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Transcriptional regulation of peroxisomal fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in rat liver by peroxisome proliferators

[catalase/fatty acid β-oxidation/clofibrate/ciprofibrate/bis(2-ethylhexyl) phthalate]

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ABSTRACT **The structurally diverse peroxisome proliferators ciprofibrate, clofibrate, and bis(2-ethylhexyl) phthalate [$(EtHx)_2$>Pht] increase the activities of hepatic catalase and peroxisomal fatty acid β-oxidation enzymes in conjunction with profound proliferation of peroxisomes in hepatocytes. In order to delineate the level at which these enzymes are induced in the liver, the transcriptional activity of specific genes for fatty acyl-CoA oxidase (FAOxase) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE), the first two enzymes of the peroxisomal β-oxidation system, and for catalase were measured in isolated hepatocyte nuclei obtained from male rats following a single intragastric dose of ciprofibrate, clofibrate, or $(EtHx)_2$>Pht. All three peroxisome proliferators rapidly increased the rate of FAOxase and PBE gene transcription in liver, with near maximal rates (9–15 times control) reached by 1 hr and persisting until at least 16 hr after administration of the compound. FAOxase and PBE mRNA levels, measured by blot-hybridization analysis and FAOxase and PBE protein content, analyzed by immunoblotting, increased concurrently up to at least 16 hr following a single dose of peroxisome proliferator. The catalase mRNA level increased about 1.4-fold, but the transcription rate of the catalase gene was not significantly affected. The results show that the peroxisome proliferators clofibrate, ciprofibrate, and $(EtHx)_2$>Pht selectively increase the rate of transcription of peroxisomal fatty acid β-oxidation enzyme genes. Whether the transcriptional effects are mediated by peroxisome proliferator–receptor complexes remains to be elucidated.**

Peroxisomes are ubiquitous cytoplasmic organelles, present in animal and plant cells, that contain enzymes capable of generating and degrading hydrogen peroxide (1–4). In mammalian liver parenchymal cells, peroxisomes are few in number and appear insignificant in the overall cytoplasmic organization. Dramatic increases in the number of peroxisomes and in the activity of the H_2O_2-generating peroxisomal fatty acid β-oxidation enzyme system occur in liver parenchymal cells of rats, mice, and certain other species exposed to several structurally dissimilar hypolipidemic drugs and certain phthalate ester plasticizers (5–9). These agents are referred to as peroxisome proliferators (7, 8). The process of xenobiotic-induced peroxisome proliferation in mammalian liver cells is receiving considerable attention in light of the evidence that peroxisome proliferators as a class are hepatocarcinogenic (10). Since these agents *per se* are nonmutagenic and non-DNA-damaging, the consistent coupling of proliferation of peroxisomes and liver tumor development with these agents led to the hypothesis that persistent proliferation of peroxisomes, vis-a-vis induction of the H_2O_2-generating fatty acid β-oxidation enzyme system, serves as an endogenous initiator of neoplastic transformation by inducing intrahepatic oxidative stress (8, 10, 11).

Induction of maximal peroxisome proliferation in liver cells is associated with an ≈2-fold increase in the activity and biosynthesis of the peroxisomal marker enzyme catalase (7, 8, 12) and up to a 20- to 30-fold increase in the activity of the peroxisomal fatty acid β-oxidation enzyme system (8, 9, 13, 14). The peroxisomal β-oxidation system is composed of three proteins (2, 13–15). The first, fatty acyl-CoA oxidase (FAOxase) is a flavoprotein that catalyzes dehydrogenation of fatty acyl-CoA leading to the production of H_2O_2. The second, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE), catalyzes the second and third reactions of the β-oxidation cycle, yielding 3-ketoacyl-CoA from enoyl-CoA. The third protein, 3-ketoacyl-CoA thiolase, catalyzes the last reaction of the β-oxidation cycle, forming acetyl-CoA (2, 13, 15). Although catalase protein does not increase in proportion to peroxisome volume density, the activity of the fatty acid β-oxidation system and the quantity of peroxisome-proliferation-associated M_r 80,000 PBE protein (16) generally change in parallel with increases in peroxisome volume density following administration of peroxisome proliferator (7, 8). The increase of fatty acid β-oxidation enzymes induced by peroxisome proliferators is due to specific increases in translatable mRNA levels in the liver (14, 17). In this report, we demonstrate that the increase in mRNAs for the first two peroxisomal fatty acid β-oxidation enzymes, FAOxase and PBE, in liver results from a rapid stimulation of FAOxase and PBE gene transcription by peroxisome proliferators.

MATERIALS AND METHODS

Animals and Peroxisome-Proliferator Administration. Groups of 3–5 male F344 rats weighing 150–200 g were given a single intragastric dose of ciprofibrate {2-[-4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropanoic acid, 250 mg/kg of body weight}, clofibrate [2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester, 500 mg/kg], or $(EtHx)_2$>Pht [bis(2-ethylhexyl) phthalate, 2.5 g/kg] and killed after 1, 4, 8, 16, and 24 hr. Control animals were given, by gavage, 0.1 ml of dimethyl sulfoxide, which was used as solvent for ciprofibrate. Additional groups of 3 rats were fed a diet containing ciprofibrate (0.025% wt/wt), clofibrate (0.5% vol/wt), or

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Abbreviations: $(EtHx)_2$>Pht, bis(2-ethylhexyl) phthalate; FAOxase, fatty acyl-CoA oxidase; PBE, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme.

$(EtHx)_2$>Pht (2% vol/wt) for 2 weeks to induce a steady state of liver peroxisome proliferation (7, 8).

Isolation of Nuclei. Highly purified nuclei were isolated from liver essentially as described by Blobel and Potter (18) except that all buffers contained 2 mM dithiothreitol and 0.5 mM spermidine·HCl (19). Purified nuclei were resuspended (2×10^8 nuclei per ml) in 40% (vol/vol) glycerol/20 mM Hepes, pH 7.6/2 mM $MgCl_2$/2 mM dithiothreitol and stored at −70°C until use.

cDNA Plasmids. The recombinant plasmids pMJ115, pMJ26, and pMJ504 contain, respectively, rat peroxisomal FAOxase, PBE, and catalase cDNA sequences in the *Pst* I site of pBR322 (20–22). cDNA probes pRSA-1 for rat serum albumin (23) and pRAF-65 for α-fetoprotein (24), which were used as controls, were obtained from H. Pitot (McArdle Laboratories, Madison, WI) and N. Fausto (Brown University, Providence, RI), respectively. Plasmids were extracted from *Escherichia coli* by the alkaline-lysis method (25).

Total Cellular RNA Isolation and Blot-Hybridization Analysis. Total liver cell RNA was prepared by the method of Chirgwin *et al.* (26). Electrophoresis of glyoxal-denatured (27) total RNA in 1% agarose gels and transfer to Biodyne A membrane (Pall Corp., Glen Cove, NY) were performed as described (28). Prehybridization and hybridization with nick-translated ^{32}P-labeled cDNA plasmids (nick-translation kit from Amersham) and washing were performed as outlined by Thomas (28). Quantitation was done by densitometric scanning.

Analysis of Nuclear Transcription. Purified liver nuclei were incubated for 30 min at 26°C in 100 μl of transcription buffer [20% glycerol/20 mM Hepes, pH 7.8/1 mM $MgCl_2$/2 mM $MnCl_2$/3 mM spermidine/5 mM dithiothreitol/75 mM $(NH_4)_2SO_4$/0.66 mM each ATP, GTP, and CTP] containing [α-^{32}P]UTP (1 mCi/ml; Amersham, 3000 Ci/mmol; 1 Ci = 37 GBq) and RNasin (700 units/ml; Promega Biotec, Madison, WI) to allow nascent RNA chains to elongate, using a modification of the method described by Mueckler *et al.* (19). In control incubations, the RNA polymerase II inhibitor α-amanitin (29) was included at 1 μg/ml. The reaction was terminated by the addition of RNase-free DNase I to a final concentration of 200 μg/ml and incubation for an additional 20 min at 26°C. After digestion with proteinase K (100 μg/ml; 30 min incubation at 42°C), the nuclear RNA was extracted with phenol and then with chloroform/isoamyl alcohol (24:1) using a modification of the procedure described by Groudine *et al.* (30). The aqueous phase, containing RNA, was passed through a Sephadex G-75 column. The RNA eluted from the column was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes of 95% ethanol. This labeled RNA was used as a probe. Linearized plasmids were denatured in 0.3 M NaOH at 60°C for 15 min and then an equal volume of 2 M ammonium acetate was added. Aliquots of DNA (1.5 μg) were spotted into nitrocellulose (presoaked in 1 M ammonium acetate) and fixed by baking for 2 hr at 80°C under vacuum. Each filter was hybridized with transcript probe (10^6 cpm) for 4 days (30). Radioactivity was detected by autoradiography using Kodak XAR-5 film at −70°C with an intensifying screen. Background levels were determined by hybridizing ^{32}P-labeled RNA to filters containing pBR322 DNA. Under the conditions of hybridization used, the signal was proportional to the concentration of the probe.

Immunoblotting Procedure. Samples of rat liver extracted with lysis buffer (31) were resolved by $NaDodSO_4$/10% PAGE and transferred to nitrocellulose paper (32). The nitrocellulose was incubated with albumin (5% solution) for 1 hr at 37°C, followed by anti-FAOxase (20) or anti-PBE (33). The antigen–antibody complexes were visualized by autoradiography after incubation with ^{125}I-labeled protein A (New England Nuclear; 70–100 μCi/μg).

RESULTS

mRNA Concentrations at the Steady State of Hepatic Peroxisome Proliferation. Blot-hybridization analysis of electrophoretically fractionated RNA was used to measure the relative abundance of mRNAs for the peroxisomal enzymes FAOxase, PBE, and catalase during the steady state of hepatic peroxisome proliferation induced by a 2-week dietary administration of ciprofibrate, clofibrate, or $(EtHx)_2$>Pht. Quantitation of the specific signals by scanning densitometry revealed approximately 15-fold and 17-fold increases in mRNAs, respectively, for FAOxase and PBE in the livers of rats given ciprofibrate (Fig. 1). In contrast, peroxisomal catalase mRNA increased only about 1.4-fold. The relative increases in mRNAs for FAOxase and PBE in clofibrate- and $(EtHx)_2$>Pht-treated livers were slightly lower than those observed in ciprofibrate-treated rats. With clofibrate and $(EtHx)_2$>Pht, the increase in FAOxase mRNA ranged from 9- to 12-fold and the increase in PBE mRNA was about 13- to 15-fold. Detailed quantitative data obtained by slot blot hybridization will be presented elsewhere. No change in the albumin mRNA level (Fig. 1*D*) was observed in the livers of rats treated with peroxisome proliferators when compared to control animals.

Time-Course of Changes in mRNA Concentrations After a Single Dose of Peroxisome Proliferators. Fig. 2 represents a typical experiment depicting the temporal expression of albumin and peroxisomal FAOxase, PBE, and catalase mRNAs in liver following a single intragastric dose of the potent hepatic peroxisome proliferator ciprofibrate. FAOxase and PBE mRNA levels began to increase 1 hr after peroxisome-proliferator administration. Ciprofibrate caused, within 4 hr, 7-fold and 15-fold increases, respectively, in FAOxase and PBE mRNAs; 20-fold and 23-fold increases, respectively, in FAOxase and PBE mRNAs were observed at 16 hr. The increase in catalase mRNA during the 16-hr period was <1.5-fold and the levels of albumin mRNA were essen-

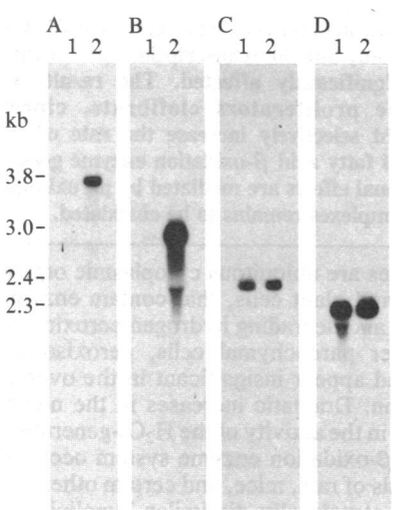

FIG. 1. Steady-state levels of mRNA as measured by blot analysis of total liver RNA from control (lanes 1) and ciprofibrate-treated (0.025% wt/wt in diet for 2 weeks) (lanes 2) rats, using cDNA probes for peroxisomal FAOxase (*A*), PBE (*B*), and catalase (*C*) and for albumin (*D*). Total liver RNA was extracted by the guanidinium thiocyanate/CsCl procedure (26). RNA (10 μg per lane) was denatured with glyoxal (27), separated by 1% agarose gel electrophoresis, and transferred to a Biodyne A nylon filter. The filters containing the RNA were hybridized to nick-translated ^{32}P-labeled cDNA probes [pMJ115 for FAOxase, pMJ26 for PBE, pMJ504 for catalase, and pRSA-1 (23) for albumin]. mRNA sizes for FAOxase [3.8 kilobases (kb)], PBE (3.0 kb), catalase (2.4 kb), and albumin (2.3 kb) are indicated.

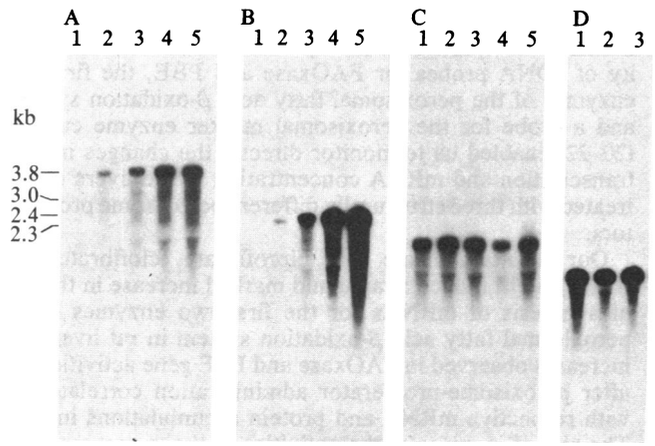

FIG. 2. Comparison by blot analysis of mRNA concentrations for peroxisomal FAOxase (*A*), PBE (*B*), and catalase (*C*) and for albumin (*D*) in total cellular RNA isolated from control rat liver (lanes 1) and livers of rats killed at 1 hr (lanes 2), 4 hr (lanes 3), 8 hr (lanes 4), and 16 hr (lanes 5) after a single dose of ciprofibrate (250 mg/kg of body weight) by gavage. Blot analysis was carried out as described in the legend to Fig. 1. Data for albumin mRNA levels at 8 hr and 16 hr are not included.

tially unchanged (Fig. 2*D*). A single dose of the hypolipidemic drug clofibrate and the plasticizer $(EtHx)_2$>Pht also caused significant increases in mRNA for FAOxase and PBE. FAOxase and PBE mRNA levels continued to increase up to 16 hr following a single dose of ciprofibrate, clofibrate, or $(EtHx)_2$>Pht (Figs. 2 and 3).

Increased Levels of FAOxase and PBE mRNAs Are Due to Increased Transcription. To ascertain whether the accumulation of peroxisomal FAOxase and PBE mRNAs in liver is a consequence of transcriptional activation, hepatic nuclei were isolated at various times after peroxisome-proliferator administration and nascent RNA was labeled by chain elongation in the presence of [α-^{32}P]UTP. The RNA transcripts initiated *in vivo* and elongated *in vitro* were hybridized to cloned FAOxase, PBE, catalase, albumin, and α-fetoprotein cDNA-containing plasmids immobilized on nitrocellulose filters. The data (Fig. 4) indicate that administration of peroxisome proliferator results in rapid and marked increases of FAOxase and PBE gene activities in rat liver. With all three peroxisome proliferators, the rate of transcription is nearly maximal by 1 hr and is maintained at the

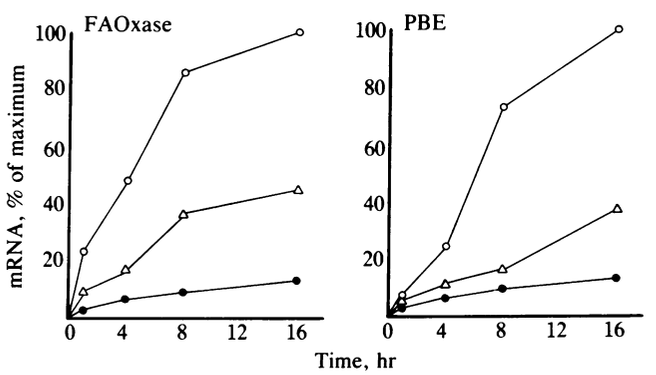

FIG. 3. Kinetics of accumulation of FAOxase (*Left*) and PBE (*Right*) mRNA in rat liver after a single intragastric dose of ciprofibrate (○), clofibrate (●), or $(EtHx)_2$>Pht (△). Blot analysis of total RNA isolated from liver was carried out as described in the legend to Fig. 1 and the resulting autoradiograms were quantitated densitometrically. The values for all time points for the three compounds are expressed as % of maximum value obtained with respective ciprofibrate mRNA at 16 hr.

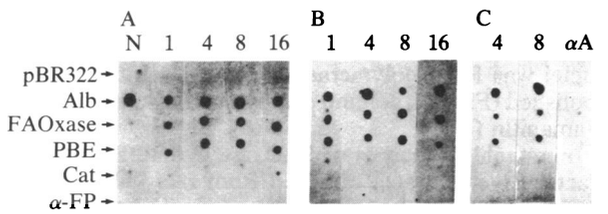

FIG. 4. Comparison of transcriptional rates for albumin (Alb), FAOxase, PBE, catalase (Cat), and α-fetoprotein (α-FP) in livers of rats treated with a single intragastric dose of ciprofibrate (*A*), clofibrate (*B*), or $(EtHx)_2$>Pht (*C*) and killed after 1, 4, 8, and 16 hr. Purified rat liver nuclei were incubated in *in vitro* transcription reaction mixtures in the presence of [α-^{32}P]UTP and the nascent labeled nuclear RNA was hybridized to filter-immobilized plasmids containing the indicated DNAs. Column N in *A* represents transcriptional activity of control rat liver nuclei, and column αA in *C* represents nuclei, from a rat killed 4 hr after ciprofibrate administration, incubated in the presence of α-amanitin. pBR322 (top row) was used as control for nonspecific hybridization. Data for 1-hr and 16-hr $(EtHx)_2$>Pht are not presented. Densitometric tracings of the ciprofibrate data for the three peroxisomal proteins are shown in Fig. 5.

maximal rate for at least 16 hr. The catalase transcription rate was not significantly affected by any of the three peroxisome proliferators; however, because of low basal activity of transcription, a subtle increase in catalase gene transcription could not be ruled out. The time-course of ciprofibrate stimulation of FAOxase, PBE, and catalase gene transcription is shown in Fig. 5. Ciprofibrate caused 9- to 15-fold increases in the transcription of the two peroxisomal β-oxidation-enzyme genes up to 16 hr after administration. The actual levels may be higher because of the very low basal level of transcription of these two genes in normal rat liver. Clofibrate and $(EtHx)_2$>Pht also caused up to a 10-fold increase in the transcription of these β-oxidation genes. The increases in FAOxase and PBE transcription rates correspond well with the magnitude of increase in the specific mRNAs presented in Figs. 2 and 3. The data thus indicate that the peroxisome proliferator-induced accumulation of peroxisomal FAOxase and PBE mRNAs in liver is due to a rapid increase in the transcription of these two genes rather than decreased degradation.

After peroxisome-proliferator administration, the albumin gene was transcribed at a rate approximately equal to that seen in the nuclei of normal rat liver. α-Fetoprotein gene transcription was not detected either in normal or in peroxi-

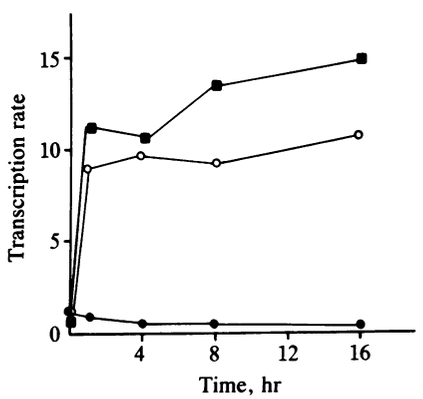

FIG. 5. Kinetics of the ciprofibrate-induced increases in the rate of transcription of peroxisomal FAOxase (○), PBE (■), and catalase (●). The change in transcription rate is presented by taking the value of control (0 hr) as 1. The data represent the average of three experiments analyzed by densitometry and have been corrected for pBR322 background.

some proliferator-treated rat liver nuclei (Fig. 4). The transcription of FAOxase and PBE genes in isolated hepatic nuclei was RNA polymerase II-dependent: it was virtually abolished (Fig. 4C, column αA) by the transcription inhibitor α-amanitin (29).

Immunoblot Analysis of FAOxase and PBE in Liver Extracts. Liver extracts obtained from rats killed at various intervals up to 24 hr following a single intragastric dose of peroxisome proliferators were analyzed by immunoblotting. Increases in the hepatic levels of FAOxase and PBE proteins were evident in rats killed 8 hr after ciprofibrate administration (Fig. 6). Clofibrate and $(EtHx)_2$>Pht treatment also increased the amounts of FAOxase and PBE in livers of rats killed after 8 hr (data not shown).

DISCUSSION

Exposure to peroxisome proliferators results in a massive hepatomegaly with significant increases in the numerical and volume densities of peroxisomes in hepatocytes (6–8, 34). Furthermore, long-term administration of these nonmutagenic agents to rats and mice results in the development of hepatocellular carcinomas (7, 8, 10). Since hypolipidemic drugs and phthalate ester plasticizers, the two major classes of peroxisome proliferators identified thus far, have a recognized and important role in our society today, an understanding of their effects on biological systems and the underlying mechanisms becomes imperative. Although the mechanisms by which peroxisome proliferators exert their peroxisome-proliferative and hepatocarcinogenic effects have not been well defined, it has been proposed that peroxisome proliferators are carcinogenic because of their ability to induce peroxisome proliferation and the resultant increases in the H_2O_2-generating fatty acid β-oxidation enzyme system (8, 10). In addition to the dramatic increases in all three proteins of the peroxisomal fatty acid β-oxidation system, increases in certain other peroxisome-associated enzymes, such as carnitine acetyl- and octanoyltransferases (35–38), acyl-CoA:dihydroxyacetone phosphate acyltransferase (39), and catalase (7, 12), also occur in livers after xenobiotic-induced peroxisome proliferation. Therefore, elucidation of the molecular mechanisms of regulation of the synthesis of peroxisomal enzymes becomes essential in understanding the role of peroxisome proliferation in the initiation of hepatocellular neoplasms (10, 11). The availability of cDNA probes for FAOxase and PBE, the first two enzymes of the peroxisomal fatty acid β-oxidation system, and a probe for the peroxisomal marker enzyme catalase (20–22) enabled us to monitor directly the changes in gene transcription and mRNA concentration in the livers of rats treated with three structurally different peroxisome proliferators.

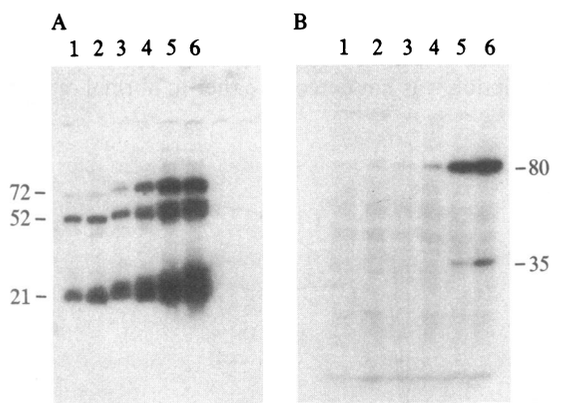

FIG. 6. Immunoblotting of rat FAOxase (A) and PBE (B) with specific antibodies. Total liver cell lysates (10 μg per lane) were resolved by $NaDodSO_4$/10% PAGE. The proteins were transferred to nitrocellulose and treated with anti-FAOxase (A) or anti-PBE (B). Antigen–antibody complexes were visualized by ^{125}I-labeled protein A and autoradiography. The positions of the three subunits of FAOxase (M_r 72,000, 52,000, and 21,000) in A and the positions of the major (M_r 80,000) and a minor (M_r 35,000) PBE band in B are indicated. Lanes 1: normal control. Lanes 2–6: liver samples of rats killed 1, 4, 8, 16, and 24 hr, respectively, after a single intragastric dose of ciprofibrate.

Our results indicate that ciprofibrate, clofibrate, and $(EtHx)_2$>Pht induce a rapid and marked increase in the rate of synthesis of mRNAs for the first two enzymes of the peroxisomal fatty acid β-oxidation system in rat liver. The increases observed in FAOxase and PBE gene activities 1 hr after peroxisome-proliferator administration correlate well with respective mRNA and protein accumulations in liver. The rate of increase of the activities of these enzymes after a single dose is slower than the rate of increase of the transcriptional products. This may be due to additional events controlling posttranscriptional processing and translation. In contrast, no substantial increase in catalase gene transcription occurs in liver nuclei obtained from rats exposed to a single dose of peroxisome proliferators. This observation is consistent with <2-fold increases in catalase mRNA and protein content (14). Despite technical limitations of transcription assays when dealing with subtle changes, it is reasonable to conclude that peroxisome proliferators do not significantly enhance catalase gene transcription.

The sequence of events occurring after peroxisome-proliferator administration indicates a coordinated regulation of transcription of genes for the first two proteins of the peroxisomal fatty acid β-oxidation system. Whether thiolase, the third enzyme of this system, is also regulated in this fashion remains to be determined. Additional studies are also needed to examine whether the high inducible peroxisomal carnitine acetyl- and octanoyltransferases (35–38) are coordinately regulated along with the β-oxidation enzyme system. Coordinate induction of a series of enzymes having closely related functions is not very common in higher eukaryotes (20). In mammalian liver, coordinate induction of the urea cycle enzymes carbamoyl phosphate synthetase 1 and ornithine carbamoyltransferase has been reported (40, 41). The coordinate induction of peroxisomal β-oxidation enzymes by structurally unrelated chemicals implies the existence of a common control mechanism (8, 42). Therefore, it would be important to determine the chromosomal assignment of the genes for these enzymes, in order to ascertain whether coordinate induction of the genes is correlated with their chromosomal location. The rapidity of transcriptional response to peroxisome proliferators suggests that these agents act directly on liver cells. This response is reminiscent of estrogen-induced transcriptional activation of ovomucoid genes (43), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced activation of cytochrome P_1-450 genes in liver (44), and estrogen-induced transcription of ornithine aminotransferase genes in rat kidney (19). Steroid hormone- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible gene activation in liver appears to be governed by ligand-specific "receptor" (22, 44, 45). The existence of peroxisome-proliferator receptor(s) has been postulated (42) to explain tissue (cell)-specific induction of peroxisome proliferation (46). Further biochemical and genetic studies of the effects of peroxisome proliferators may lead to a better understanding of the mechanisms and implications of peroxisome proliferation.

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