DEGRADATION OF THE PEROXISOME-ASSOCIATED POLYPEPTIDE IN CLOFIBRATE-TREATED MOUSE LIVER

Takafumi WATANABE and Tetsuya SUGA
Department of Clinical Biochemistry, Tokyo College of Pharmacy, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

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1. Introduction

In 1977, Reddy and Kumer reported the proliferation of M_r 80 000 polypeptide in clofibrate-treated rat liver and suggested that this polypeptide might be a protein component of peroxisomes [1]. This finding is significant for studies of the biogenesis and turnover of hepatic peroxisomes because no other protein component is available as a marker of the peroxisomal membrane. In addition, studies of the physiological role of rat liver peroxisomes during the last few years, have shown that the peroxisomes contain enzymes capable of catalysing β-oxidation of long chain fatty acids, as well as catalase, carnitine acetyltransferase and several H_2O_2-generating oxidases [2–6]. Enoyl-CoA hydratase, one of the enzymes of fatty acid oxidizing system has been purified [7] from hepatic peroxisomes and from the M_r of the purified enzyme suggested that the peroxisome-associated polypeptide of M_r 80 000 first reported in [1] in clofibrate treated rat liver is this enzyme. In [8] we reported the effects of simfibrate, a simple derivative of clofibrate, on the amount of the M_r 80 000 polypeptide, and on the rate of the synthesis of this peptide in the liver, and showed that simfibrate stimulated the rate of biosynthesis of the polypeptide [8]. Here, the effects of clofibrate on the degradation rate of the hepatic peroxisome-associated M_r 80 000 polypeptide is presented.

2. Materials and methods

2.1. *Materials*

D,L-[4,5-^{3}H]Leucine (30 Ci/mmol) was obtained from Amersham RadioChemicals. L-Carnitine–HCl, acetyl-CoA, NAD, palmitoyl-CoA and CoA were obtained from Sigma Chemical Co. St Louis, MO DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) and DTT (dithiothreitol) were obtained from Wako Pure Chemicals, Osaka. Other chemicals, all of reagent grade, were obtained from commercial sources.

2.2. *Animals and treatments*

Male mice of the ddY-strain (28–31 g) were used. Thirty animals were divided into 2 groups. After fasting overnight, mice were fed, ad lib. for 3 weeks on Oriental laboratory chow with and without 0.25% (w/w) clofibrate. After 3 weeks clofibrate-treatment, mice were injected intraperitoneally with 0.15 mCi D,L-[4,5-^{3}H]leucine/10 g body wt, and then sacrificed at 0 (2 h), 2, 4, 6 and 8 days after this injection. After perfusing with ice-cold 0.25 M sucrose, the liver was removed and 10% (w/w) homogenate was prepared in 0.25 M sucrose.

Homogenates were used for assays of the peroxisomal enzyme activities and for studies of decay of [^{3}H]Leu in the peroxisomal protein component.

2.3. *Assay methods*

The enzyme activities of catalase, D-amino acid oxidase (DAAO) and urate oxidase were determined as in [9]. Unit enzyme activities of catalase and urate oxidase were defined as the amounts which caused a decrease of 1 unit A_{240} in 1 s and at A_{290} in 1 min at 25°C, respectively. Monoamine oxidase activity was determined as in [10] with modifications, using *m*-nitro benzylamine as substrate [11]. One unit of the activity was defined as the amount of the enzyme which produced 1 nmol *m*-nitrobenzaldehyde from the substrate/min under the assay condition. This enzyme served as a marker enzyme for mitochondria. The activity of cyanide-insensitive FAOS was determined spectrophotometrically from the palmitoyl-

CoA-dependent reduction of NAD at 340 nm in the presence of 1 mM KCN as in [3]. The activity of CAT (carnitine acetyltransferase) was determined spectrophotometrically using DTNB as in [12]. These enzyme activities were assayed using the homogenate fraction.

2.4. *Studies on the peroxisome proliferation-associated polypeptide*

The liver homogenate was centrifuged at 3300 × *g* for 10 min and the supernatant was recentrifuged at 12 500 × *g* for 20 min at 4°C. The final pellet was suspended in 0.25 M sucrose–20 mM glycylglycine buffer (pH 7.4) (SG buffer) (equiv. 5 ml/g original tissue), which corresponds to 'the light mitochondrial fraction (LM fraction)' in [13]. The separation of peroxisomes and mitochondria was carried out by sucrose density-gradient centrifugation of the fraction. Sucrose solutions were discontinuously layered from the bottom of 60 ml tubes: 8 ml 53.9% (w/w; density (*d*) = 1.26 g/ml); 17 ml 45.8% (*d* = 1.215 g/ml); 15 ml 37.4% (*d* = 1.17 g/ml) and 10 ml 23.3% sucrose (*d* = 1.10 g/ml) in 20 mM glycylglycine (pH 7.4). Centrifugation was carried out for 2.5 h at 24 000 rev./min at 4°C using Hitachi RPS-25-2 rotor. Samples were collected in 4 ml fraction from the bottom of the tubes by a micropump. The peroxisome-rich fractions were collected and the protein content of the fraction was adjusted to 0.8 mg/ml after concentration with Amicon-5Minicon.

The decay of [^{3}H]Leu from protein components of the peroxisomes was as follows: The concentrated peroxisome fractions (0.1 ml) were subjected to sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [1]. After electrophoresis the gels were stained with Coomassie brilliant blue and the band corresponding to the M_r 80 000 polypeptide was separated off. The gel slices were put into 10 ml minivials solubilized in 0.5 ml NCS at 50°C overnight and counted in 5 ml toluene scintillator (DPO 3 g–dimethyl POPOP 75 mg/500 ml toluene).

3. Results and discussion

Certain hypolipidemic drugs, such as clofibrate, induce hepatomegaly and proliferation of hepatic peroxisomes in rat, accompanied by a significant increase in the activity of catalase, one of the marker enzymes of peroxisomes [14–16]. However, until now, reports of biochemical effects of these drugs on hepatic peroxisomal enzymes of the mouse have been few. Table 1 shows the effects of clofibrate on some peroxisomal enzymes and on some enzymes related to fatty acid metabolism. The activities of cyanide-insensitive fatty acyl-CoA oxidation system (FAOS) and carnitine acetyltransferase (CAT) were markedly increased from 0.36 units/g liver to 4.1 units/g liver and from 544 units/g liver to 8950 units/g liver, respectively, in clofibrate-treated animals. This manner of alteration in the hepatic peroxisomal enzyme activities in the treated mouse is quite similar to that observed in the rat, suggesting that clofibrate may affect the metabolism of both animals in a similar manner. To isolate peroxisomes the 'LM (light mitochondrial) fraction obtained from the liver homogenate was subjected to sucrose density gradient centrifugation. Fig.1 shows the typical pattern of the centrifugation. Catalase is the marker enzyme of peroxisomes and monoamine oxidase is of mitochondria. Peroxisomes and mitochondria were satisfactorily separated in accordance with those densities. Monoamine oxidase activity in the peroxisomal fraction (fractions 2,3) was only 8.5% of the total activity. Fig.2 shows SDS–polyacrylamide gel electrophoretic analysis of the protein components of the peroxisomal fraction after sucrose density-gradient centrifugation of the LM fraction obtained from clofibrate-treated and control animals. The staining-density of the protein band indicated by arrow was markedly higher than that of control, and the M_r was established to be ~78 000–80 000 from its relative mobility to the marker proteins on SDS–polyacrylamide gel electrophoresis. No other protein components seem to be

Table 1
Effects of clofibrate on the peroxisomal enzymes of the mouse liver

Enzymes	Activity (units/g liver)		
	Control	Clofibrate	
Catalase	25.5 ± 3.4	35.1 ± 2.8	$P < 0.01$
DAAO	0.94 ± 0.15	0.44 ± 0.21	$P < 0.01$
Uricase	4.11 ± 0.48	4.32 ± 0.71	n.s.
FAOS	0.36 ± 0.12[a]	4.11 ± 0.98[a]	$P < 0.01$
CAT	544 ± 61[b]	8950 ± 694[b]	$P < 0.01$

[a] Unit = μmol/min; [b] unit = nmol/min

Values represent means ± SD. Statistical evaluations were performed by Student's *t*-test: n.s. means not significant ($P > 0.05$)

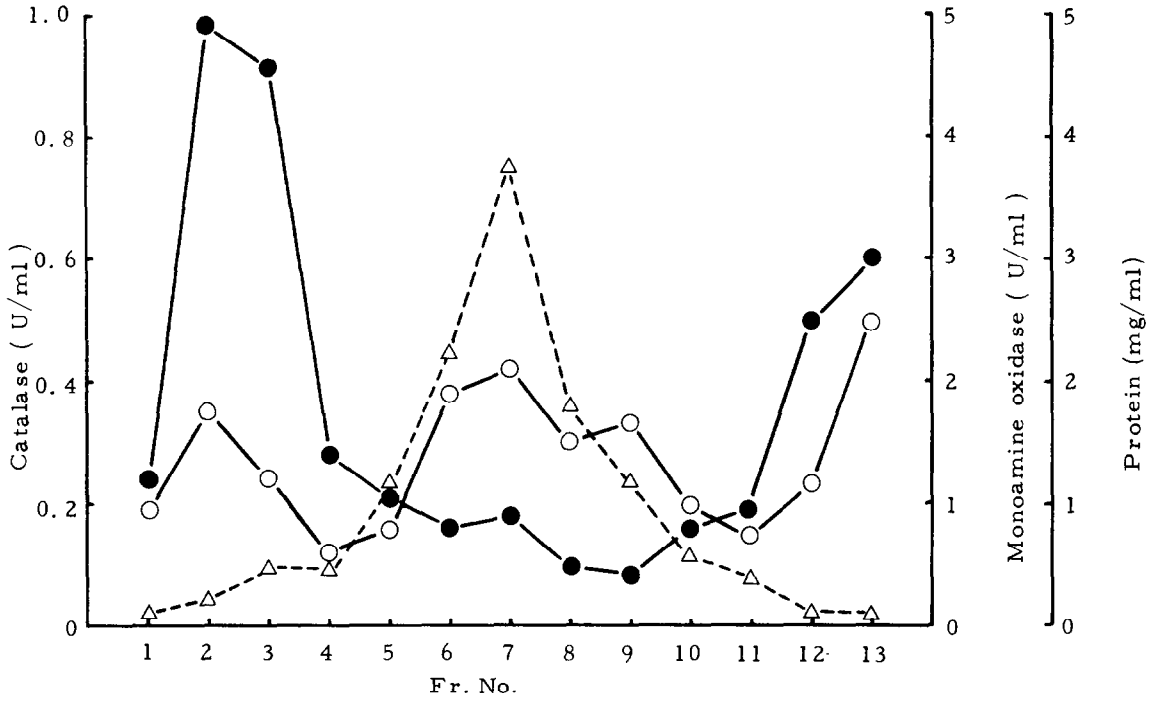

Fig.1. Sucrose density gradient centrifugation of the light mitochondrial fraction of the livers of the clofibrate-treated mouse. The light mitochondrial fraction (equiv. to 1.0 g liver) was layered on top of the gradients: (•), catalase; (△), monoamine oxidase; (○) protein.

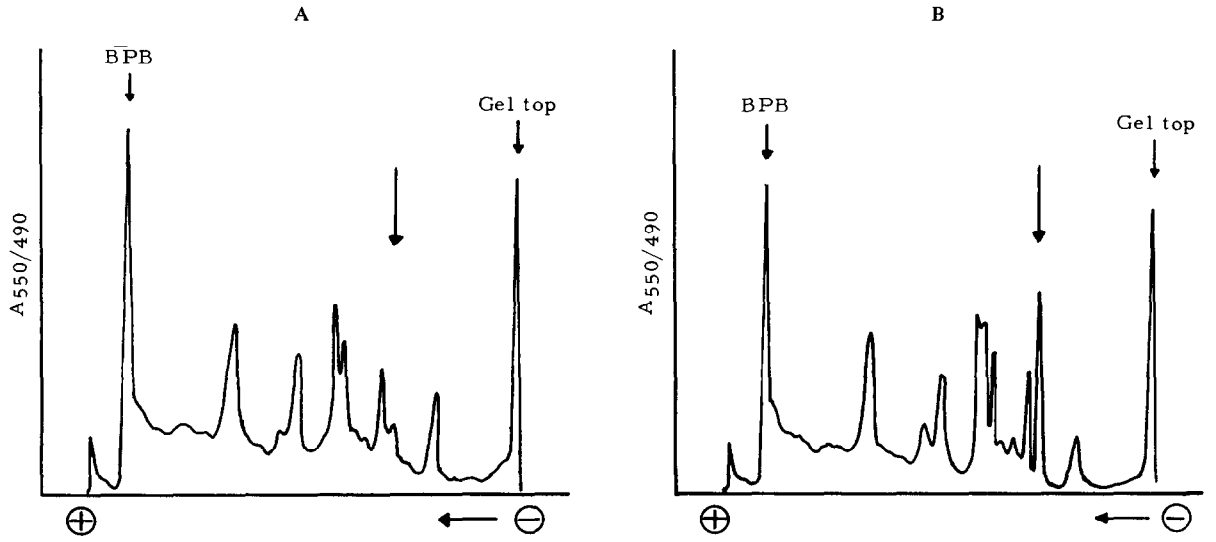

Fig.2. Densitometry of stained gel. The gel was scanned by Shimazu CS-900 dual beam chromatoscanner as difference between 550 nm (λ_S) and 490 nm (λ_R) after staining: (A) control; (B) clofibrate-treated.

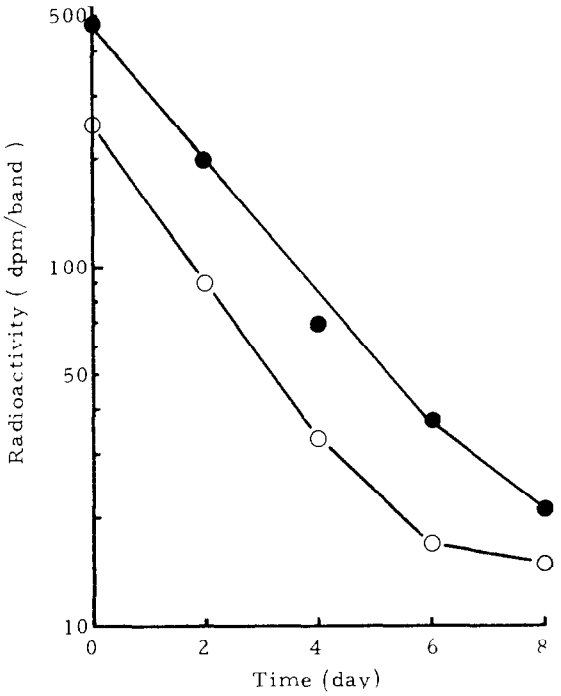

Fig.3. Decay of the radioactivity of [^{3}H]Leu from the peroxisomal M_r 80 000 polypeptide. Mice fed a 0.25% clofibrate diet for 3 weeks were injected with [^{3}H]Leu and sacrificed at 0 (2 h), 2, 4, 6 and 8 days after. SDS–polyacrylamide gel electrophoresis of the peroxisomal fraction prepared from the LM fraction of the liver was performed as in section 2 and the radioactivity in the band corresponding to peroxisomal M_r 80 000 polypeptide was determined in a liquid scintillation counter. Each value plotted in the figure is the mean of 3 independent expt: (○) control; (●) clofibrate-treated.

changed in amount. Fig.3 shows the time course of decay of the radioactivity of [^{3}H]leucine in the peroxisome proliferation-associated M_r 80 000 polypeptide. At 0 time (= 2 h after injection), the radioactivity incorporated into the peptide of clofibrate-treated animal was increased by ~3.8-fold. In course of time, the radioactivity in the peptide was gradually decreased and the half-life of disappearance of the radioactivity from the peptide was 38.4 h for treated and 32.4 h for control. These findings suggest that, in the clofibrate animal, the increase in the amount of this peptide is primarily due to the enhancement of the biosynthesis of the peptide. This is supported by our previous finding that, in simfibrate-treated animals the initial incorporation rate of [^{3}H]leucine into this polypeptide was ~3-times higher at 2 h, compared with control animals [8]. Although, until now, intracellular localization of this M_r 80 000 polypeptide has not been established, the suggestion in [1] that this peptide is a component of the peroxisomal membrane is remarkably interesting. The characterization of clofibrate-inducible membrane protein of hepatic peroxisomes of rat is reported in [17] and the increased peptides shown to have M_r 40 000 and 80 000 [17].

Further studies are required to elucidate whether the polypeptide is a structural protein component maintaining the granule structure or a functional protein such as an enzyme which contributes to the physiological functions of the granule.

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