Comparison of Hepatic Peroxisome Proliferative Effect and Its Implication for Hepatocarcinogenicity of Phthalate Esters, Di(2-ethylhexyl) Phthalate, and Di(2-ethylhexyl) Adipate with a Hypolipidemic Drug

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Peroxisome proliferation is inducible in hepatocytes of rodent and nonrodent species by structurally dissimilar hypolipidemic drugs and certain phthalate ester plasticizers. The induction of peroxisome proliferation appears to be a tissue specific response limited largely to the hepatocyte. Peroxisome proliferation is associated with increases in the activity of the H$_2$O$_2$-generating peroxisomal fatty acid $\beta$-oxidation system and in the amount of peroxisome proliferation-associated 80,000 MW polypeptide (PPA-80). Chronic administration of these non-DNA damaging and nonmutagenic peroxisome proliferators to rats and mice results in the development of hepatocellular carcinomas. Comparative morphometric and biochemical data from rats treated with varying dose levels of ciprofibrate, a hypolipidemic drug, and di(2-ethylhexyl) phthalate, and di(2-ethylhexyl) adipate, the widely used plasticizers, indicate that the hepatocarcinogenic potency of these agents is correlatable with their ability to induce peroxisome proliferation, peroxisomal $\beta$-oxidation and PPA-80. Available evidence strongly favors the role of peroxisome proliferation-associated oxidative stress in the induction of liver tumors by peroxisome proliferators.

Introduction

The cytoplasmic organelle peroxisome (microbody), characterized morphologically by a single limiting membrane and a finely granular or homogeneous matrix, is a ubiquitous structure in animal and plant cells (1–3). In normal hepatic parenchymal cells, peroxisomes are few in number and appear somewhat insignificant in the overall cytoplasmic organization. Twenty years ago, the hypolipidemic agent clofibrate was the first xenobiotic shown to induce a marked proliferation of peroxisomes in liver parenchymal cells (4). In recent years, peroxisome proliferation and induction of peroxisome associated enzymes in the livers of rodents exposed to a variety of structurally dissimilar hepatic peroxisome proliferators have been extensively studied (5–9). The induction of peroxisomes is associated with a severalfold increase in the activity of the peroxisomal fatty acid $\beta$-oxidation system (8,10–15) and a twofold increase in the activity of catalase (7,8,16). In addition, long-term exposure to these peroxisome proliferators results in the induction of hepatocellular carcinomas in rats and mice (8,17–22). The lack of mutagenicity of these agents (8,23–25) combined with consistent coupling of proliferation of H$_2$O$_2$-generating peroxisomes led to the hypothesis that persistent proliferation of peroxisomes serves as an endogenous initiator of neoplastic transformation by enhancing oxidative stress (19,26). Since the morphological observation of hepatic peroxisome proliferation is considered a useful marker of potentially carcinogenic nonmutagenic compounds, we have undertaken a comparative examination...

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of peroxisome proliferative effects of some known carcinogenic peroxisome proliferators in order to correlate the extent of peroxisome proliferation with hepatocarcinogenicity. In this report, possible mechanisms of induction of peroxisome proliferation and peroxisome proliferation-induced liver carcinogenesis are also considered.

**Ciprofibrate- and Phthalate Ester-Induced Peroxisome Proliferation and Correlation with Hepatocarcinogenic Potency**

Several structurally diverse hypolipidemic agents and the widely used phthalate ester plasticizers constitute two major classes of chemicals capable of inducing peroxisome proliferation in liver (7,8). Clofibrate and other hypolipidemic agents are several orders of magnitude more effective in inducing hepatomegaly, peroxisome proliferation, peroxisome proliferation-associated 80,000 molecular weight polypeptide (PPA-80) and peroxisomal fatty acid β-oxidation system when compared to phthalate ester plasticizers di-(2-ethylhexyl) phthalate (DEHP) and di-(2-ethylhexyl) adipate (DEHA). For example, clofibrate, when administered at 0.25% dietary level, induces considerable proliferation of peroxisomes in rat liver (4,5). With DEHP, a level of 2% is necessary to induce nearly similar levels of peroxisome proliferation (28,29). A systematic comparison of peroxisome proliferative response and associated enzyme changes in the livers of rats and mice fed various concentrations of known carcinogenic peroxisome proliferators such as DEHP, DEHA, clofibrate, and ciprofibrate is necessary in order to correlate morphological and biochemical changes in peroxisomes with the hepatocarcinogenic potency of these chemicals. To address this issue, comparative studies were performed in groups of F344 male rats fed selected dose levels of two plasticizers (DEHP and DEHA) and a potent hypolipidemic drug (ciprofibrate) (Fig. 1). In these studies DEHP and DEHA were added to the diet at 0.25, 0.5, 1, and 2% (w/w) and ciprofibrate was fed at 0.001, 0.01, and 0.02% (w/w).

After 30 days of treatment, a dose-dependent increase in liver weight was noted with ciprofibrate and DEHP (Fig. 2), whereas with DEHA the increase in liver weight was significant only at the 2% level. Of particular interest to note is that the increase in liver weight observed in rats fed ciprofibrate at the 0.01% level was comparable to that induced by DEHP at the 2% level, suggesting an approximately 200-fold difference in the hepatomegalic potency of these two agents in rats. Likewise, the hepatomegalic effect of ciprofibrate is estimated to be >1000 times that of DEHA.

A close relationship between hepatomegalic and peroxisome proliferative effects of these three agents is noted (Figs. 3–6). In normal rat liver peroxisomes are few and randomly distributed (Fig. 3A). Ciprofibrate at the 0.001% level in the diet caused a perceptible increase in the number of these organelles (Fig. 3B). At this lower dose level, peroxisomes appeared as focal clusters. At

![Figure 1](image1.png)

Figure 1. Chemical structures of: (1) di-(2-ethylhexyl) phthalate; (2) di-(2-ethylhexyl) adipate, and (3) ciprofibrate (2-(4-(2,2-dichlorocyclopropyl)phenoxy)2-methylpropionic acid).

![Figure 2](image2.png)

Figure 2. Changes in liver weight of rats fed diets containing ciprofibrate, di-(2-ethylhexyl) phthalate (DEHP) or di-(2-ethylhexyl) adipate (DEHA) for 30 days at the concentrations shown. Bar N represents normal rats. The values represent mean ± SD.
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FIGURE 3. Liver parenchymal cells of (A) normal rat and rats treated with different concentrations of ciprofibrate for 30 days: (B) 0.001%, (C) 0.01%, and (D) 0.02%. M, mitochondria; P, peroxisomes. OsO₄; stained with lead citrate. All electron micrographs, approx. × 4650.

0.01% (Fig. 3C) and 0.02% (Fig. 3D), these organelles were distributed throughout the hepatocyte cytoplasm and displayed remarkable variation in size. DEHP at the 0.25% dietary level caused a slight increase in peroxisome number and volume density (Fig. 4A). At this lower dose, level peroxisomes were irregular and displayed matrical
striations (Fig. 4A). DEHP at higher dose levels caused a marked increase in peroxisome number (Figs. 4C and D). The size of hepatic peroxisomes also increased in rats fed DEHP at 2% level (Fig. 4C vs. Fig. 4D).
exerted no perceptible effect on peroxisome number at 0.25% and 0.5% dose levels (Figs. 5A and B), but at the 1% and 2% levels, the peroxisome proliferation was evident (Figs. 5C and D). However, even at the 2% dietary level, DEHA induced only a moderate degree of peroxisome proliferation in rat liver cells. Quantitative mor-
phometric changes in peroxisome volume density presented in Figure 6 confirm the qualitative ultrastructural observations.

Several studies have clearly established that the induction of peroxisome proliferation is associated with a significant increase in the activities of the peroxisomal marker enzyme catalase and the peroxisomal fatty acid β-oxidation system (8). Table 1 presents the results of changes in catalase and peroxisomal 14C-palmitoyl-CoA oxidation in the livers of rats treated with varying concentrations of DEHP, DEHA, or ciprofibrate. Changes in the fatty acid β-oxidation system appeared to parallel the alterations in peroxisome volume density. As expected, the increase in specific activity of catalase was about 2-fold at the maximum level of peroxisome proliferation (Table 1).

The xenobiotic-induced increase in peroxisome population is associated with specific changes in the composition of hepatocyte proteins (30). Of particular interest is the remarkable increase in the content of PPA-80 (27,30), which has been identified as the bifunctional protein of the peroxisomal fatty acid β-oxidation system (31). To further evaluate the relative potency of DEHP, DEHA, and ciprofibrate as peroxisome proliferators, post-nuclear fractions of liver were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). A dose-dependent increase in the amount of PPA-80 was clearly evident with ciprofibrate, DEHP, and DEHA. Furthermore, these data also demonstrate that ciprofibrate is more potent than DEHP in inducing PPA-80. Of the three agents, DEHA is the weakest inducer of PPA-80 even at the 2% dietary level. Figures 8 and 9 present representative densitometric tracings. As shown in Figure 9, DEHP at the 2% dietary level appeared to induce more than twice as much of an increase in PPA-80 content as did DEHA administered at the same dose level. Immunoblot analysis with anti PPA-80 antibodies confirmed the dose-dependent increases in the amounts of PPA-80 (Fig. 10B), whereas immunoblots with ant catalase antiserum showed only subtle changes in the amounts of catalase (Fig. 10A).

These observations clearly indicate that ciprofibrate, at 0.02 and 0.01% dietary concentrations, induces remarkable increases in the peroxisome population and PPA-80 content in rat liver. This compound induces nearly a 100% incidence of liver tumors in rats when administered at 10 mg/kg body weight (i.e., 0.02–0.025% dietary level) for 60 weeks (32). Unpublished observations show that this compound is also hepatocarcinogenic when given at 5 mg/kg dose level (i.e., at ~ 0.01% dietary level). It is also pertinent to point out that DEHP at 0.6 and 1.2% dietary level induced hepatocellular neoplasms in only about 10–15% of the rats (21). The difference in tumor incidence appears to correlate well with the degree of induction of peroxisome proliferation (see Fig. 4) and PPA-80 at these dose levels. The relatively low incidence of tumors in rats and mice fed DEHA similarly reflects the relatively weak peroxisome proliferative effect of this agent observed at the 2% dietary level (see Fig. 5).

### Table 1. Changes in liver catalase and peroxisomal β-oxidation.

| Group   | Dose, % in diet* | Catalase, units/mg protein | [1-14C]-palmitoyl-CoA oxidation, μmole/min/g liver* |
|---------|------------------|----------------------------|---------------------------------------------------|
| Normal  |                  | 42 ± 3                     | 1.1 ± 0.01                                        |
| Ciprofibrate | 0.001     | 52 ± 2                     | 5.8 ± 2.5                                         |
|         | 0.01          | 76 ± 3                     | 12.9 ± 0.5                                        |
|         | 0.02          | 98 ± 3                     | 14.6 ± 0.2                                        |
| DEHP    | 0.25           | 48 ± 3                     | 4.4 ± 0.1                                         |
|         | 0.50           | 55 ± 2                     | 6.1 ± 0.3                                         |
|         | 1.0            | 58 ± 3                     | 5.9 ± 0.9                                         |
|         | 2.0            | 70 ± 3                     | 10.7 ± 1.0                                        |
| DEHA    | 0.25           | 46 ± 3                     | 2.9 ± 0.9                                         |
|         | 0.50           | 49 ± 2                     | 2.8 ± 0.1                                         |
|         | 1.0            | 57 ± 4                     | 3.7 ± 0.1                                         |
|         | 2.0            | 63 ± 3                     | 6.8 ± 0.4                                         |

*The compound was mixed in the powdered rat chow at the level (% w/w) indicated. Male F344 rats were maintained on these diets for 30 days before sacrifice.

*The values are mean ± SD of 3 to 4 animals in each group.
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FIGURE 7. Relative potency of two plasticizers and ciprofibrate to induce peroxisome proliferation associated polypeptide (PPA-80). SDS-polyacrylamide slab gel electrophoretic patterns of post-nuclear fractions of liver of normal rat (N), and rats fed diets containing ciprofibrate: (lane 1) 0.001%; (lane 2) 0.01%; (lane 3) 0.02% in diet; di-(2-ethylhexyl) phthalate: (lane 4) 0.25%, (lane 5) 0.5%, (lane 6) 1.0%, (lane 7) 2.0% in diet; and di-(2-ethylhexyl) adipate: (lane 8) 0.25%, (lane 9) 0.5%, (lane 10) 1.0%, (lane 11) 2.0% in diet for 30 days. The arrows indicate the position of the peroxisome proliferation associated 80,000 molecular weight polypeptide, which represents the bifunctional (enoyl-CoA hydratase-dehydrogenase) enzyme of the peroxisomal fatty acid β-oxidation enzyme system. Approximately 30 μg protein was loaded in each slot.

Tissue Specificity in the Induction of Peroxisome Proliferation

Peroxisomes are present in all cell types (3). They appear prominent in hepatic parenchymal cells and in proximal convoluted tubular epithelium of kidney (1). These organelles in nonhepatic cells are sometimes referred to as microperoxisomes because of their small size (33). Available evidence indicates that peroxisome proliferators exert their peroxisome proliferative and PPA-80 inducing effects mostly in liver parenchymal cells and to a lesser extent in other hepatic cells, and to an even lesser extent in nonhepatic cells. However, the presence of peroxisomes in nonhepatic cells is not uniform throughout the animal. For example, peroxisomes are not present in cardiac muscle (33), and only a limited number of peroxisomes are observed in the kidneys of rats fed diets containing peroxisome proliferators (4). The peroxisomes in liver parenchymal cells are larger than those in nonhepatic cells (33), and are therefore referred to as "macroperoxisomes." Peroxisomes in the kidneys, on the other hand, are very small and are referred to as microperoxisomes (33).

FIGURE 8. Densitometric scans of one-dimensional SDS-polyacrylamide slab gel electrophoretic patterns of post-nuclear fractions obtained from the livers of (A) normal and (B) ciprofibrate-treated (0.001% w/w in diet) rats. The vertical arrow indicates the position of peroxisome proliferation associated polypeptide (~ molecular weight 80,000), which is the bifunctional enzyme of the peroxisomal fatty acid β-oxidation system. Approximately 30 μg protein was loaded in each slot.

FIGURE 9. Densitometric scans of one-dimensional SDS-polyacrylamide slab gel electrophoretic patterns of post-nuclear fractions obtained from the livers of (A) di(2-ethylhexyl) phthalate-treated (2% w/w in diet) and (B) di(2-ethylhexyl) adipate-treated (2% w/w in diet) rats. The vertical arrow indicates the position of peroxisome proliferation associated polypeptide. See Fig. 8 for patterns of normal and ciprofibrate-treated rat livers. Approximately 30 μg protein was loaded in each slot.
FIGURE 10. Immunoblotting of electrophoretically separated polypeptides of postnuclear fractions of liver of normal rats (N) and rats fed diets containing different concentrations of ciprofibrate (lanes 1–3), di(2-ethylhexyl) phthalate (DEHP) (lanes 4–7) or di(2-ethylhexyl) adipate (DEHA) (lanes 8–11). For dose levels see legend for Fig. 7. Electrophoretically separated (~10 μg per lane in panel B and ~50 μg per lane in panel A) proteins were transferred to nitrocellulose and immunblotted with antiserum for rat catalase (A), or with peroxisome proliferation associated 80,000 molecular weight polypeptide (B). The immune complexes were detected by autoradiography following treatment with 125I-labeled protein. In panel A, the intensity of catalase of bands in treated animals is slightly increased when compared to that of normal (N). In general, the maximum increase in catalase protein is about two-fold. In Panel B peroxisome proliferation associated 80,000 molecular weight protein (bifunctional protein of peroxisomal β-oxidation) is barely detectable in normal post-nuclear fractions. This protein is induced in a dose related fashion in treated rats. The relative potency of the three peroxisome proliferators can be assessed by comparing the intensity of the bands in lane 2 (ciprofibrate 0.01%) with lane 6 (DEHP 2%) and lane 1 (ciprofibrate 0.001%) with lane 10 (DEHA 1%). This suggests that ciprofibrate is at least 200 times more potent than DEHP and 1000 times more potent than DEHA.

extent in the proximal tubular epithelium of the kidney (8,11). Although slight increases in the activities of catalase, fatty acyl-CoA oxidase, and/or the peroxisomal fatty acid β-oxidation enzyme system were reported in certain nonhepatic tissues, including small intestinal mucosa, following treatment with clofibrate or other peroxisome proliferators (34,35), recent ultrastructural studies as well as high-resolution two-dimensional analy-
sis of proteins of small intestine, heart, skeletal muscle, testis, adrenal, brain, and lung of rats fed ciprofibrate, a potent carcinogenic hepatic peroxisome proliferator; failed to demonstrate peroxisome proliferation and induction of PPA-80 in these nonhepatic tissues (36). These results clearly show that ciprofibrate-induced—and by implication other peroxisome proliferator-induced—peroxisomal gene expression is largely liver specific.

Peroxisome proliferation was also inducible by clofibrate and DEHP, in hepatocytes transplanted in interscapular fat pads (37) or in the anterior chamber of the eye (38). The magnitude of increase in peroxisome volume density in transplanted hepatocytes at these extrahepatic sites was comparable to the increase in the volume density of these organelles in the homotopic liver cells (37). The absence of peroxisome proliferation in brown fat cells or epithelial cells of the eye, which are adjacent to hepatocytes at these transplantation sites, further supports the contention that peroxisome proliferative response is a hepatocyte specific phenomenon. Another piece of evidence in favor of tissue specificity in the induction of peroxisome proliferation comes from the observation of peroxisome proliferation in transdifferentiated hepatocytes in rat and hamster pancreas (39,40). In these studies, drug-induced peroxisome proliferation was observed in pancreatic hepatocytes, but not in adjacent pancreatic acinar and endocrine cells (39,40). Therefore, it is reasonable to conclude that hepatocytes, irrespective of their location in the body, recognize peroxisome proliferators and respond to their stimulating effect on peroxisome-specific gene expression.

Mechanism of Peroxisome Proliferation

Tissue-specific biological response resulting from the administration of structurally dissimilar peroxisome proliferators suggests that the interaction of these agents with a receptor(s) might be the mechanism responsible for peroxisome proliferation. Lalwani et al. (41) have described specific binding of [3H]-nafenopin, a peroxisome proliferator, to liver cytosol. The presence of such a binding protein(s) in hepatocytes (41) and its relative absence in other tissues can account for the difference in tissue inducibility of peroxisomes. The induction of peroxisome proliferation in primary liver cell cultures by these peroxisome proliferators under serum free conditions provides further support for the peroxisome proliferator—receptor interaction (42). Physicochemical characterization of peroxisome proliferator specific receptor(s) is essential for elucidation of the biochemical mechanism by which a peroxisome proliferator—receptor complex activates specific genes.

Hydropolipidemic peroxisome proliferators such as clofibrate, ciprofibrate, and methyl clofenapate do not require extensive metabolic transformation. Compounds which are in the form of ethyl or methyl esters (i.e., clofibrate and methyl clofenapate) are converted into free acids and usually excreted as glucuronide conjugates. Compounds which are in the acid form (i.e., ciprofibrate and nafenopin) are present in the circulation as such and are excreted as glucuronide conjugates. The receptor-binding moiety, therefore, appears to be the acid form of these hypolipidemic agents. The plasticizer DEHP undergoes extensive metabolic conversion (43), and it is not certain whether the metabolite monoethylhexyl phthalate or the metabolite 2-ethylhexanol is the moiety capable of inducing peroxisome proliferation (29).

Mechanism of Peroxisome Proliferator-Induced Liver Carcinogenesis

Reddy et al. (19) proposed that hepatic peroxisome proliferators constitute a novel class of chemical carcinogens. Several studies have now established that compounds capable of inducing peroxisome proliferation are hepatocarcinogenic in both mice and rats when administered chronically in the diet (8). None of these carcinogenic peroxisome proliferators has been shown to be mutagenic in bacterial assays (20,23–25,44). Furthermore, no carcinogenic peroxisome proliferators, including hypolipidemic drugs and phthalate ester plasticizers displayed any capacity to covalently modify or damage cellular DNA either in vivo or in vitro (23,24,44–48). Recently, we examined, using the [32P]-postlabeling assay, the ability of clofibrate, ciprofibrate, Wy-14,643, and DEHP to form DNA adducts in liver (49). With this highly sensitive method, which is capable of detecting one adduct in $10^9$–$10^{16}$ normal nucleotides, we found no peroxisome proliferator–DNA adducts (49). It is, therefore, reasonable to conclude that, with peroxisome proliferators, formation of a DNA adduct may not be a necessary step for carcinogenesis.

As discussed elsewhere (8,19,26), the carcinogenicity of these nonmutagenic and non-DNA adduct-forming peroxisome proliferators appears to be related to biologically active products of the proliferated peroxisomes rather than a direct chemical effect. Evidence supporting the role of peroxisome proliferation mediated oxidative stress as a possible hepatocarcinogenic mechanism includes: (a) consistent association between the induction of peroxisome proliferation and hepatocellular carcinomas (17–21); (b) sustained and specific induction of H$_2$O$_2$-generating peroxisomal fatty acid $\beta$-oxidation system and PPA-80 (8,11–15); (c) increased intracellular levels of H$_2$O$_2$ in livers with peroxisome proliferation (15,50,51); (d) increased accumulation of lipofuscin in livers following chronic induction of peroxisome proliferation (26); (e) marked inhibition of peroxisome proliferator-induced carcinogenesis by antioxidants ethoxyquin and butylated hydroxyanisole (32); and (f) DNA damaging capability of hypolipidemic drug induced liver peroxisomes (50). In addition, the recent identification of an oxidase in peroxisomal membrane capable of oxidizing glycerol phos-
phate, xanthine, or aldehydes (52) further supports the mechanism of peroxisome proliferation associated oxidative stress. This peroxisomal membrane oxidase is capable of generating $O_2^-$ and $H_2O_2$, and, as pointed out recently, the increased generation of $H_2O_2$ and $O_2^-$ by proliferated peroxisomes may lead to $OH$\(^{-}\) (hydroxy radical) formation (50). Although these reactive oxygen species are known to interact with cellular macromolecules including DNA, considerable work is needed to identify the mechanism by which these oxygen radicals cause liver cancer. Whether oxidative stress resulting from sustained proliferation of peroxisomes leads to DNA damage or alters the expression of oncogenes and growth factors is not known.

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