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Some Peroxisomal Proliferators Enhance Membrane Fluidity of Liver Peroxisomes

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The membrane fluidity of liver peroxisomes from normal and clofibrate-treated rats was investigated by using a fluorescence probe, 1-anilinonaphthalene-8-sulfonate (ANS). The excitation maximum of the probe was changed from 347 to 380 nm and the emission maximum from 493 to 463 nm in the presence of peroxisomes, and fluorescence intensity was enhanced more than 50 times. These results suggest that ANS was bound to the peroxisomal membrane. Fluorescence depolarization (*P*-value) was determined with such ANS-labeled peroxisomes. The *P*-values of peroxisomes from both normal and clofibrate-treated rats gradually decreased with increasing temperature, but that of clofibrate-treated peroxisomes was always smaller than that of normal peroxisomes. *P*-values were observed at 37 °C for 60 min. The average *P*-value of normal peroxisomes was 0.270, while that of clofibrate-treated peroxisomes was 0.248. These results indicate that the membrane fluidity of liver peroxisomes is increased by the treatment with clofibrate. Treatment of rats with another inducer of liver peroxisome proliferation, di(2-ethylhexyl)phthalate, had an effect similar to that of clofibrate. However, alloxan-treated (diabetic) rats showed no change of peroxisomal membrane fluidity.

Keywords——peroxisome; peroxisome proliferator; peroxisomal membrane; membrane fluidity; clofibrate

The peroxisomal membrane seems to be quite porous, since small molecules such as sucrose, lactate, amino acid, and urate can freely penetrate it.[1] Not only small molecules but also large molecules such as catalase, the main peroxisomal matrix enzyme, can be lost by leakage through the membrane during the preparation of the particles, even when the native morphology of the membrane is apparently retained.[2] The molecular basis of such porosity is still unknown. Clofibrate has been shown to elevate liver catalase levels in rat[3,4] as well as to cause a considerable increase in the number of peroxisomes.[5,6] However, the increase of catalase is greater in the supernatant fraction than in the peroxisome-rich fraction.[4,7] Most catalase is located in peroxisomes in the native state, because the enzyme has been reported not to be release from hepatocytes by treatment with digitonin, which easily solubilize cytosol lactate dehydrogenase.[8] Thus, clofibrate may modify the peroxisomal membrane. This paper describes the alterations in membrane fluidity of hepatic peroxisomes in rats treated with drugs (mainly clofibrate) that increase peroxisomal β-oxidation activity, generally accompanied with a proliferation of the organelle.

Experimental

Chemicals——Usual laboratory chemicals and solvents were from Wako Pure Chemicals (Tokyo, Japan). Hepes (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid), Mops (4-morpholine-propanesulfonic acid), and EGTA (ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid) were from Dotite (Kumamoto, Japan). ANS (1-anilinonaphthalene-8-sulfonate) was from Sigma (St. Louis, MO, U.S.A.). Percoll was from Pharmacia (Uppsala, Sweden).

Purification of Liver Peroxisomes——Triton WR-1339 was i.p. injected into Wistar male rats (250 g b.w.) at a

dose of 100 mg. Four days after the injection, the light mitochondrial fraction was prepared from rat liver homogenate in 0.25 M sucrose containing 10 mM Hepes (pH 7.2) and 1 mM EGTA. The fraction was suspended in 0.25 M sucrose containing 2 mM Mops (pH 7.2), 5 mM EGTA, and 0.1% ethanol to 1 g liver/ml, and then centrifuged in a stepwise gradient of sucrose of density 1.15, 1.18, 1.20, 1.21, 1.23, and 1.25 containing 2 mM Mops (pH 7.2), 1 mM EGTA, and 0.1% ethanol at 30000 rpm for 30 min in Hitachi RPV-50T vertical rotor. The peroxisome-rich fraction was obtained from the gradient, and further centrifuged in a Percoll gradient, starting with 50% Percoll solution containing 0.25 M sucrose, 2 mM Mops (pH 7.2), 1 mM EGTA and 0.1% ethanol. The centrifugation was performed at 27000 rpm for 30 min using the above rotor. The peroxisome-rich fraction was obtained from *d* 1.035 to *d* 1.052 (density marker beads were used). This fraction was used as purified peroxisomes in fluorescence depolarization studies. Marker enzyme activities of the suspension were determined by the methods described in the cited references: peroxisomal fatty acyl-CoA oxidizing system (FAOS),[9] catalase,[10] urate oxidase,[11] cytochrome c oxidase,[12] glucose-6-phosphatase,[10] and acid phosphatase.[13] Protein was determined by the method of Bradford[14] or Lowry *et al.*[15]

Fluorescence-Labeling of Peroxisomes with ANS——Purified peroxisomes (*ca.* 0.75 mg protein) were mixed with 3 ml of 20 mM ANS in 0.25 M sucrose–20 mM Tris–HCl buffer (pH 7.4) at 5 °C for 5 min, and then the mixture was centrifuged at 25000 × *g* for 15 min to remove free ANS. The intensity of fluorescence was determined with Hitachi fluorescence spectrometer, model RF-501 (Tokyo, Japan), using 10^{-6} M quinine sulfate in 0.1 N H_2SO_4 as a standard.

Determination of Fluorescence Depolarization (*P*-Value)——The *P*-value of ANS-labeled peroxisomes prepared above was measured with a Union fluorescence depolarization spectrophotometer, model FS-501 (Osaka, Japan). Excitation was performed with a polarized 380 nm beam generated from a mercury arc, and the emitted light was detected simultaneously in two independent cross-polarized channels equipped with cut-off filters for wavelengths below 450 nm. The *P*-value was obtained according to the following equation:

$$P=\frac{I_v-I_h}{I_v+I_h}$$

where I_v and I_h are the emission intensities polarized vertically and horizontally to the direction of polarization of the exciting light, respectively.[16-18]

Administration of Peroxisome Proliferators——Clofibrate, di(2-ethylhexyl)phthalate (DEHP) and alloxan were used as peroxisome proliferators. Clofibrate-treated rats were fed on a diet containing 0.25% clofibrate *ad libitum* for 2 weeks. DEHP-treated rats were similarly given a diet containing 2% DEHP for 2 weeks. With alloxan, the chemical was dissolved to 150 mg/ml in physiological saline. One milliliter of the solution was intraperitoneally administered to the rats, and 5 d after the treatment rats were sacrified. All rats were intraperitoneally injected with Triton WR-1339 at a dose of 100 mg 4 d before killing.

Results and Discussion

Peroxisome Preparation

The main enzyme activities of the purified peroxisomal preparation are listed in Table I. The extent of purification appears to be about 30- to 40-fold over the original homogenate. By applying the method of Leighton *et al.*,[10] we concluded that more than 93% of the total

TABLE I. Purification of Peroxisomes of Rat Liver

	Specific enzyme activities					
	Catalase[a]	FAOS[b]	Urate ox.[c]	Cyto. c ox.[d]	G-6-P[e]	Acid Pase[f]
Homogenate	35.82	3.52	9.68	10.6	111	42.5
	(1)	(1)	(1)	(1)	(1)	(1)
Light mit. fr.	296.4	7.22	64.8	18.8	187	213.5
	(8.3)	(2.1)	(6.7)	(1.8)	(1.7)	(5.0)
Peroxisomes	1060	43.3	402.5	n.d.	35.98	n.d.
	(29.6)	(12.3)	(42.6)	(—)	(0.3)	(—)

The light mitochondrial fraction from rat liver homogenate treated with Triton WR-1339 was centrifuged in a sucrose density gradient and further in a Percoll gradient using a vertical rotor. The details are present in Materials and Methods. Values are means of 3 experiments. *a*) U/mg protein. *b*) Fatty acyl CoA oxidizing system, U/mg protein. *c*) Urate oxidase, mU/mg protein. *d*) Cytochrome c oxidase, U/mg protein. *e*) Glucose-6-phosphatase, mU/mg protein. *f*) Acid phosphatase, mU/mg protein.

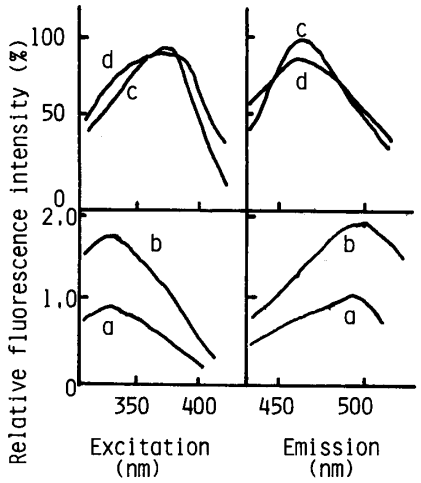

Fig. 1. Fluorescence Spectra of ANS

(a) and (b): ANS (10^{-3} M) was dissolved in (a) water, (b) isotonic Tris–HCl buffer (pH 7.4). (c): ANS (10^{-5} M) in ethanol. (d): Purified peroxisomes (0.5 mg) were incubated in ANS (10^{-3} M) in isotonic Tris–HCl buffer (pH 7.4), then the mixture was centrifuged to remove free ANS. Data are shown as the relative fluorescence intensity with respect to the peak intensity of (c).

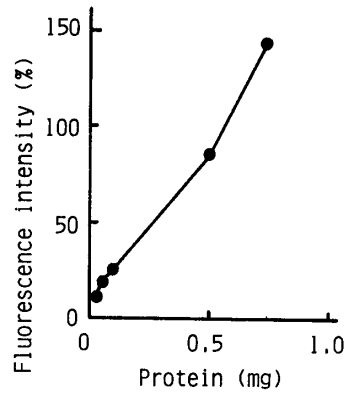

Fig. 2. Dependence of the Binding Amount of ANS on Peroxisome Concentration

Peroxisomes (0.1—0.75 mg protein) were incubated with 10^{-3} M ANS in isotonic Tris–HCl buffer (pH 7.4), then centrifuged.

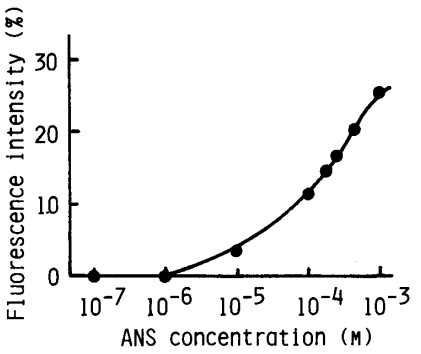

Fig. 3. Dependence of the Binding Amount of ANS to Peroxisomes on ANS Concentration

Peroxisomes (0.1 mg protein) were incubated with ANS (10^{-7}—10^{-3} M) in isotonic Tris–HCl buffer (pH 7.4), then centrifuged.

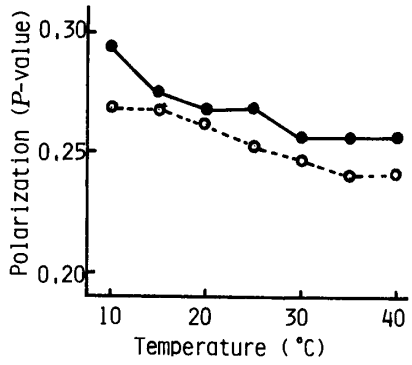

Fig. 4. Temperature Dependence of the Fluorescence Polarization of ANS-Labeled Peroxisomes

ANS-labeled peroxisomes were obtained from normal rats (●—●), or clofibrate-treated ones (○---○), and the *P*-values of the peroxisomes were determined at various temperatures from 10 to 40 °C.

protein of this fraction is contributed by peroxisomes, with microsomes accounting for about 6.5%, and the sum for mitochondrial and lysosomal contamination accounting for less than 1%.

Changes of Fluorescence Excitation and Emission Maxima on Binding

The excitation and emission maxima of ANS fluorescence are at 347 and 493 nm, respectively, in water or Tris–HCl buffer (pH 7.4), while they are at 380 and 463 nm, respectively, in ethanol due to its hydrophobicity (Fig. 1). The values for ANS-labeled peroxisomes were 380 and 463 nm, and the fluorescence intensity was enhanced more than 50 times, as the case of ethanol (Fig. 1).

Alteration in Binding of ANS

Binding of ANS to peroxisomal membrane increased with increasing peroxisomal

TABLE II. *P*-Values of ANS-Labeled Peroxisomes from the Liver of Rats Treated with Peroxisome Proliferators

Proliferators	Conditions	*P*-Value	(Control)
Clofibrate	0.25% diet, 2 weeks	0.248	(0.270)
DEHP[a)]	2% diet, 2 weeks	0.190	(0.260)
Alloxan	150 mg/kg b.w. 5 d after	0.275	(0.272)

a) Di(2-ethylhexyl)phthalate.

content (from 0.1 to 0.75 mg as protein) (Fig. 2). When the ANS concentration was increased in the incubation mixture with peroxisomes, ANS binding to the peroxisomal membrane increased almost linearly at more than 10^{-4} M ANS (Fig. 3).

These results (Figs. 1—3) show that ANS sedimented by centrifugation is ANS bound to the peroxisomal membrane, but not free ANS.

Determination of *P*-Values of ANS-Labeled Peroxisomes

Figure 4 shows changes in *P*-values of ANS-labeled peroxisomes at various temperatures. The *P*-values of normal and clofibrate-treated peroxisomes decreased with increasing temperature, but the *P*-value of clofibrate-treated peroxisomes was always smaller than that of normal peroxisomes. Changes in *P*-values of ANS-labeled peroxisomes at 37 °C during 60 min. The *P*-value of normal peroxisomes lay in the range of about 0.287 to 0.258, and the average was 0.270 ± 0.009. That of clofibrate-treated peroxisomes lay in the range of about 0.257 to 0.237, and the average was 0.248 ± 0.008 (data not shown). Fluorescence depolarization should reflect membrane fluidity.[16,18)] Therefore, the results indicate that the peroxisomes from rat liver treated with clofibrate probably show increased membrane fluidity as compared with those from normal rat liver.

Table II shows the *P*-values of ANS-labeled peroxisomes from the liver of rats treated with two other peroxisomal proliferators DEHP,[19)] and alloxan.[20)] As in the case of clofibrate treatment, hepatic peroxisomes from DEHP-treated rats had higher membrane fluidity than those from normal rats. Alloxan-treated (diabetic) rats showed no increase in peroxisomal membrane fluidity.

It is not known why the membrane fluidity of peroxisomes was enhanced by clofibrate (and DEPH), but this effect may also be responsible for the elevation of peroxisomal FAOS activity. Membrane fluidity of peroxisomes is closely associated with phospholipid composition; an increase of unsaturated fatty acids at the C-2 position of phosphatidylcholine and/or a decrease of cholesterol content of the membrane enhance membrane fluidity. Crane and Masters[21)] reported that clofibrate perturbs the phospholipid composition of the peroxisomal membrane, and docosahexaenoic acid (C 22:6) content in peroxisomal membrane phospholipid was considerably increased. This may account for the enhancement of peroxisomal membrane fluidity.

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