EFFECTS OF SOME HYPOLIPIDEMIC DRUGS AND PHTHALIC ACID ESTERS ON FATTY ACID BINDING PROTEIN IN RAT LIVER

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Effects of clofibric acid, probucol, niceritrol, di-(2-ethylhexyl) phthalate (DEHP) and dibutylphthalate (DBP) on the binding capacities of fatty acid binding protein (FABP) and ligandin fractions in rat liver were investigated. The binding capacity of FABP fraction in male rat liver to oleic acid was increased remarkably on clofibric acid-feeding for 7 d. The administration of either probucol or niceritrol had little influence on the binding capacity of FABP. Both DEHP and DBP increased the binding capacity of FABP in liver of male rats, but the extent of the increase was somewhat less significant in DEHP- or DBP-fed rats compared to that in clofibric acid-fed rats. Sex-related difference in the increase in the binding capacity of FABP fraction to oleic acid by either clofibric acid or DEHP was observed and the extent of the increase in female rats feeding clofibric acid or DEHP was about half of male rats. Effects of any drugs and agents tested here on the binding capacity of ligandin in liver to bromosulphophthalein (BSP) were little if any. The increase in the binding capacity of FABP fraction, but not ligandin in liver was confirmed *in vivo* by intravenous administration of BSP to rats which had been fed DEHP. In spite of marked increase in the binding capacity of FABP fraction in liver from clofibric acid-fed rats, no remarkable increase in the incorporation of radioactive oleic acid into esterified lipids was observed *in vivo*.

Keywords — fatty acid binding protein; ligandin; clofibric acid; probucol; niceritrol; phthalic acid esters; rat liver

INTRODUCTION

Fatty acid binding protein is a cytoplasmic protein with molecular weight of approx. 12000¹⁾ and has been shown to be present in various tissues including liver, myocardium, skeletal muscle, intestinal mucosa, adipose tissue and kidney.²⁾ This protein was initially called Z protein and was thought to be involved in the translocation of various organic anions from plasma to tissues.³⁾ Recently, the evidence showing a high affinity of the protein for not only long-chain fatty acids but also long-chain acyl-CoA was provided⁴⁾ and it has been considered that hepatic FABP plays a role in the uptake of fatty acid from plasma or in the direction of cellular fatty acid toward esterification,⁵⁾ especially triacylglycerol.⁶⁾ Fleischner *et al.* reported that clofibrate or nafenopin, which are well known hypolipidemic drugs and derivatives of clofibric

acid, increased hepatic level of FABP significantly.^{7,8)} Therefore, it is of interest to study the effect of hypolipidemic drugs or chemicals, which are not derivatives of clofibric acid, on the levels of FABP in liver to obtain the information concerning a role of FABP in hypolipidemic action of the drugs and chemicals. In this context, we studied the effects of three hypolipidemic drugs, clofibric acid, probucol and niceritrol, and two phthalic acid esters, DEHP and DBP, on the binding capacity of hepatic FABP to oleic acid. The effects of these chemicals on the binding capacity of ligandin, which is known to be another cytoplasmic protein having a high affinity for various organic anions, were also studied.

MATERIALS AND METHODS

Materials—[1-¹⁴C]Oleic acid (57.0 Ci/mol)

was obtained from New England Nuclear Corp., (Boston, MA). Clofibrilic acid, serum albumin, chymotrypsinogen A, cytochrome *c* and bromosulphophthalein were purchased from Sigma Chemical Co., (St. Louis, MO); di-(2-ethylhexyl) phthalate from Tokyo Kasei Kogyo Co., (Tokyo, Japan); dibutylphthalate from Wako Pure Chemical Co., (Osaka, Japan); Triton WR-1339 from Nakarai Chemicals (Kyoto, Japan); Sephadex and blue dextran from Pharmacia Fine Chemical Co., (Uppsala, Sweden). Probucol [4,4'-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol)] was kindly gifted by Dow Chemical (Indianapolis, Indiana) and Daiichi Pharmaceutical Co., (Tokyo, Japan). Niceritrol (pentaerythritol tetranicotinate) was kindly donated by Sanwa Kagaku Kenkyusho Co., (Nagoya, Japan).

Animals—Male and female rats of Wistar strain, weighing 150–180 g and 140–150 g, respectively, were used. Rats of control group were fed *ad libitum* on a commercial diet. Rats of drug- or chemical-treated groups were fed *ad libitum* for 7 d on a diet containing one of the following drugs or chemicals; 0.5% (w/w) clofibrilic acid; 0.5% (w/w) probucol; 1.5% (w/w) niceritrol; 2% or 4% (w/w) DEHP and 2% (w/w) DBP. Rats were decapitated. Livers were isolated immediately and were perfused with ice-cold 0.9% NaCl solution until traces of blood were washed out. Livers were homogenized with 1.5 volumes of ice-cold 0.25 M sucrose. The homogenates were centrifuged at $20000 \times g$ for 15 min and the resulting supernatant was recentrifuged at $105000 \times g$ for 120 min. The supernatant fraction was separated from the pellet and surface lipid, and was stored at -35°C .

Binding Assay of FABP—The concentration of available FABP-associated fatty acid binding receptors in cytosol was measured by the binding of [$1\text{-}^{14}\text{C}$]oleic acid to FABP fraction of cytosol with chromatography on Sephadex G-50 essentially according to Ockner *et al.*⁶⁾ with some modifications as follows. The incubation mixture contained 120 nmol of [$1\text{-}^{14}\text{C}$]oleic acid (45 nCi), 7.5 mg of cytosolic protein and 3 mg of

Triton WR-1339. The final volume was adjusted to 1.5 ml with 0.154 M KCl-0.01 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 30 min. After cooling on ice, 1 ml of this mixture was subjected to gel filtration on Sephadex G-50 column (2.2×30 cm) equilibrated with 0.154 M KCl-0.01 M potassium phosphate buffer (pH 7.4) at 4°C . Elution was performed with the same buffer at a flow rate of 20 ml/h and fractions of 2 ml were collected. An aliquot of each fraction was mixed with toluene/Triton X-100-based scintillator, and radioactivity was measured by a liquid scintillation spectrometer. The use of Triton WR-1339 instead of organic solvents or propylene glycol gave good recoveries of oleic acid from the column without affecting the amounts of oleic acid bound to FABP. Typical elution profile was shown in Fig. 1A. The first peak, corresponding to the void volume, includes micelle of Triton and oleic acid. There are many reports in which FABP concentrations were measured, but in most cases the assay was performed without assessing the sensitivity of the assay system used. To obtain linearity between amounts of oleic acid bound to FABP and the concentrations of FABP in samples, experimental conditions, especially column size, amounts of oleic acid and cytosolic protein, should be chosen strictly and a column of Sephadex never be used over 10 times. Under the experimental conditions described above, the assay was responsive linearly to changes in FABP concentrations as shown in Fig. 1B.

Binding Assay of BSP to Ligandin—Binding of BSP to cytosolic ligandin was assayed by the method described by Levi *et al.*³⁾ except for the use of Sephadex G-75 column in size of 2.5×45 cm.

Preparation of Injection Solution of [$1\text{-}^{14}\text{C}$]Oleic Acid and Treatment of Rats—The injection solution of [$1\text{-}^{14}\text{C}$]oleic acid was prepared essentially according to Göransson and Olivecrona.⁹⁾ On analysis, it became evident that more than 99.9% of the radioactivity was in free fatty acid fraction. Under light ether anesthesia 0.3 ml of

the serum containing 1 μ Ci of labeled oleic acid was injected into tail vein of rat. At 10 min after the injection, the rat was decapitated and liver was isolated immediately. The liver was perfused with ice-cold 0.9% NaCl solution to remove blood. Lipids were extracted by the method of Bligh and Dyer.¹⁰ The lipid obtained was fractionated into individual phospholipid components by one-dimensional thin-layer chromatography on silica gel according to Skipski *et al.*¹¹ Neutral lipids were separated by one-dimensional thin-layer chromatography on silica gel and the developing solvent system was petroleum ether/ether/acetic acid (80:30:1, v/v).¹² Lipids were visualized by brief exposure

to iodine vapor. Classes were identified with *R_f* values relative to authentic lipid standards. The silica gel was scraped off into counting vials. Lipid was extracted with methanol and mixed with toluene-based scintillator. The radioactivity was measured by a liquid scintillation spectrometer.

Analytical Procedures—Triacylglycerol was extracted two times, from silica gel after the separation with thin layer chromatography (TLC), with Folch's lower phase according to Kondrup¹³ and organic solvent was evaporated off. Triacylglycerol obtained was emulsified with 40 μ l of bovine serum albumin solution (fatty acid free bovine serum albumin, 30

FIG. 1A. Gel Filtration Chromatography of [1-¹⁴C]Oleic Acid with Hepatic Cytosol of Male Rat

Binding of oleic acid to cytosolic protein was determined by Sephadex G-50 gel filtration as described in the text. The numbered arrows indicate the positions at which markers of molecular weight were eluted; 1, blue dextran; 2, cytochrome c.

FIG. 1B. Relationship between Oleic Acid binding to FABP and FABP Concentration in Cytosol

FABP fractions obtained by gel filtrations of Sephadex G-50 of hepatic cytosol of male rats were combined and concentrated with Diaflow membrane YM 5 (Amicon Corp.). Graded amounts of the partially purified FABP were added to cytosol in order to increase the concentration of FABP as indicated in the figure. The resulting cytosol was subjected to binding assay for total FABP as described in the text.

mg/ml) and measured enzymatically with a commercial triacylglycerol assay kit (TG-Five kainos, Kainos Lab., Inc., Tokyo, Japan).

Lipid phosphorus was determined according

to Rouser *et al.*¹⁴⁾ after separating with TLC.

Protein concentration in cytosol was determined by the method of Lowry *et al.*¹⁵⁾ with bovine serum albumin as standard.

TABLE I. *Effects of Various Hypolipidemic Drugs and Chemicals on the Binding Capacity of Hepatic FABP to Oleic Acid and on Liver Weight of Rats*

Treatment		Oleic acid bound to FABP			Liver weight (% of body weight)	Cytosolic protein (mg/g liver)
		nmol/mg cytosolic protein	nmol/g liver	nmol/ liver		
Male rat						
None	(3)	1.45 ± 0.11	50.0 ± 9.0	599 ± 110	6.04 ± 0.16	34.5 ± 3.7
0.5% Clofibrilic acid	(4)	6.61 ± 1.86	284.2 ± 43.2	4643 ± 928	8.83 ± 0.69	34.6 ± 1.1
0.5% Probucol	(4)	2.10 ± 0.20	73.2 ± 9.6	972 ± 112	6.13 ± 0.19	33.0 ± 0.8
1.5% Niceritol	(4)	1.66 ± 0.35	55.0 ± 13.2	706 ± 148	5.93 ± 0.31	34.1 ± 2.7
2 % DEHP	(4)	4.83 ± 0.27	210.4 ± 12.1	3855 ± 322	9.39 ± 0.32	43.9 ± 1.4
4 % DEHP	(3)	4.79 ± 0.26	315.0 ± 16.2	4297 ± 644	8.82 ± 0.71	40.0 ± 1.5
2 % DBP	(4)	3.96 ± 1.03	177.7 ± 48.3	2668 ± 710	7.81 ± 0.23	45.2 ± 3.0
Female rat						
None	(3)	2.36 ± 0.05	86.3 ± 7.0	769 ± 74	5.31 ± 0.12	36.5 ± 3.4
0.5% Clofibrilic acid	(3)	4.27 ± 0.42	177.3 ± 22.6	2175 ± 388	7.45 ± 0.62	40.0 ± 1.5
2 % DEHP	(3)	5.45 ± 0.33	190.9 ± 15.3	2410 ± 217	9.37 ± 0.05	35.0 ± 1.1

Rats were fed a control diet or a diet containing one of drugs or chemicals as indicated in Table for 7 d. Each value represents the mean ± S.D. Value in parenthesis represents number of rats used. Binding of oleic acid to FABP was assayed as described in the text.

TABLE II. *Effects of Various Hypolipidemic Drugs and Chemicals on the Binding Capacity of Hepatic Ligandin of Rats*

Treatment		BSP bound to ligandin		
		nmol/mg cytosolic protein	nmol/g liver	nmol/ liver
None	(3)	1.01 ± 0.11	35.0 ± 7.1	420 ± 87
0.5% Clofibrilic acid	(4)	0.97 ± 0.39	35.7 ± 13.0	580 ± 222
0.5% Probucol	(3)	1.34 ± 0.05	48.0 ± 4.3	637 ± 60
1.5% Niceritol	(3)	1.15 ± 0.06	38.0 ± 1.6	491 ± 20
2 % DEHP	(3)	0.75 ± 0.08	31.9 ± 3.4	621 ± 44
2 % DBP	(3)	0.69 ± 0.05	35.4 ± 6.5	476 ± 39

Male rats were fed on a diet indicated in Table for 7 d. Each value represents the mean ± S.D. Value in parenthesis represents number of rats used. Binding of BSP to ligandin was assayed as described in the text.

RESULTS AND DISCUSSION

The results in Table I show the binding capacity of FABP fraction in hepatic cytosol of male rats to oleic acid when administered with hypolipidemic drugs or phthalic acid esters. Clofibrate is well known to increase the concentration and total content of FABP in male rat liver.^{7,8)} In accordance with the results of the earlier reports,^{7,8)} the binding capacity of FABP fraction in cytosol per g liver from rats fed clofibric acid, which is considered to be an active form of clofibrate,¹⁶⁾ was increased approx. 5.7 times compared to that of control rats. This increase in the binding capacity was not accompanied with the increase in the concentration of cytosolic protein. Moreover, the total binding

capacity of FABP fraction in liver was also increased approx. 7.7 times on clofibric acid-feeding. Probucol and niceritrol are used as hypolipidemic drugs^{16,17)} and are unrelated structurally to clofibric acid and to each other. In contrast to clofibric acid, however, the administration of these drugs changed scarcely the binding capacity of FABP fraction in liver, suggesting that both probucol and niceritrol may lower triacylglycerol by different mechanism(s) from that of clofibric acid and also suggesting that the increase in binding capacity of FABP fraction in liver is not always obligatory on hypolipidemic action of drugs.

Reddy *et al.* presented the evidence showing that DEHP-feeding caused a substantial reduc-

FIG. 2. Elution Profiles of BSP from Cytosol of Control and DEHP-treated Rat after Intravenous Administration

Male rats fed a control diet or a diet containing 2% (w/w) DEHP for 7 d, were injected intravenously 6 μ mol of BSP per 100 g body weight under light ether anesthesia. They were killed at 5 min after the injection, and the livers were isolated and fractionated as described in the text. Cytosol corresponding to 1 g liver was eluted from a Sephadex G-75 column (2.5 $\times$ 45 cm) at a flow rate of 20 ml/h. A, control rat; B, DEHP-treated rat. Y and Z in figure indicate the positions at which ligandin and FABP were eluted, respectively.

tion in serum triacylglycerol level.¹⁸⁾ They also demonstrated that the hypolipidemic effect of DEHP was associated with marked proliferation of peroxisomes.¹⁸⁾ Clofibrate is also a well known proliferator of peroxisomes¹⁹⁾ and the participation of FABP in peroxisomal fatty acid oxidation was suggested.²⁰⁾ Thus, it is of interest to examine the effects of phthalic acid esters on

TABLE III. *Effect of DEHP-feeding on the Distribution of BSP to FABP after Intravenous Administration*

Treatment	Ligandin	FABP
	BSP bound (nmol)	
None	13.1 (14.5–11.7)	0.3 (0.4–0.2)
2% DEHP	19.7 (26.4–12.9)	2.6 (3.4–1.7)

Experimental conditions were the same as in Fig. 2. Each value represents the mean and range (parenthesis) of two separate experiments and the value was obtained by integrating the amounts of BSP eluted in the fractions of peaks corresponding to ligandin and FABP, respectively.

TABLE IV. *Effects of Clofibric Acid- and DEHP-feeding on the Incorporation of Labeled Oleic Acid into Hepatic Lipid in Vivo*

Treatment	Lipid content $\mu\text{mol/liver}$	Radioactivity incorporated	
		dpm/liver	dpm/ μmol
Control (4)			
Triacylglycerol	42.2 $\pm$ 12.0	415140 $\pm$ 66600	8835 $\pm$ 2403
Phosphatidylethanolamine	86.5 $\pm$ 2.3	31080 $\pm$ 2220	335 $\pm$ 5
Phosphatidylcholine	174.5 $\pm$ 10.3	42180 $\pm$ 8880	229 $\pm$ 42
Total		530580 $\pm$ 106560	
Clofibric acid (4)			
Triacylglycerol	51.1 $\pm$ 13.0	479520 $\pm$ 97680	9232 $\pm$ 1323
Phosphatidylethanolamine	179.8 $\pm$ 7.9	48840 $\pm$ 15540	280 $\pm$ 83
Phosphatidylcholine	290.8 $\pm$ 28.4	99900 $\pm$ 19980	355 $\pm$ 63
Total		701520 $\pm$ 108780	
DEHP (2)			
Triacylglycerol	17.3	268620	15052
Phosphatidylethanolamine	113.4	59940	542
Phosphatidylcholine	273.0	150960	561
Total		552780	

Rats were fed a control diet or a diet containing 0.5% (w/w) clofibric acid or 2% (w/w) DEHP for 7 d. Experimental conditions were described in the text. Values in Table represent mean $\pm$ S.D. Numbers without S.D. represent the mean of two separate experiments. These individual values did not differ by more than 30%. Numbers in parentheses represent numbers of rats used.

the binding capacity of FABP fraction in the liver. As shown in Table I, similar results to that from clofibric acid-fed rats were obtained from DEHP-fed rats; DEHP-administration also caused the striking increase in the binding capacity of FABP fraction in the liver of male rats. DBP-feeding also increased the binding capacity of FABP fraction in male rat liver as well as DEHP-feeding did, although the ability of DBP to increase the binding capacity of FABP fraction was less significant than that of DEHP. These results suggest strongly that the effect of phthalic acid esters on the increase in binding capacity of FABP in liver may not be so dependent on alkyl chains.

Since the peroxisome proliferation with clofibrate-feeding is known to occur more significantly in male rat liver than in female rat liver,²¹⁾ effects of clofibric acid and DEHP on the binding capacity of FABP fraction in liver of female rats were also examined. In accordance with the results presented by Ockner *et al.*,²²⁾ the binding capacity of FABP fraction per g liver of control female rat was higher by approx. 170% compared to that of control male rat as shown in Table I. Moreover, the ratio of the increase in binding capacity in treated female rat liver to control was about half compared to that of male rat liver when treated with these peroxisome proliferators.

In association with the increase in the binding capacity of FABP fraction, a significant increase in liver weight also occurred on the feeding of clofibric acid, DEHP and DBP. On the other hand, no enlargement of liver was observed by feeding the drugs, which did not increase the binding capacity of FABP fraction, probucol and niceritrol. It is of interest to note that hepatic peroxisome proliferation is usually associated with an increase in liver size.^{23,24)}

It is well known that rat hepatocytes contain ligandin (Y protein) which is another cytoplasmic protein, having a high affinity to various organic anions.³⁾ According to Fleischer *et al.*, the concentration of ligandin was raised by 280% on phenobarbital-administration.²⁵⁾ We

therefore examined the effects of these hypolipidemic drugs on the binding capacity of ligandin fraction in male rat livers to BSP. As described in Table II, no appreciable changes in the binding capacity of ligandin fraction in liver was observed by the treatment of any hypolipidemic drugs or phthalic acid esters tested.

Intravenously administered BSP is known to bind not only ligandin but also FABP (Z protein).³⁾ Therefore, in order to confirm the increase of FABP *in vivo*, BSP was administered intravenously into rats, which had been fed a control or DEHP-containing diet, and hepatic cytosol obtained were subjected to chromatography on Sephadex G-75. Elution profiles of BSP bound to cytosolic protein were shown in Fig. 2 and the data, calculated to enable comparison, were described in Table III. In good agreement with the results obtained from *in vitro* experiments, BSP bound to FABP fraction increased strikingly without appreciable affect on the amounts of BSP bound to ligandin. A similar increase in the amounts of BSP bound to FABP was observed in rats which had been fed clofibric acid, although urina sanguinis was observed in the rats when BSP was administered (data not shown).

It is understood that FABP participates in the incorporation of fatty acid into esterified lipid in physiologically normal conditions.^{5,6)} Nevertheless it is reported that clofibrate inhibited the formation of hepatic triacylglycerol from radioactive glycerol *in vivo*.²⁶⁾ To make clear the relationship between the increase in the binding capacity of FABP fraction and the incorporation of fatty acid into esterified lipid, we examined the effects of clofibric acid and DEHP on the incorporation of [1-¹⁴C]oleic acid into glycerides as acyl moiety *in vivo*. As shown in Table IV, the contents of phosphatidylcholine and phosphatidylethanolamine in liver were increased by the feeding of clofibric acid or DEHP and the content of triacylglycerol in liver decreased remarkably by the administration of DEHP, but not of clofibric acid. In spite of the marked increase in the binding capacity of FABP fraction,

however, the incorporation of [1-¹⁴C]oleic acid into phosphatidylcholine increased slightly in the liver of rats which had been fed clofibrilic acid, resulting in the rise of specific radioactivity of phosphatidylcholine only approx. 150% compared to control liver. Furthermore, no appreciable changes in specific radioactivities of triacylglycerol and phosphatidylethanolamine were observed when fed clofibrilic acid. The incorporation of radioactive oleic acid into both phosphatidylcholine and phosphatidylethanolamine increased when fed DEHP, resulting in the marked rise in specific radioactivities of both phosphatidylcholine and phosphatidylethanolamine. On the other hand, the incorporation of [1-¹⁴C]oleic acid into triacylglycerol decreased in the liver of rats which had been fed DEHP, nevertheless the specific activity of triacylglycerol increased because of the extreme reduction of content of triacylglycerol in liver, suggesting the increase in the turn over of triacylglycerol. These results may imply that the increase in the binding capacity of FABP does not always relate to the increase in the incorporation of fatty acid into esterified lipid in liver when the rats are not physiologically normal by feeding hypolipidemic drugs as in the present work. Recently the possible participation of FABP in peroxisomal β -oxidation of fatty acid has been suggested.²⁰⁾ We showed in the present work that phthalic acid esters, which are unrelated structurally to the derivatives of phenoxyacetic acid such as clofibrilic or nafenopin, also increase the concentration of FABP in liver and that FABP was less inducible in female rat liver which is known to proliferate peroxisomes less compared to male rat liver upon the treatment of peroxisome proliferator. In addition, the hypolipidemic drugs like probucol and niceritol, which act without peroxisome proliferation, did not increase the binding capacity of FABP fraction. These facts probably suggest that FABP concentration may be increased in association with peroxisome proliferation and that FABP may act as a director of fatty acid to peroxisomes when the capacity of fatty acid oxidation is strikingly raised by the

treatment of peroxisome proliferators.

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