systems [4–7].

Previous studies have demonstrated that the early hepatic changes in rats on administration of clofibrate and DEHP include hepatomegaly, proliferation of peroxisomes, and more recently, the induction of a microsomal isoenzyme of cytochrome P-450 (termed cytochrome P-452), the latter specifically catalysing the hydroxylation of fatty acids [8]. The relationship between the induction of microsomal and peroxisomal enzymes following hypolipidaemic challenge is thought to be closely linked and previous preliminary studies have demonstrated a good correlation between the induction of these lipid metabolising enzymes [9, 10]. Although the significance of these early changes in lipid metabolising enzymes remains to be elucidated, it appears to be related to the more toxicologically profound changes following chronic exposure to these agents.

The present study sets out to compare the hepatic dose–response characteristics of the above lipid metabolising enzymes in the rat to clofibrate and DEHP. Furthermore, with the recent development of a specific and sensitive assay for the direct determination of cytochrome P-452 isoenzyme levels in our laboratories [5], we have now attempted to correlate the induction of this latter isoenzyme with the induction of peroxisomal, KCN-insensitive palmitoyl-CoA oxidation.

Our dose–response studies indicate that for both clofibrate and DEHP, the induction of the specific cytochrome P-452 fatty acid hydroxylase is intimately associated with the process of peroxisome proliferation.

MATERIALS AND METHODS

Chemicals. The hypolipidaemic agents were obtained from the following sources; Clofibrate was a gift from I.C.I. p.l.c., Pharmaceuticals Division (Macclesfield, U.K.); and DEHP purchased from Lancaster Synthesis, Morecambe, U.K. (98+% pure).

Lauric acid and NADPH were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 1-^{14}C Lauric acid was supplied by the Radiochemical

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§ Abbreviations used: DEHP, di-(2-ethylhexyl)phthalate; ELISA, enzyme-linked immunosorbent assay; clofibrate, ethyl 2-(*p*-chlorophenoxy) isobutyrate.

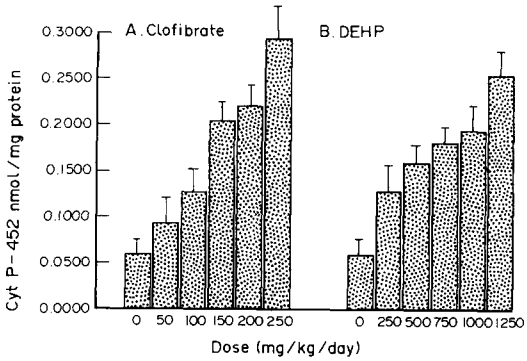

Fig. 1. Dose–response relationships for the induction of the hepatic, microsomal cytochrome P-452 isoenzyme by (A) clofibrate and (B) DEHP in the rat.

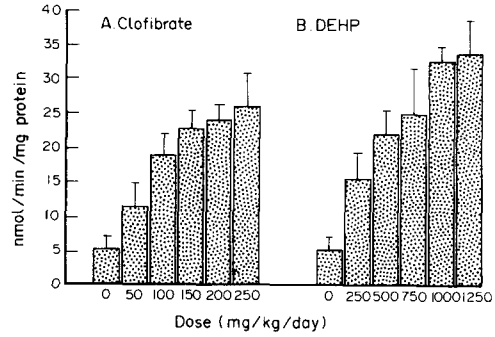

Fig. 3. Induction of peroxisomal palmitoyl CoA oxidation by clofibrate and DEHP in the rat. Palmitoyl-CoA oxidation is given in nmol/min/mg protein.

Centre (Amersham, Bucks., U.K.) and all other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and drug pretreatment. Male Wistar albino rats (150–200 g body weight, University of Surrey breeders) were separately pretreated from low to high dose levels with the fibrate type of inducer, clofibrate and the phthalate ester plasticizer di-(2-ethylhexyl)phthalate (DEHP). The doses used were: Clofibrate, 250 (1030), 200 (824), 150 (618), 100 (412) and 50 (206) mg/kg; DEHP, 1250 (3200), 1000 (2560), 750 (1920), 500 (1280) and 250 (640) mg/kg, the numbers in parenthesis referring to μmol/kg. Compounds were dissolved in peanut oil as the vehicle and administered via gastric intubation. Control animals were given peanut oil at 5 ml/kg via the same route. The animals were dosed once daily for 3 days and then killed at the start of the fourth day, i.e. 24 hr after the last dose. Whole homogenate samples were taken for the measurement of peroxisomal palmitoyl-CoA oxidation, and liver microsomes were prepared by the calcium precipitation method as previously described [8].

Enzyme assays. All enzyme assays are described in detail in the accompanying paper according to the methods of Omura and Sato [11], Voller *et al.* [12], Parker and Orton [13], Gray *et al.* [14] and Lowry *et al.* [15].

RESULTS

The immunochemically determined specific cytochrome P-452 concentrations increase in a dose-dependent manner subsequent to both clofibrate and DEHP pretreatment (Fig. 1). Similarly, the associated cytochrome P-452-driven lauric acid hydroxylase is induced by both compounds in a dose-dependent manner and clearly demonstrates the specificity of induction of the 12-hydroxylase following pretreatment (Fig. 2).

Peroxisomal palmitoyl CoA-oxidation also exhibits dose-dependent induction following the administration of these two structurally dissimilar hypolipidaemic agents (Fig. 3). It is interesting to note that the maximal induction of these parameters occurs at different dose levels for the two inducers. Clofibrate, a relatively weak fibrate type inducer, is, however, apparently 4–6 times more potent than the phthalate ester DEHP, when considered on a μmol/kg/day basis.

A correlation matrix for clofibrate- and DEHP-inducible enzyme changes (Table 1) reveals the extent of microsomal and peroxisomal inter-relationship under dose-dependent conditions.

Clearly measurements of total cytochrome P-450 do not correlate well with any other parameter and hence do not provide a good index of specific micro-

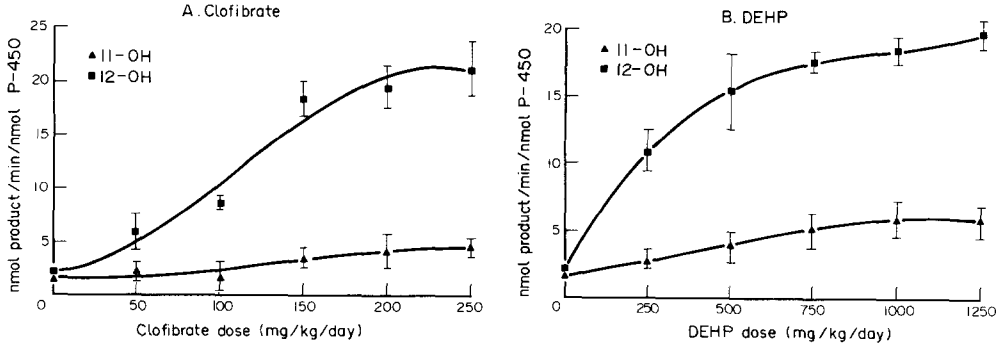

Fig. 2. Influence of clofibrate and DEHP on lauric acid hydroxylase activities in rat liver microsomes. A: clofibrate, B: DEHP, 11-OH and 12-OH refer to the 11-hydroxy and 12-hydroxy metabolites of lauric acid respectively.

Table 1. Correlation matrix for the association between induction of microsomal and peroxisomal fatty acid oxidising enzymes*

Parameter	Total cyt. P-450 (specific content)	Cyt. P-452	LA 11-OH	LA 12-OH	Palmitoyl-CoA oxidation
Clofibrate induction					
Total cyt. P-450		0.648	0.465	0.583	0.763
Cyt. P-452			0.946	0.970	0.930
LA 11-OH				0.956	0.841
LA 12-OH					0.952
DEHP induction					
Total cyt. P-450		0.776	0.544	0.744	0.624
Cyt. P-452			0.931	0.949	0.965
LA 11-OH				0.953	0.984
LA 12-OH					0.967

* For a correlation matrix with 6 observations (dose levels) the correlation coefficient in a linear regression analysis is significant (95% probability) if $r > 0.811$.

somal or peroxisomal changes. Levels of the cytochrome P-452 isoenzyme correlate highly with gross liver changes such as hepatomegaly (data not shown), and also subcellular enzyme changes such as the 11- and 12-hydroxylation of lauric acid, as well as peroxisomal palmitoyl-CoA oxidation (Table 1).

DISCUSSION

The data presented in this paper demonstrates the dose-dependent, differential induction and correlation of microsomal and peroxisomal lipid metabolising enzymes by clofibrate and DEHP.

Clofibrate has been determined in this study to be a more potent inducer of the microsomal fatty acid hydroxylase compared to DEHP. In any consideration of potencies of inducing agents it must be recognised that the important parameter is the concentration of the inducer that actually reaches the site of action. In comparing the relative potencies of clofibrate and DEHP in their abilities to induce the enzymes of fatty acid metabolism, the differential absorption of these two compounds after oral administration must be taken into consideration. In this context it has been shown that significant amounts of DEHP can be absorbed at high doses in adult rats [16], whereas at low doses DEHP is almost completely hydrolysed in the intestine to the monoester, MEHP [17]. MEHP is then absorbed and can be further converted to the specific metabolite(s), including 2-ethylhexanol which cause peroxisome proliferation in addition to MEHP itself [18]. By comparison, clofibrate itself (acid form) appears to be solely responsible for the previously noted hepatic enzyme changes and thus obscures any definitive statement regarding relative potencies of the biologically active material. Accordingly, it must be emphasised that our dose–response studies simply reflect the overall *in vivo* response to clofibrate and DEHP and do not take into account the ultimate chemical species responsible for induction.

The co-induction of the microsomal fatty acid hydroxylase and the peroxisomal β-oxidation system as indicated by the induction of palmitoyl-CoA oxidation may be the result of disturbances in the metabolism of lipids [19, 20]. Following clofibrate treatment there is an accumulation of lipid in the liver in a similar fashion to that observed in animals receiving high fat diets [21]. Administration of DEHP also results in the accumulation of small droplets of lipid in the periportal zone of the liver lobule. DEHP also inhibits cholesterol biosynthesis together with a reduction in fatty acid and triglyceride synthesis [22, 23].

The induction of fatty acid- and β-oxidation metabolising systems may therefore represent an adaptive response to the early changes seen following hypolipidaemic challenge. The resulting hepatotoxicity following chronic exposure to clofibrate and DEHP may therefore be the result of lipid metabolising systems in the liver being continuously overloaded due to the sustained presence of high concentrations of the inducing agent, ultimately leading to alterations in lipid homeostasis as mechanistically enunciated in the accompanying paper.

In conclusion, our results demonstrate that the early enzyme changes in lipid metabolism following clofibrate or DEHP administration are dose-dependent and that there is a close association between the induction of microsomal cytochrome P-452, its associated laurate 12-hydroxylase activity and the peroxisomal fatty acid β-oxidation pathway.

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