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DELAYED EFFECTS OF CIPROFIBRATE ON RAT LIVER PEROXISOMAL PROPERTIES AND PROTO-ONCOGENE EXPRESSION

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Abstract—Peroxisome proliferators (PPs) are non-genotoxic carcinogens in rodents. Their reversible effects on rat liver have been studied with ciprofibrate and fenofibrate. We found that with the hypolipemic drug fenofibrate a pause of 28 days is sufficient for a return to normal status, whereas with the highly potent PP ciprofibrate, the stimulation of ACO mRNA levels remains after its withdrawal. We investigated the effects of the renewal of the treatment with PPs on other peroxisomal parameters and proto-oncogene expression using Wistar rats. Interestingly, c-myc expression was enhanced even upon drug withdrawal, and was more stimulated by the second exposure to ciprofibrate, while c-fos expression was unaltered. However, only slight differences in c-Ha-ras expression were observed. Therefore, the effects of PPs in the Wistar rats are not totally reversible within 28 days following withdrawal, depending on the drug used. These delayed effects of ciprofibrate could be a key to our understanding the hepatocarcinogenic effect of PPs in rodents.

Key words: rat liver; peroxisomes proliferators; carcinogenesis; oncogenes; ciprofibrate; fenofibrate

Peroxisome proliferators (PPs) are a group of many compounds with diverse structures. This family is characterized by the phenomena they provoke, particularly in rodent liver: peroxisome proliferation, hepatomegaly, and later liver tumors [1]. The increase in the size and number of peroxisomes in liver is associated with an increase in the activities of the peroxisomal fatty acid β-oxidation enzymes [2]. The transcription of their genes is stimulated by PPs via a nuclear receptor called peroxisome proliferator activated receptor (PPAR), a member of the steroid hormone receptor superfamily [3], but the mechanism by which PPs activate PPAR is not known. The hepatocarcinogenic effects of PP have not been related to PPAR and remain unexplained. PPs do not appear to react with DNA, are non-genotoxic, and do not induce UDS (unscheduled DNA synthesis) in hepatocytes [4]. Activation of various proto-oncogenes has been observed, but the studies have sometimes been contradictory (see Discussion). In this study, we investigated the phenomenon of the delayed effects of PPs in the Wistar rat strain treated with ciprofibrate or fenofibrate, in this so-called intermittent treatment, where the animals were exposed to the PPs twice for 14 day periods, separated by a pause of 28 days.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing approximately 250 g were purchased from Iffa Credo, France. They were kept at constant temperature with alternate periods of light and darkness, and had free access to water and food.

Chemicals and treatment

Ciprofibrate and fenofibrate were generously provided by Sterling Winthrop and Groupe Fournier (Dijon, France), respectively.

Wistar rats were fed with equilibrated food in pellets (Aliments UAR, Villemoisson/Orge, France) containing either ciprofibrate (200 mg per kg of food: 200 ppm) or fenofibrate (3 g per kg of food: 3000 ppm). These compounds were solubilized in acetone and mixed with the food. The acetone was then eliminated by overnight evaporation. Control animals were fed with acetone-treated pellets. A group consisted of four control rats, four ciprofibrate-treated rats, and four fenofibrate-treated rats. Three groups of twelve rats were treated as follows:

- 2 weeks: 2 weeks of treatment;
- 6 weeks: 2 weeks of treatment followed by a 4-week pause;
- 8 weeks: same as for 6 weeks, followed by a new 2-week treatment.

In case of possible mortality during the study, one rat was added to each group. Since no deaths were observed, these rats were kept for another 8-week break, giving a time point at 16 weeks.

Animals were killed by decapitation. The liver was excised and two grams were immediately frozen in liquid nitrogen for RNA preparation, with the remainder used for subcellular fractionation.

Subcellular fractionation

Mitochondria, peroxisomes, and microsomes were prepared as described by Cherkaoui Malki *et al.* [5].

Abbreviations: ACO, acyl-CoA oxidase; Cipro, ciprofibrate group; Feno, fenofibrate group; PCoA, palmitoyl-CoA oxidase; PPs, peroxisome proliferators; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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RNA preparation

Total RNA isolation and hybridization were described by Cherkaoui Malki *et al.* [6], except when specified otherwise in the legends. Gels were stained with ethidium bromide for normalization, and autoradiographs scanned for quantitation. Rat acyl-CoA oxidase probe (ACO) was kindly provided by Dr T. Osumi (Himeji, Japan). Probes for β-actin (normalization), c-myc, c-fos, and c-Ha-ras were purchased from Oncor, Gaithersburg, MD, U.S.A.

SDS-PAGE

Protein concentrations were determined using the dye-binding method of Bradford [7]. Proteins of the purified peroxisomal fractions were dissociated by heating at 95°C for 5 min in 200 mM Tris-HCl (pH 8.8), 500 mM sucrose, 5 mM EDTA, 1% methionine, 2% SDS, 5 mM DTT, 0.01% bromophenol blue, alkylated by adding 50 mM iodoacetamide and incubating the mixture for 10 min in the dark at room temperature, and finally centrifuged for 5 min at 10,000 g. Electrophoresis was carried out in gradient slab gels (90 × 70 × 0.75 mm), 7 to 15% polyacrylamide, 0.1% SDS with a discontinuous buffer system [8]. Molecular weight markers were purchased from BioRad (Hercules, CA, U.S.A.).

Enzyme assay

Peroxisomal cyanide insensitive palmitoyl-CoA oxidase activity was assayed according to Lazarow & de Duve [9].

Table 1. Effect of intermittent treatment on biochemical parameters

	2 weeks			6 weeks		
	Control	Ciprofibrate	Fenofibrate	Control	Ciprofibrate	Fenofibrate
Hepatosomatic	3.27 ± 0.16	7.28 ± 0.37	7.04 ± 0.24	2.67 ± 0.21	5.01 ± 0.27	3.02 ± 0.16
Index	–	×2.23	×2.15	–	×1.88	×1.13
(*DF* = 6)		($p < 0.001$)	($p < 0.001$)		($p < 0.001$)	($p < 0.05$)
PCoA activity	53.36 ± 3.17	268.23 ± 53.56	246.99 ± 26.3	23.76 ± 3.23	201.89 ± 51.43	66.56 ± 21.86
(*DF* = 5)	–	×5.03	×4.63	–	×8.50	×2.80
		($p < 0.001$)	($p < 0.001$)		($p < 0.005$)	($p < 0.05$)
Mitochondria	22.02 ± 1.84	22.95 ± 7.42	30.44 ± 3.39	18.29 ± 4.23	23.97 ± 3.07	22.19 ± 1.91
(mg/g of liver)	–	×1.04	×1.38	–	×1.31	×1.21
(*DF* = 6)			($p < 0.005$)			
Peroxisomes	0.79 ± 0.42	2.31 ± 0.47	2.49 ± 0.34	0.68 ± 0.15	1.61 ± 0.14	0.92 ± 0.25
(mg/g of liver)	–	×2.89	×3.10	–	×2.37	×1.35
(*DF* = 6)		($p < 0.005$)	($p < 0.001$)		($p < 0.001$)	
Microsomes	26.71 ± 6.34	17.49 ± 3.46	17.20 ± 2.19	16.06 ± 2.01	14.74 ± 1.93	18.05 ± 2.61
(mg/g of liver)	–	×0.65	×0.64	–	×0.92	×1.12
(*DF* = 6)		($p < 0.05$)	($p < 0.05$)			
ACO mRNA	–	×3.4	×3.5	–	×5.6	×1.1
c-fos mRNA	–	×1.7	×1.6	–	×1.1	×0.9
c-myc mRNA	–	×2.9	×1.4	–	×3.2	×0.4

	8 weeks			16 weeks		
	Control	Ciprofibrate	Fenofibrate	Control	Ciprofibrate	Fenofibrate
Hepatosomatic	2.57 ± 0.12	6.06 ± 0.49	5.16 ± 0.13	1.96	4.10	2.92
Index	–	×2.36	×2.0	–	×2.09	×1.49
(*DF* = 6)		($p < 0.001$)	($p < 0.001$)			
PCoA activity	17.59 ± 5.00	270.24 ± 16.19	228.36 ± 22.84	42.03	73.08	24.2
(*DF* = 5)	–	×15.36	×12.98	–	×1.74	×0.57
		($p < 0.001$)	($p < 0.001$)			
Mitochondria	22.08 ± 7.18	40.22 ± 1.96	34.55 ± 2.94			
(mg/g of liver)	–	×1.82	×1.56			
(*DF* = 6)		($p < 0.005$)	($p < 0.05$)			
Peroxisomes	0.91 ± 0.44	2.91 ± 0.62	2.99 ± 0.63			
(mg/g of liver)	–	×2.90	×2.28			
(*DF* = 6)		($p < 0.005$)	($p < 0.005$)			
Microsomes	14.27 ± 1.53	9.47 ± 2.73	10.64 ± 0.26			
(mg/g of liver)	–	×0.66	×0.75			
(*DF* = 6)		($p < 0.01$)	($p < 0.05$)			
ACo mRNA	–	×3	×2.5			
c-fos mRNA	–	×1.1	×1.2			
c-myc mRNA	–	×8.5	×1.8			

Results are indicated as mean ± standard deviation. Significant variations are indicated with bold numbers and *p* value underneath (*DF* = degree of freedom). In each case the increase (*x*) compared to the control is indicated. Hepatosomatic index is measured as a percentage of the ratio between liver weight and body weight. PCoA activity was measured in the purified peroxisomal fraction as described in Materials and Methods. This activity is expressed as nmol of NADH produced per minute and per milligram of protein. mRNA expression is indicated in fold induction over the control.

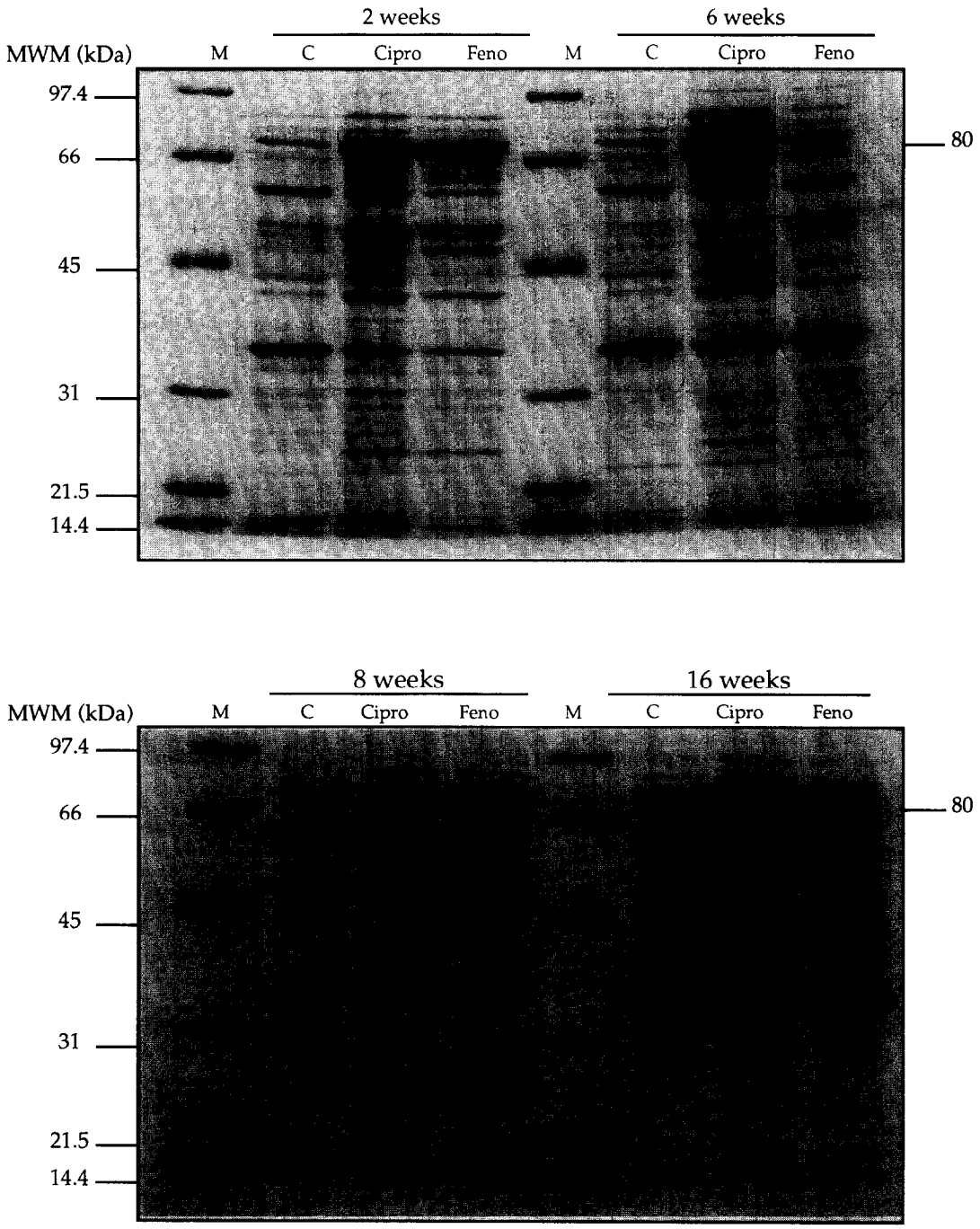

Fig. 1. Peroxisomal polypeptide pattern during treatment of Wistar rat strain. 10 µg of protein were loaded in each lane. Proteins were stained with Coomassie Brilliant Blue. MWM: Molecular weight markers; M: Marker lane.

RESULTS

Peroxisome proliferation

We investigated different parameters of peroxisome proliferator effect. The first, the hepatosomatic index, increased under PP treatment (Table 1). For 16 weeks, all measurements carried out after PP treatment showed a strong increase in the liver/body weight ratio: two times the control. It is important to note that after the withdrawals (6 and 16 weeks), the hepatosomatic index of the cipro group did not return to a normal level (Table 1), as compared with the control and the feno groups. Morphometric analysis of the reversal of hepatic pleiotropic effects were previously studied for ciprofibrate [10] and for other PPs [11].

The second parameter is PCoA activity in the peroxisomal fraction. As with the hepatosomatic index, there was an increase in PCoA activity following PP treatment (Table 1). After 2 weeks of treatment (2 and 8 weeks), PCoA activity was stimulated approximately 5-fold, as

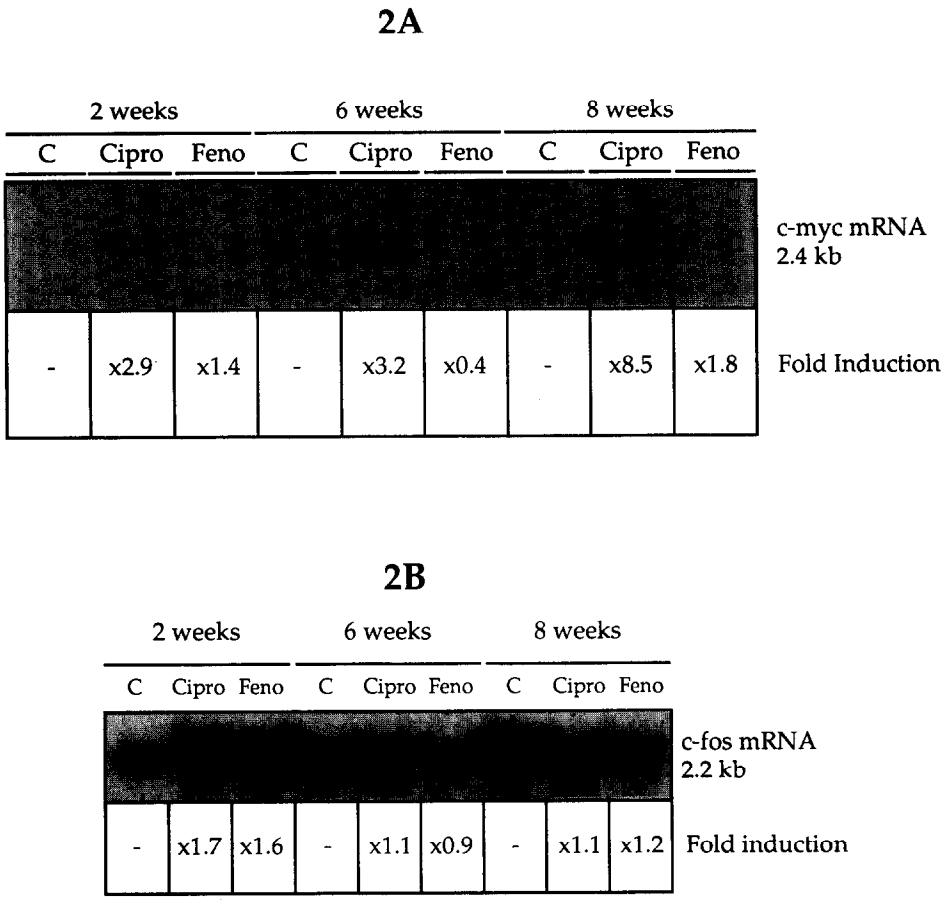

Fig. 2. Proto-oncogene c-myc (A) and c-fos (B) expression during treatment of Wistar rat strain. Total RNA (20 µg) were separated by electrophoresis and blotted on GeneScreenPlus (NEN) as described by the manufacturer. Prehydridization and hybridization were performed in 5 × SSC; 1% SDS; 50% formamide; 5% dextran sulfate; 1 × Denhardt's; 50 µg/ml heparin at 42°C overnight. Washing was performed at 42°C: 2 × 15 min with 2 × SSC; 0.1% SDS; 2 × 15 min with 0.5 × SSC; 0.1% SDS; 30 min with 0.5 × SSC; 0.1% SDS for c-myc (A) and at 42°C: 2 × 15 min with 2 × SSC; 0.1% SDS; 2 × 30 min with 0.5 × SSC; 0.1% SDS; 2 × 15 min with 0.1 × SSC; 0.1% SDS for c-fos (B). The autoradiographs were exposed to Hyperfilm (Amersham) (A) for 4 days at −70°C for c-myc and (B) overnight at −70°C for c-fos.

compared to the control (Table 1). Following the pause (6 and 16 weeks), the PCoA activity in the feno group returned to near normal levels (Table 1), while the activity of the cipro group was still strongly stimulated approximately 8.5-fold (Table 1: 6 weeks). This indicates that ciprofibrate action on peroxisome proliferation persists following a 4-week interruption.

In order to measure the induction of peroxisomal-protein-encoding genes by PPs, we estimated the mRNA expression of the marker gene ACO during the experiment. The expression of ACO mRNA was measured by hybridization with a rat liver ACO probe. The transcription of the ACO gene was highly stimulated by the PP treatments (Table 1). At the end of the 4-week pause, the cipro group did not return to the control level, whereas the feno group did.

The different subcellular fractions collected contained a varying protein content. As expected, an increase in the protein content of de Duve's light fraction (containing mitochondria, peroxisomes, lysosomes, and microsomes) was observed after treatment (data not shown). As seen in Table 1, this was due to an increase in the protein content in the peroxisomal fraction. An increase in the protein content of the mitochondrial fraction was also observed (approx. 50%), especially after renewal of treatment (Table 1: 8 weeks). Interestingly, the protein content of the microsomal fraction decreased as compared to control (Table 1: microsomes).

Peroxisomal polypeptide content

We then studied the peroxisomal polypeptide composition by SDS-PAGE. The electrophoretic pattern of the peroxisomal proteins (Fig. 1) shows several bands of different intensities depending on the group. After 2 weeks of treatment, there were significant changes in the profile of the peroxisomal proteins from PP-treated rats as compared to control, but there were no qualitative differences between ciprofibrate or fenofibrate, especially the 80 kDa band, as described earlier [12]. After the pause, the profile of peroxisomal proteins of fenofibrate-treated rats was similar to the control. In contrast, the peroxisomal protein pattern from ciprofibrate-treated rats showed slight changes with the 2-week profile, but did not return to a pre-treatment pattern (6 weeks: cipro vs control). Furthermore, after an 8-week break, there were still some remaining bands identical to the 2-week profile.

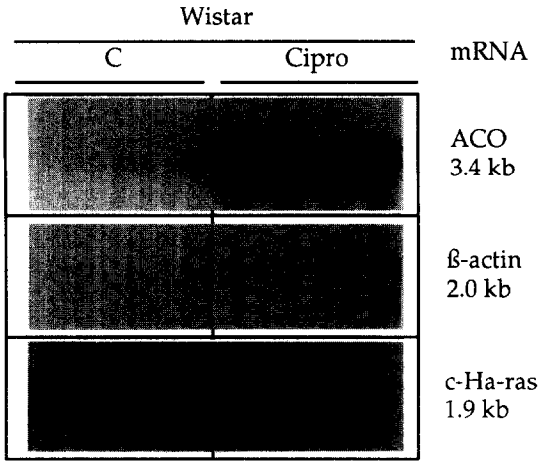

Fig. 3. Comparison of different mRNA expression in Wistar rat strain treated for two weeks with ciprofibrate. Total RNA (10 μg) were separated by electrophoresis and blotted on HybondN (Amersham) as described by the manufacturer. Prehydridization and hybridization were performed in RapidHyb (Amersham) at 65°C for 2 hours. Washing was performed 2 × 20 min with 2 × SSC; 0.1% SDS at RT; 2 × 15 min with 0.1 × SSC; 0.1% SDS at 65°C. (c-Ha-ras was hydridized under the conditions described for c-myc.) The autoradiographs were exposed to XAR film (Kodak) overnight at −70°C.

Proto-oncogene expression

Although earlier studies have shown a stimulation of the transcription of proto-oncogenes such as c-myc [6, 13], c-Ha-ras [6, 14], and c-fos/c-jun [15] in rat or mouse liver, the reversibility effect remained to be investigated. Thus, we analyzed the expression of different proto-oncogenes during an intermittent treatment. The expression of c-myc was found to be stimulated by a 2-week treatment with ciprofibrate (Fig. 2A). Interestingly, this expression increased even after a 4-week withdrawal of the drug. In addition, the level of c-myc mRNA was highly stimulated (8.5-fold) after the second period of treatment. c-fos levels were stimulated 2-fold by the first treatment and not at all by the second treatment (Fig. 2B). No significant changes were observed for c-Ha-ras (Fig. 3).

DISCUSSION

The aims of this experiment were to study the potential reversibility of peroxisome proliferation and the reversibility of proto-oncogene activation observed in previous reports but with the Wistar strain, less sensitive to carcinogens than the Fisher F344 strain. Ciprofibrate is known to have a half-life in rats of 25 hours (α-phase) and 82 hours (β-phase), and fenofibrate, a half-life of 22 hours [16]. Generally, a drug is considered to be completely eliminated from the organism after 7 half-lives. Thus, we conjectured that neither ciprofibrate nor fenofibrate would remain in the blood after the 4-week pause. However, our findings show that the ciprofibrate effect remains even after the 4-week interruption. It is significant that after a longer break (8 weeks, but only for a single animal), the hepatosomatic index and PCoA activity remain 2-fold higher than the control. These variations are confirmed at the different levels of peroxisomal polypeptide pattern and mRNA level. These results are to be compared with those of Wadell *et al.* [17] obtained in mice, showing that ciprofibrate persists in the gall-bladder, intestine, fat, and liver after 27 days of pause, as observed by whole-body autoradiography.

The partitioning of the proteins in the cell is modified during treatment, from the microsomal fraction to the mitochondrial and peroxisomal fractions. These results suggest that after the PP action, the cell increases the biogenesis of peroxisomes and mitochondria, perhaps by slowing down the synthesis of proteins in other compartments (endoplasmic reticulum, golgi, and cytosol).

The ciprofibrate hepatocarcinogenic effect in rodents could be due to long-term modification. As revealed by the peroxisomal polypeptide pattern, the peroxisomes are still altered even after 8 weeks of pause. Some specific mechanism may reduce the turn-over of certain peroxisomal proteins. It is possible that a biological switch is turned on by ciprofibrate and turns off some time after the drug has been eliminated. This could be related to the role of peroxisome proliferator nuclear receptors (PPARs) [3]. Our results show that ACO mRNA is still expressed at a higher level than the control after the first break of 4 weeks. However, we have shown that the transcription rate (assessed by run-on assays) is nevertheless not sufficient to explain this high level of mRNA [18]. Some specific post-transcriptional regulation is therefore hypothesized as existing.

We did not observe a strong stimulation of proto-oncogene expression in two different experiments involving two rat strains, although the Fisher F344 strain does appear to be more sensitive than the Wistar strain. There is some controversy concerning the ability of PPs to induce the expression of proto-oncogenes: Bentley *et al.* [19] showed induction of c-raf, c-fos, and c-Ha-ras, but not c-myc in the liver of rats treated with nafenopin, another PP; Cherkaoui-Malki *et al.* [6] showed induction of c-myc and c-Ha-ras in the liver of rats treated with ciprofibrate; Hsieh *et al.* [13] showed induction of c-myc in rats treated with BR-931, a derivate of Wy-14,643. Recently, Ledwith *et al.* [15] have shown that the activation of fos and jun was independent of peroxisome proliferation, and that induction occurs within the first hour of treatment with Wy-14,643 on cultured cells NIH3T3. Their explanation is that Wy-14,643 has strong effects on cell proliferation and tumorigenesis, and c-fos and c-jun inductions are known to be universal responses to mitogenic stimuli. From our work, c-myc expression appears to be highly stimulated by the second exposure to ciprofibrate. This stimulation increases with the duration of treatment even after a 4-week pause, though only with ciprofibrate. In contrast to fenofibrate, this could be due to the potency of ciprofibrate and/or the accumulation of possible alterations.

Ciprofibrate might initiate mechanisms in the cell that cannot be turned off following withdrawal of the drug, and this phenomenon could play a role in the hepatocarcinogenic effect observed after long-term treatment of rodents with ciprofibrate.

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