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EFFECTS OF LONG-TERM ADMINISTRATION OF CLOFIBRIC ACID ON PEROXISOMAL β-OXIDATION, FATTY ACID-BINDING PROTEIN AND CYTOSOLIC LONG-CHAIN ACYL-CoA HYDROLASES IN RAT LIVER

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Abstract—Long-term effects of *p*-chlorophenoxyisobutyric acid (clofibric acid) on inductions of peroxisomal β-oxidation, fatty acid-binding protein and cytosolic acyl-CoA hydrolases in rat liver were studied. Male rats were fed clofibric acid at a dietary concentration of 0.25% for 22 weeks. The induction of peroxisomal β-oxidation activity lasted throughout the long-term treatment of rats, the activity being a half that of rats treated with clofibric acid for 2 weeks. Cytosolic long-chain acyl-CoA hydrolase I and II were both induced by the long-term and the short-term treatment of age-matched rats with clofibric acid, although the ability to induce hydrolase I decreased greatly by aging of rats. There was little difference in the inducing effect on fatty acid-binding protein between the long-term treatment and the short-term treatment. These results suggest that the inductions of peroxisomal β-oxidation, fatty acid-binding protein and two cytosolic long-chain acyl-CoA hydrolases are essential responses of rats to clofibric acid (but not the brief events which occur in only the first stage of the continuous treatment with clofibric acid).

A number of compounds with hypolipidemic properties have been identified as peroxisome proliferators for the liver of rodents [1] and the hypolipidemic property of the compounds has been considered to be due to proliferate peroxisomes which contain a β-oxidation system, differing from the mitochondrial β-oxidation system in its response to KCN [2]. Recent studies demonstrated that peroxisome proliferators could include not only peroxisomal enzymes, but also may enzymes and protein which are localized in organellae other than peroxisomes [3–7]. Fatty acid-binding protein is one such protein. The protein has been known to be localized in hepatic cytosol [8, 9] and to act as a carrier for fatty acid [10, 11] and as a metabolic regulator for lipid metabolisms [12–16]. We found [4, 17] that two long-chain acyl-CoA hydrolases, which are not found in cytosol of control rats, appeared in hepatic cytosol after the administration of peroxisome proliferators to rats, although the precise physiological role of these hydrolases has not been clarified. Thus, the administration of peroxisome proliferators causes the inductions of peroxisomal β-oxidation enzymes, fatty acid-binding protein and long-chain acyl-CoA hydrolases (most information about these inductions is obtained from the studies on relatively short-term treatment of animals with peroxisome proliferators). To consider the action of clofibric acid and the physiological roles of the induced peroxisomal enzymes, acyl-CoA hydrolases and fatty acid-binding protein, it would be important to study whether the inductions last concomitantly during the treatment period. In the present work, we studied the effects of long-term administration of clofibric acid on the inductions of peroxisomal β-oxidation, fatty acid-burning protein and cytosolic long-chain acyl-CoA hydrolases in rat liver. Hypolipidemic and hepatomegalic effects of the long-term treatment with clofibric acid were also studied.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleic acid (57.0 Ci/mole) was obtained from New England Nuclear Co. (Boston, MA). Palmitoyl-CoA, myristoyl-CoA, clofibric acid and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO); NAD and CoA from Oriental Yeast Co. (Tokyo, Japan); Sephadex G-100 and aminohexylamino-Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were of analytical grade. Oleoyl-aminohexylamino-Sepharose was prepared as previously described [3].

Animals. Male rats of Wistar strain were used. Rats were fed *ad libitum* a commercial diet containing 54.6% digestible carbohydrate, 20.8% protein, 4.5% fat and 3.4% fiber together with all necessary vitamins and minerals. The proportion of fatty acid in the dietary fat was 11.5% palmitic acid, 1.8% palmitoleic acid, 3.6% stearic acid, 21.3% oleic acid, 51.9% linoleic acid and 9.9% linolenic acid by mole %. Clofibric acid was simply added to the commercial ground diet and the diet was pelleted and dried below 30°. The first group of rats aged 5

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weeks at the initiation of treatment was fed a diet containing 0.25% clofibric acid for 22 weeks. The second group of rats aged 5 weeks at the initiation of treatment was fed a diet containing 0.25% clofibric acid for 2 weeks or a diet containing 0.5% clofibric acid for 1 weeks. The third group of rats aged 25 weeks at the initiation treatment was fed a diet containing 0.25% clofibric acid for 2 weeks. For control experiments age-matched rats, which had been fed the commercial control diet, were used; young control rats were 6 or 7-week-old and aged control rats were 27-week-old at the day when they were killed. All animals were exposed to an altering light cycle (light from 8:00 a.m. to 18:00 p.m.) and rats were killed between 10:00 a.m. and 11:00 a.m.

Rats were decapitated and livers were isolated. The livers were perfused with cold 0.9% NaCl and rinsed in cold 0.25 M sucrose. A part of the liver was frozen at −30° until peroxisomal enzymes were assayed. For analyses of fatty acid-binding protein and long-chain acyl-CoA hydrolases, the other part of the liver was homogenized with 1.5 vol. of 0.25 M sucrose and cytosol was obtained from the homogenates by differential centrifugations as described previously [3, 4, 17].

Enzyme assays. Cyanide-insensitive palmitoyl-CoA oxidation was assayed by the method of Lazarow and de Duve [2] with some minor modifications as described previously [18]. Long-chain acyl-CoA hydrolases were assayed after gel filtration of cytosol through a Sephadex G-100 column as described previously [4, 17, 18], except for the use of myristoyl-CoA as the substrate.

Assay for fatty acid-binding protein. [1-^{14}C]Oleic acid-binding capacity of fatty acid-binding protein in hepatic cytosol was measured according to Ockner *et al.* [19] with some modifications as described previously [3, 20]. The concentration of fatty acid-binding protein was determined by affinity chromatography with oleoyl-aminohexylamino-Sepharose as described previously [3].

Other analytical procedures. The concentration of

Table 1. Effects of long-term administration of clofibric acid on concentration of serum neutral lipid and liver weight

Animals	Clofibric acid	Concentration of serum lipid (μmole/ml serum) Trigylyceride	Cholesterol	Liver wt Wt (g)	Relative wt (% of body wt)
Young rats	−	0.93 ± 0.16	2.00 ± 0.15 (7)	7.1 ± 0.4	5.6 ± 0.1 (3)
	+ 2 weeks	0.64 ± 0.18*	0.92 ± 0.20† (8)	18.8 ± 3.5	10.7 ± 1.1 (8)
	+ 22 weeks	1.00 ± 0.27‡§	0.80 ± 0.28† (10)	20.9 ± 1.4	6.6 ± 0.4 (5)
Aged rats	−	2.38 ± 0.77	2.06 ± 0.35 (4)	15.8 ± 2.2	4.2 ± 0.2 (3)
	+ 2 weeks	0.83 ± 0.24§	0.88 ± 0.18§ (8)	20.9 ± 1.7	5.8 ± 0.2 (4)

Each value represents mean ± S.D. Numbers in parentheses are the numbers of animals used. The rats were fed a control diet or a diet containing 0.25% clofibric acid for 2 weeks or 22 weeks. The amounts of triglyceride and cholesterol were determined as described in the text.

* $P < 0.01$, relative to young rat control.
† $P < 0.001$, relative to young rat control.
‡ Not significant relative to young rat control.
§ $P < 0.001$, relative to aged rat control.

Table 2. Effect of long-term administration of clofibric acid on cyanide-insensitive palmitoyl-CoA oxidation in rat liver

Animals	Clofibric acid		Cyanide-insensitive palmitoyl-CoA oxidation × 10^3 units/mg protein	Units/g liver	Units/liver
Young rat	−	(6)	5.80 ± 1.10	0.66 ± 0.12	6.6 ± 2.6
	+ 2 weeks	(3)	42.18 ± 2.70*	6.84 ± 0.43*	149.3 ± 26.3*
	+ 22 weeks	(8)	28.51 ± 8.28*†§¶	3.46 ± 1.04*†‡‖	72.3 ± 22.8*†‡‖
Aged rats	−	(5)	4.04 ± 1.24	0.43 ± 0.11	7.1 ± 0.9
	+ 2 weeks	(8)	40.79 ± 7.15†	6.56 ± 1.15†	145.8 ± 19.9†

Each value represents mean ± S.D. Numbers in parentheses are the numbers of animals used. The rats were fed a control diet or a diet containing 0.25% clofibric acid for 2 weeks or 22 weeks. The enzyme activity in post-nuclear supernatant of livers was assayed as described in the text. One unit of the activity was defined as the amount of enzyme required to reduce 1 μmole of NAD per min.

* $P < 0.001$ relative to young control rat.
† $P < 0.001$ relative to aged control rat.
‡ $P < 0.001$ relative to aged rat treated for 2 weeks.
§ $P < 0.01$ relative to aged rat treated for 2 weeks.
‖ $P < 0.001$ relative to young rat treated for 2 weeks.
¶ $P < 0.05$ relative to young rat treated for 2 weeks.

serum cholesterol was determined by the method of Allain *et al.* [21]. The concentration of serum triglyceride was determined as described previously [22]. The protein concentration was determined by the method of Lowry *et al.* [23].

RESULTS AND DISCUSSION

Table 1 shows the effects of administration of clofibric acid to rats on serum lipid concentrations and liver weight under various experimental conditions. The concentration of serum triglyceride was decreased significantly by the administration of clofibric acid to young rats for 2 weeks. The short-term treatment of aged rats with clofibric acid caused a marked reduction of the concentration of serum triglyceride, despite the fact that the concentration of serum triglyceride of aged control rats was 2.6 times that of young control rats. The administration of clofibric acid rats for 35 weeks did not change significantly the concentration of serum triglyceride, when compared to the concentration of serum triglyceride of young control rats, whereas the concentration of serum triglyceride after the long-term treatment was almost the same as that of the aged rats which had been treated with clofibric acid for a short term. The concentration of serum cholesterol was decreased markedly, regardless of age of rats and duration of the administration of clofibric acid. In agreement with the earlier studies [18, 24], a great enlargement of liver was observed in both young rats and aged rats after the short-term treatment with clofibric acid. The long-term treatment also produced a significant hepatomegaly. These results may suggest that hypolipidemic and hepatomegalic effects of clofibric acid last throughout the administration period.

Table 2 shows the changes in activity of cyanide-insensitive palmitoyl-CoA oxidation when rats were fed clofibric acid. The short-term treatment of aged rats with clofibric acid increased markedly the activity of cyanide-insensitive palmitoyl-CoA oxidation to almost the same extent as was observed in young rats. The long-term administration of clofibric acid to rats also gave a similar inducting effect on the activity of cyanide-insensitive palmitoyl-CoA oxidation, whereas the extent of the increase in the activity was about a half of those of the rats which had been treated with clofibric acid for short term. Cyanide-insensitive palmitoyl-CoA oxidizing activity is known to be responsible for peroxisomal β-oxidation [2] and proliferated peroxisomes are known to contain a polypeptide with a molecular weight of about 80,000 at higher concentration [25]. Subcellular fractionation study on liver from the long-term treated rats demonstrated that the activity of cyanide-insensitive palmitoyl-CoA oxidation was localized in light mitochondrial fraction and that the distribution pattern of the activity was consistent with that of urate oxidase, a marker enzyme for peroxisomes (results not shown). Electrophoretic study showed that the concentration of the polypeptide of a molecular weight of about 80,000 in the mitochondrial fraction was increased after the long-term treatment of rats with clofibric acid (results not shown). These findings strongly suggest that the

Table 3. Effect of long-term administration of clofibric acid on fatty acid-binding protein in rat liver

Conditions		Oleic acid bound to fatty acid-binding protein			Concentration of fatty acid-binding protein		
		nmoles/mg cytosolic protein	nmoles/g liver	μmoles/ liver	μg/mg cytosolic protein	mg/g liver	mg/liver
Control	(4)	1.57 ± 0.42	58.9 ± 17.2	0.65 ± 0.12	49.27 ± 8.81	1.86 ± 0.46	20.7 ± 4.5
0.5% Clofibric acid 1 week	(3)	3.91 ± 0.49*	160.6 ± 20.2†	2.74 ± 0.46†	107.06 ± 9.64†	4.42 ± 0.74‡	74.9 ± 9.2†
0.25% Clofibric acid 22 weeks	(4)	3.78 ± 0.75‡	126.7 ± 24.3‡	2.83 ± 0.59†	123.17 ± 9.76†	4.01 ± 0.23†	88.5 ± 6.2†

Each value represents mean ± S.D. Numbers in parentheses are the numbers of animals used. The rats were fed a control diet, 0.5% clofibric acid for 1 week or 0.25% clofibric acid for 22 weeks. Binding of [1-^{14}C]oleic acid to fatty acid-binding protein and concentration of fatty acid-binding protein were determined as described in the text.

* $P < 0.05$ relative to control.
† $P < 0.001$ relative to control.
‡ $P < 0.01$ relative to control.

Table 4. Effect of long-term administration of clofibric acid on inductions of liver cytosolic hydrolases

Animals	Administration of clofibric acid	Hydrolase I		Hydrolase II	
		Units/g liver	Units/ liver	Units/g liver	Units/ liver
Young rats	2 weeks (2)	3.05	58.6	1.20	23.1
		2.14	43.0	0.92	18.4
	22 weeks (4)	1.19 ± 0.33	25.7 ± 7.3	0.86 ± 0.06	18.7 ± 2.1
Aged rats	2 weeks (4)	1.62 ± 0.21	33.6 ± 3.1	1.25 ± 0.04	26.0 ± 1.6

The activity of hydrolase I and II were determined as described in the text. The rats were fed a diet containing clofibric acid for 2 weeks or 22 weeks. Livers of the rats which had been fed control diet contained no activity of either hydrolase I or II. Where shown, values are mean ± S.D. Numbers in parentheses are the numbers of animals used.

cyanide-insensitive palmitoyl-CoA oxidizing activity of rats treated with clofibric acid for a long term is attributable to peroxisomal β-oxidation, as was confirmed for rats treated for a short term.

Thus, the continuous feeding of clofibric acid for a long term maintained the induced activity of peroxisomal β-oxidation in rat liver during the treatment, although the activity was a half of that in liver of the young rats which had been treated with clofibric acid for a short term. The ability of aged rats to induce the activity of peroxisomal β-oxidation was almost the same as that of young rats. Therefore, the reduced induction of peroxisomal β-oxidation in liver after the long-term treatment does not seem to be due to the aging of the rats. The precise reason for the low activity of peroxisomal β-oxidation remains to be elucidated. The present results are essentially in accordance with the findings of Reddy *et al.* [26] who found that long-term administration of methyl-2-[4-(*p*-chlorophenyl)phenoxy]-2-methylpropionate to rats induced peroxisomal β-oxidation in both tumor part induced by the compound and non-tumorous part of liver, because the compound is related structurally to clofibric acid.

Fleischner *et al.* [27] reported that the administration of clofibrate (ethyl ester of clofibric acid) to rats increases concentration of fatty acid-binding protein in liver. More recent studies [3, 28] presented the evidence suggesting the involvement of fatty acid-binding protein in peroxisomal β-oxidation. However, these findings were all derived from the studies on short-term administration of peroxisome proliferators to rats. Table 3 shows the effects of the long-term treatment of rats with clofibric acid on hepatic fatty acid-binding protein. On the long-term administration of clofibric acid, the amount of [1-^{14}C]oleic acid bound to cytosolic fatty acid-binding protein increased markedly. The increase by the long-term treatment in the amount of [1-^{14}C]oleic acid bound to liver cytosolic fatty acid-binding protein was almost the same as that by the short-term administration of clofibric acid. The concentration of fatty acid-binding protein was measured by affinity chromatography. The concentration of fatty acid-binding protein in liver was increased by the long-term administration of clofibric acid, as was observed with the short-term administration of clofibric acid. The increase in the oleic acid-binding capacity of cytosol was parallel to the increase in the concentration of fatty acid-binding protein in cytosol. In contrast to peroxisomal β-oxidation, however, there was no significant difference in [1-^{14}C]oleic acid-binding capacity and concentration of hepatic fatty acid-binding protein between the short-term administration and the long-term administration of clofibric acid to rats.

In agreement with our previous findings [4, 17, 18], the short-term administration of clofibric acid to young rats caused inductions of two long-chain acyl-CoA hydrolases in hepatic cytosol; hydrolase I with a molecular weight of about 80,000 and hydrolase II with a molecular weight of about 40,000 (Table 4). The ability of aged rats to induce hydrolase I was much lower than that of young rats. By the long-term administration of clofibric acid, both hydrolase I and II were induced, as was the case with the short-term administration. However, the long-term administration induced hydrolase I to much less extent than the short-term administration did. There was no difference in the induction of hydrolase II between young rats and aged rats, but the induction of hydrolase II in liver of rats affter the long-term administration was somewhat lower compared to those of young rats and aged rats after the short-term treatment. We have previously reported [29] that the induction of hydrolase I by clofibric acid is dependent on the state of androgen. Accordingly, a plausible explanation for the lower ability of aged rats to induce hydrolase I would be that hepatocytes of aged rats responded less to androgen, or the state of androgen was altered in aged rats. In contrast to hydrolase I, the ability of aged rats to induce hydrolase II was not decreased by aging. These results are well consistent with our previous findings, that is the induction of hydrolase II is independent of the state of androgen [29]. The long-term administration of clofibric acid induced hydrolase II to somewhat a lesser extent than the short-term administration. This lower ability of the long-term treated rats to induce hydrolase II may be responsible for toxic effect of the long-term treatment with clofibric acid on cellular machinery to induce the enzyme.

Finally, the present study provided the evidence that although the extents of the inductions of peroxisomal β-oxidation enzymes and cytosolic acyl-CoA hydrolases are varied by the duration of the treat-

ment with clofibric acid and the age of rats used, the inductions of these enzymes and fatty acid-binding protein continued during the administration of clofibric acid being lasting. Based on these results, it seems to be conclusive that these inductions of the four markers are not a brief response of rats to clofibric acid in only the first stage of the administration of the drug, but that they are concomitant and essential for the response of rats to clofibric acid.

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