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EFFECTS OF CHRONIC ADMINISTRATION OF THE PEROXISOME PROLIFERATOR, CLOFIBRATE, ON CYTOSOLIC ACETYL-CoA HYDROLASE IN RAT LIVER

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Abstract—The hypolipidemic drug ethyl chlorophenoxyisobutyrate (clofibrate) is known to induce peroxisome proliferation and to be carcinogenic after long term administration to rats and mice. We examined the effects of treatment with this drug for periods of up to 18 months on cytosolic ATP-stimulated and ADP-inhibited acetyl-CoA hydrolase in rat liver. In male Donryu albino rats on a diet containing 0.5% clofibrate, the enzyme activity increased to about 2- and 3-fold the initial level per milligram liver protein and cytosolic protein, respectively, and 2-fold per milligram DNA in 3 days, and then remained at this level for up to 18 months. The increased activity in rats receiving clofibrate for 3 months returned to control level within a week when clofibrate was withdrawn. The change in enzyme activity paralleled the change in the amount of enzyme protein determined by immunoblotting with antibody against purified acetyl-CoA hydrolase from rat liver cytosol. No liver tumors were detected macroscopically after administration of clofibrate for 18 months. However, our results suggest that cytosolic acetyl-CoA hydrolase could be an extraperoxisomal marker enzyme induced by this type of drug.

In rat liver, an extra-mitochondrial acetyl-CoA hydrolase (EC 3.1.2.1), which is stimulated by ATP and inhibited by ADP, hydrolyses acetyl-CoA to acetate and CoASH [1, 2]. This enzyme, which shows extreme cold lability [3, 4], has been demonstrated in the post-microsomal supernatant fraction (possibly the cytosol) of rat liver [1, 2, 5]. This cytosolic enzyme is distinct from mitochondrial acetyl-CoA hydrolase because the latter is not stimulated by ATP and does not cross-react with antibody against purified acetyl-CoA hydrolase from rat liver cytosol [5]. We observed that the cytosolic enzyme activity that is stimulated by ATP and inhibited by ADP increased in various metabolic states [5, 6] and after a single injection of ethyl chlorophenoxyisobutyrate (clofibrate) or α-(*p*-chlorophenoxy)isobutyric acid [5]. Clofibrate is a hypolipidemic drug, known to cause hepatomegaly [7], proliferation of hepatic peroxisomes [7] and an increase in fatty acid β-oxidation in both peroxisomes [8] and mitochondria [8] by changing the activities of related enzymes [9]. On the other hand, drugs that induce peroxisome proliferation are also known to be hepatocarcinogenic in rats and mice on long term administration [10, 11]. Very recently, ATP-stimulated acetyl-CoA hydrolase activity was detected in the liver peroxisomal fraction of rats [12]. Interestingly, in contrast to the cytosolic enzyme, the specific activity of the isolated peroxisomal fraction is only marginally increased by treatment with clofibrate and the activity in this fraction is about 1/30 of that of the cytosolic enzyme [12]. Thus, it was of great interest to study the change in activity of the clofibrate-inducible cytosolic (extra-peroxisomal and extra-mitochondrial) acetyl-CoA hydrolase in rat liver during long-term administration of this drug. The results should be of special significance in relation to the time course of hepatocarcinogenesis induced by this drug, which is not mutagenic in the Ames test and does not cause DNA modifications [11].

In the present work, we investigated the changes in the activity of cytosolic acetyl-CoA hydrolase in the liver of rats given a diet containing clofibrate for up to 18 months. We also examined whether these changes in enzyme activity corresponded to changes in the amount of enzyme protein and/or in the molecular structure of the enzyme protein by immunoblotting.

MATERIALS AND METHODS

Materials. CoASH, DL-dithiothreitol, ATP, ADP and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Clofibrate and all other chemicals used were purchased from Wako Pure Chemical Industries. Acetyl-CoA was prepared from CoASH and acetic anhydride as reported by Simon and Shemin [13].

Animals and clofibrate administration. Male albino Donryu rats, weighing approximately 100 g, were maintained in an air-conditioned room at approximately 25° with alternating 12 hr periods of light (6:00–18:00) and dark [5]. They were supplied *ad lib.* with powdered Oriental laboratory chow with or without 0.5% (w/w) clofibrate [10] for 18 months.

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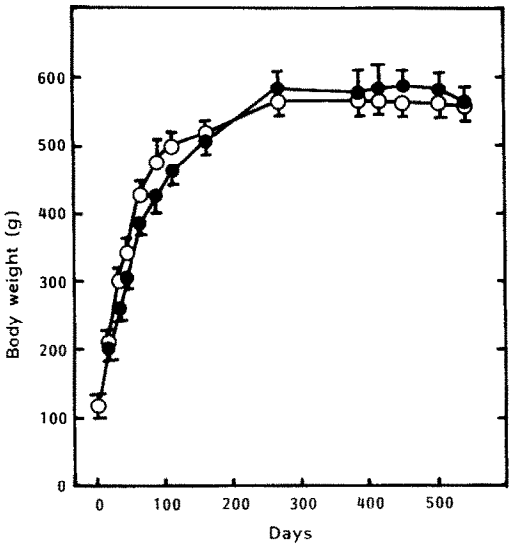

Fig. 1. Time courses of change in body weight of rats given a diet with or without clofibrate. Rats were maintained on a diet with (●) or without (○) 0.5% (w/w) clofibrate for 18 months. Points and bars are means ± SE (N = 3–20).

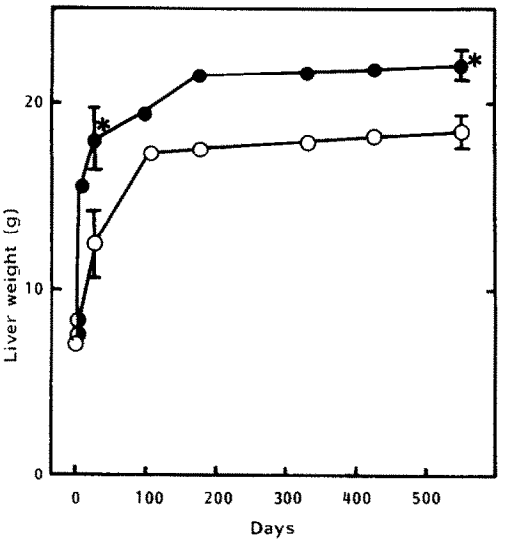

Fig. 2. Time courses of change in liver weight of rats given a diet with or without clofibrate. Rats were maintained on a diet with (●) or without (○) 0.5% (w/w) clofibrate and were killed on the indicated days. Points and bars are means ± SE (N of 28 days and 552 days are 6 and 3, respectively). The points without bars are means for duplicate determinations in two animals. *Significant difference from the value for controls (without clofibrate treatment) at $P < 0.05$.

The amounts of food consumed and body weights of both experimental and control rats were measured daily throughout the experiment. Rats were killed by decapitation under anesthesia with diethyl ether at the times indicated in the figures.

Preparation of liver homogenates. Liver homogenates were prepared as described previously [6].

Assay of acetyl-CoA hydrolase. Enzyme activity was routinely assayed spectrophotometrically at 25° by determining the rate of formation of CoASH from acetyl-CoA using 5,5'-dithiobis(2-nitrobenzoic acid) [14]. Acetyl-CoA hydrolase activity in crude preparations was estimated by subtracting the activity with 2 mM ADP from that with 2 mM ATP [5]. The activity in homogenates was assayed in the presence of 0.025% (v/v) Triton X-100 [5].

Immunoblotting assay. Antibodies against purified acetyl-CoA hydrolase from rat liver cytosol were raised in a rabbit as described previously [5, 15]. Sodium dodecyl sulfate–gel electrophoresis of proteins in 10–15% gradient polyacrylamide gel, and transfer of the separated proteins to polyvinylidene difluoride sheets were performed with a PhastSystem (Pharmacia LKB). Before addition of antibody, the sheets containing transferred protein were blocked by treatment for 30 min at 37° with 2% goat serum diluted with phosphate-buffered saline (10 mM phosphate, pH 7.6, 0.15 M NaCl). Then, the sheets were incubated with anti-cytosolic acetyl-CoA hydrolase overnight at 4°. The reaction products were developed by the avidin–biotin–peroxidase complex method [16], and the intensities of bands on the sheets were determined with a chromatoscanner (Shimadzu dual-wavelength flying-spot scanner, type CS-9000).

Protein determination. Protein was determined by the modification by Hartree [17] of the Lowry method [18] with bovine serum albumin as a standard.

DNA determination. DNA was determined by the method of Labarca and Paigen [19], based on the enhancement of fluorescence seen when bisbenzimidazole binds to DNA. Native calf thymus DNA was used as a standard.

Statistical methods. Student's *t*-test was used to determine the significance of observed differences between experimental groups.

RESULTS

Effect of clofibrate treatment on food intake and body and liver weights

Rats weighing about 100 g were fed *ad lib.* on laboratory chow with or without 0.5% (w/w) clofibrate. Food intake of clofibrate-treated rats was similar to that of control rats, although the latter was slightly less later in the experimental period. The body weight gain of clofibrate-treated animals was slightly less than that of controls until about day 200 of treatment, but then the body weight of the treated animals became similar to that of the controls, although the rate of body weight gain of control rats became attenuated earlier than that of treated ones (Fig. 1). Increase in the liver weight of clofibrate-treated animals was significantly more than that of control rats (Fig. 2). No tumors were detectable macroscopically in the livers of rats during administration of either the diet containing clofibrate or control diet for 18 months.

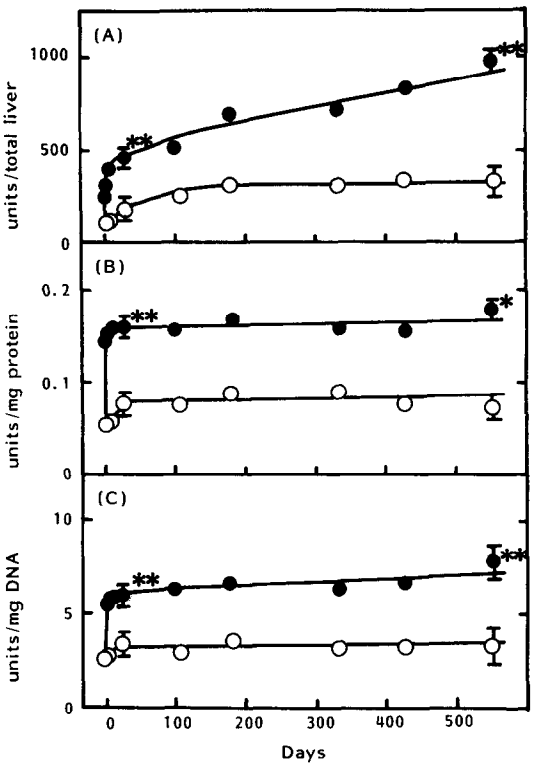

Fig. 3. Time courses of change in acetyl-CoA hydrolase activity in rats given a diet with or without clofibrate. The rats were maintained on a diet with (●) or without (○) 0.5% (w/w) clofibrate and were killed on the indicated days. Activity was determined at 25° immediately after homogenation of the liver as described in Materials and Methods. Points and bars are as described for Fig. 2. *, **Significant differences from the values for control animals (without clofibrate treatment) at $P < 0.05$ and $P < 0.01$, respectively.

Enhancement of cytosolic acetyl-CoA hydrolase activity by adminstration of clofibrate

Figure 3 shows the time course of change in cytosolic acetyl-CoA hydrolase activity in rat liver induced by a diet containing clofibrate. On administration of the diet, the enzyme activity in the liver increased to about twice the initial level per milligram protein or milligram DNA in 3 days, and then remained at this increased level for at least 18 months.

As the liver weight increaed during treatment with clofibrate (Fig. 2), after 18 months, the activity per total liver was about 10 times that initially (Fig. 3), but it was thrice that observed in the control rat when compared at the same periods after feeding. The increased enzymatic activity in clofibrate-treated rats returned to the control level within a week when the diet was changed to a diet without clofibrate 3 months after the start of the experiment (Table 1).

Correlation between enzyme activity and enzyme protein concentration

Next, we examined whether the change in the activity of acetyl-CoA hydrolase in the liver of rats given a diet containing 0.5% clofibrate for 18 months was associated with a change in the amount of enzyme protein.

Figure 4A (lanes 1–5) shows the blots obtained with purified cytosolic acetyl-CoA hydrolase, and Fig. 4B shows that the areas of the stained bands in Fig. 4A (lanes 1–5) increased linearly with up to at least 1 ng of enzyme protein.

The protein content of acetyl-CoA hydrolase in the supernatant fraction was measured by this quantitative immunoblotting assay (Fig. 4). Table 2 shows the relationship between the enzyme activity and amount of enzyme protein in the supernatant fraction. The amount of enzyme protein and the enzyme activity in the liver cytosol (not homogenate) of rats given a diet containing 0.5% clofibrate for 18 months (Fig. 4, lane 7) were about three times those in the liver of rats given a normal diet for 18 months (Fig. 4, lane 6). Thus, change in enzyme activity was associated with change in the amount of immunoreactive enzyme protein.

DISCUSSION

Compounds that induce peroxisome proliferation, such as clofibrate, have been found to be hepatocarcinogens in rats and mice when admin-

Table 1. Changes in acetyl-CoA hydrolase activity in rats transferred from a diet with to a diet without clofibrate

Diet	Acetyl-CoA hydrolase (U/mg protein of homogenate)
Normal diet (3 months)	0.079
Clofibrate diet (3 months)	0.160
Clofibrate diet (3 months) → normal diet (7 days)	0.083

The rats were maintained on a diet with or without 0.5% (w/w) clofibrate for 3 months, and then the diet containing clofibrate was changed to that without clofibrate for 7 days. Activity was determined at 25° immediately after homogenation of the liver as described in Materials and Methods.

Values are means for duplicate determinations in two separate experiments.

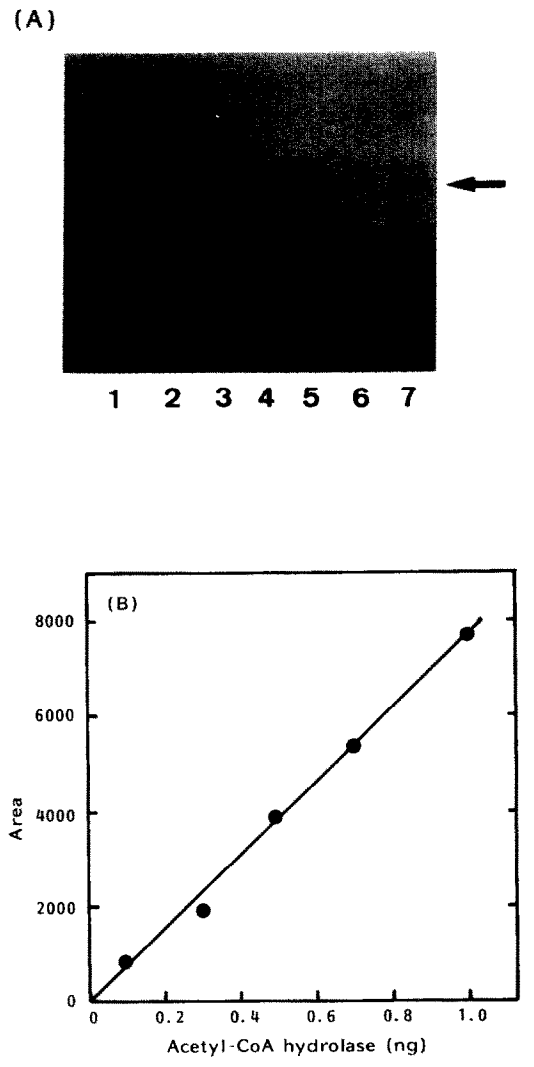

Fig. 4. Quantitative immunoblotting assay of acetyl-CoA hydrolase. (A) Immunoblots of increasing quantities of purified enzyme (lane 1, 0.1 ng; lane 2, 0.3 ng; lane 3, 0.5 ng; lane 4, 0.7 ng; lane 5, 1.0 ng) and supernatant fractions of the livers of rats with (lane 7, 2 μg of protein) or without (lane 6, 2 μg of protein) clofibrate treatment for 18 months. (B) Typical standard curve obtained with increasing quantities of the purified enzyme.

istered for long periods [10, 11]. These drugs have been classified as non-genotoxic chemical carcinogens because they are not mutagenic, and do not cause DNA damage or DNA modification [11, 20]. They are also known to induce preneoplastic and neoplastic lesions in the liver of rodents without inducing γ-glutamyltranspeptidase or glutathione *S*-transferase (placental form), which are used for detection of preneoplastic changes in hepatocarcinogenesis induced by genotoxic carcinogens [21]. Previously, we reported that the change in the activity of cytosolic acetyl-CoA hydrolase during 3′-methyl-4-dimethyl aminoazobenzene hepatocarcinogenesis in rat liver was biphasic [22]: the activity decreased to about one third of the initial level in week 2, returned to the control level in week 7, and then decreased again to about one tenth of the control level in week 20 when tumor nodules occupied more than 90% of the liver. Interestingly, this biphasic change in enzyme activity was inversely associated with the well-known change in γ-glutamyltranspeptidase activity [22, 23]. On the other hand, in the present study, we found that the activity of cytosolic acetyl-CoA hydrolase in the liver increased about 2-fold on day 3 after administration of clofibrate, and then remained at this high level for at least 18 months during further treatment with this drug (Fig. 3). No tumors were detected macroscopically in the liver during this experimental period.

Peroxisomes in liver cells contain enzymes involved in the β-oxidation of long chain fatty acids, several hydrogen peroxide-generating oxidases and catalase [24], and the activities of these enzymes increase in association with peroxisome proliferation induced by clofibrate [24]. Interestingly, acetyl-CoA hydrolase is mainly localized in the liver cytosol (with about 5% as much in the kidney) [5], but it is induced by administration of drugs that induce peroxisome proliferation. Thus, our results suggest that change in cytosolic acetyl-CoA hydrolase during clofibrate treatment may be a useful extraperoxisomal indicator of the effect of the drug. The mechanisms of induction of the enzyme activity and enzyme protein as well as those of carcinogenesis by compounds inducing peroxisome proliferation are unknown. There are, however, some recent reports that a peroxisome proliferator binding protein is present in rat liver and that peroxisome proliferators can modulate specific gene transcription, suggesting that

Table 2. Comparison of activities and protein contents of acetyl-CoA hydrolase in rat liver

Diet	Specific activity (U/mg protein of cytosol)	Relative specific activity (%)	Relative amount of enzyme protein (%)
Normal diet	0.103	100	100
Clofibrate diet	0.387	376	340

Rats were maintained on a diet with or without 0.5% (w/w) clofibrate for 18 months. Activity in the liver cytosol (supernatant of 105,000 *g*) was determined at 25°. Amounts of enzyme protein were determined by immunoblotting assay using the calibration curve in Fig. 4.

Values are means for duplicate determinations in two separate experiments.

these compounds act by a mechanism similar to that of steroid hormones [25]. More recently, Issemann and Green [26] succeeded in cloning a peroxisome proliferator receptor as a member of the steroid hormone receptor superfamily of ligand-activated transcription factors that is specifically activated by peroxisome proliferators. These results suggest that this specific receptor may be a direct mediator of the effects of this class of agents, i.e. peroxisome proliferation and induction of cytosolic acetyl-CoA hydrolase, and be involved in the development of liver tumors in rodents.

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