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Metabolic alterations by clofibric acid in the formation of molecular species of phosphatidylcholine in rat liver

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Abstract

The mechanism by which *p*-chlorophenoxyisobutyric acid (clofibric acid) induces striking changes in the proportion of the molecular species of phosphatidylcholine (PC) in rat liver was studied. Treatment of rats with clofibric acid strikingly increased the content of 1-palmitoyl-2-oleoyl (16:0–18:1) PC, but decreased the contents of 1-palmitoyl-2-docosahexaenoyl (16:0–22:6), 1-stearoyl-2-arachidonoyl (18:0–20:4), and 1-stearoyl-2-linoleoyl (18:0–18:2) PC; the drug did not change the content of 1-palmitoyl-2-arachidonoyl (16:0–20:4) PC. The mechanism underlying these changes has been investigated with regard to the *in vivo* formation of the molecular species of PC by: (i) *de novo* synthesis, (ii) reacylation, and (iii) methylation of phosphatidylethanolamine (PE). We found that (i) the incorporation of [^{3}H]glycerol, which was injected intravenously, into 16:0–18:1 diacylglycerol (DG) and 16:0–18:1 PC was increased markedly by clofibric acid feeding without changing the substrate specificity of CDP-choline:DG cholinephosphotransferase, (ii) the *in vivo* formation of 16:0–18:1 and 16:0–20:4 PC from 1–16:0-[^{3}H]glycerophosphocholine (GPC), which was injected intraportally, was increased markedly by clofibric acid feeding, and (iii) the incorporation of [^{14}C]ethanolamine, which was injected intravenously into 16:0–22:6, 18:0–22:6, and 18:0–20:4 PC, was decreased by clofibric acid feeding; the extent of the decrease in 16:0–20:4 PC was less than that of 18:0–20:4 PC. It was concluded, therefore, that (i) clofibric acid selectively increased the content and proportion of 16:0–18:1 PC by enhancing both the CDP-choline pathway and the remodeling of the pre-existing PC molecule, and (ii) the drug kept the content of 16:0–20:4 PC unchanged by stimulating the remodeling of the pre-existing PC molecule, whereas the formation of other more long chain, polyunsaturated molecular species, such as 16:0–22:6, 18:0–22:6, and 18:0–20:4, was decreased owing to the suppression of PE methylation.

Keywords: Clofibric acid; Phosphatidylcholine; Molecular species; Metabolic alteration; Liver; Rat

1. Introduction

Lipid bilayers are fundamental structures of biological membranes of which the major constituents are phospholipids. Phospholipids are comprised of hydrocarbon chains attached to a glycerophosphate backbone via acyl, alkyl, or alkenyl linkages. The molecular diversity of phospholipids is dictated by the combination of different carbon chain lengths, the number of double bonds, and the types of linkages of hydrocarbon chains. As a result, a mammalian cell contains more than one thousand molecular species of phospholipids [1]. Since the composition of these phospholipids plays an important role in controlling cellular homeostasis through the regulation of membrane fluidity and signal transduction within cells, it is necessary for cells to keep the composition regulated. It is therefore pharmacologically important to know whether drugs alter the composition of these phospholipids.

Only a few xenobiotics have been reported to affect the composition of phospholipids. None of them, however, has been investigated with regard to the mechanism by which the compounds change the composition of the numerously diverse phospholipids. One of the xenobiotics is *p*-chlorophenoxyisobutyric acid (clofibric acid), a hypolipidemic drug and a peroxisome proliferator. Limited information, however, is available about the mechanism by which this drug changes the composition of the molecular species of phospholipids. Our previous studies showed that the admin-

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Abbreviations: DG, diacylglycerol; GPC, glycerophosphocholine; PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

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istration of clofibric acid to rats causes striking changes in the composition and the content of the molecular species of PC in the liver [2,3]. Individual molecular species of PC are generated in the liver by three routes: *de novo* synthesis, remodeling of *de novo* synthesized PC, and methylation of PE [4,5]. In the liver, clofibric acid induces 1-acylglycerophosphocholine (1-acyl-GPC) acyltransferase [6], increases the activity of CTP:phosphocholine cytidylyltransferase [7], and decreases the activity of PE methyltransferase [7,8]. However, although the changes in the activities of these enzymes by clofibric acid were investigated extensively, the data, which were obtained previously from *in vitro* experiments assaying the enzyme activities, did not provide a satisfying answer to the question of how the drug changes the formation of individual molecular species of PC. Hence, the metabolic alterations induced by clofibric acid in the biosynthesis of individual molecular species of PC were investigated in the liver *in vivo.*

2. Materials and methods

2.1. Materials

[1(3)-^{3}H]Glycerol (500 Ci/mol) was purchased from Amersham. [1,2-^{14}C]Ethanolamine (100 Ci/mol) was obtained from ICN Biochemicals. CDP-[*methyl*-^{14}C]choline (55.5 Ci/mol), dipalmitoyl-[*choline-methyl*-^{3}H]PC (43 Ci/mmol), and [1-^{14}C]oleic acid (18:1)[1] (57 Ci/mol) were from NEN Life Science Products Inc. Clofibric acid, linoleic anhydride, phospholipase C (from *Clostridium welchii*), and snake venom (from *Crotalus adamanteus*) were obtained from the Sigma Chemical Co. 1–16:0-GPC and 1–18:0-GPC were obtained from Avanti Polar Lipids. Tween 20 was from Wako Chemicals. 1-Palmitoyl-[*choline-methyl*-^{3}H]GPC (1–16:0-[^{3}H]GPC) was prepared enzymatically from dipalmitoyl-[*choline-methyl*-^{3}H]PC using phospholipase A_2 of snake venom [9] and was purified by TLC on silica gel G plates (Merck), which were developed with chloroform:methanol:water (65:54:4, by vol.). The radiochemical purity of 1–16:0-[^{3}H]GPC was 99.9%. 16:0–18:2 and 18:0–18:2 DG were prepared by the hydrolysis of 16:0–18:2 and 18:0–18:2 PC, respectively, with phospholipase C, according to Wood and Snyder [10]. 16:0–18:2 and 18:0–18:2 PC, which were utilized for the preparation of the corresponding DGs, were synthesized from 1–16:0-GPC and 1–18:0-GPC, respectively, and linoleic anhydride by the method of Gupta *et al.* [11]. The DGs were purified by TLC on silica gel G plates, which were developed with benzene:chloroform:methanol (16:3:1, by vol.) as described by Ishidate *et al.* [12]. The purity of each DG was confirmed by TLC, and the presence of acyl moieties was confirmed by GLC as described in the section entitled "Lipid analyses." The purities of all DGs used were greater than 99%, based on results from fatty acid analyses.

2.2. Preparation of microsomes

Male Wistar rats were fed a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days, and then were decapitated. Livers were isolated and perfused with ice-cold 0.9% NaCl. Livers were homogenized in 3 vol. of 0.25 M sucrose/1 mM EDTA/10 mM Tris–HCl buffer, pH 7.4. The homogenates were centrifuged at 18,000 *g* for 20 min at 4°, and the supernatants were recentrifuged under the same conditions. The resulting supernatants were centrifuged at 105,000 *g* for 60 min at 4°. The pellets were resuspended in 0.25 M sucrose/10 mM Tris–HCl buffer, pH 7.4, and the suspensions were recentrifuged under the same conditions. The microsomal pellets obtained were resuspended in a small volume of 0.25 M sucrose/10 mM Tris–HCl buffer, pH 7.4. The concentrations of protein in the microsomal suspensions were determined by the method of Lowry *et al.* [13] with BSA as a standard.

2.3. Enzyme assays

CDP-choline:DG cholinephosphotransferase in hepatic microsomes, which were isolated from the livers of control rats and rats treated with clofibric acid, was assayed by the method of Ishidate *et al.* [12] using CDP-[*methyl*-^{14}C]choline and a mixture of 16:0–18:1 and 16:0–18:2 DGs in a Tween dispersion with various ratios as substrates. The molecular species of PC formed were analyzed by reverse-phase HPLC according to Patton *et al.* [14], as described in the section entitled "Analyses of molecular species."

2.4. In vivo metabolism of radiolabeled compounds

Male Wistar rats (160–200 g) were fed a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. [^{3}H]Glycerol was dissolved in 0.9% NaCl at a concentration of 100 μCi/0.2 mL after the evaporation of ethanol. The injection solution (10 μCi/0.2 mL) of [^{14}C]18:1 was prepared essentially according to Göransson and Olivecrona [15] with some modifications as described previously [16]. [^{14}C]Ethanolamine was dissolved in 0.9% NaCl at a concentration of 2.23 μCi/0.2 mL. Under light anesthesia with diethyl ether, one of the solutions (0.2 mL/rat) was injected into the exposed right jugular vein. For the determination of the incorporation of [^{3}H]glycerol into specific molecular species of PC, rats were killed at 5, 10, 30, and 120 min after administering [^{3}H]glycerol. To measure the formation of molecular species of PC containing [^{14}C]18:1, rats were killed at 10 min after injection. To estimate the methylation

[1] The numerical designation of fatty acids indicates their carbon chain lengths and number of double bonds: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; and 22:6, docosahexaenoic acid. The use of a hyphen between two numerical designations indicates that a particular phosphoglyceride contains both fatty acids.

of PE derived from [^{14}C]ethanolamine, rats were killed at 120 min after injection.

1–16:0-[^{3}H]GPC was dissolved in 0.9% NaCl at a concentration of 4 μCi/0.2 mL for the measurement of the incorporation of 1–16:0-[^{3}H]GPC into hepatic PC. Under light anesthesia with diethyl ether, rats were injected with 0.2 mL of a 1–16:0-[^{3}H]GPC solution through the portal vein. At 30 sec after injection, the rats were killed, and their livers were isolated and immediately frozen with liquid nitrogen.

2.5. Lipid analyses

Hepatic lipid was extracted by the method of Bligh and Dyer [17]. Phospholipids were separated by TLC on silica gel G plates, which were developed with chloroform:methanol:acetic acid:water (50:37.5:3.5:2, by vol.) according to Holub and Skeaff [18]. After visualizing by spraying 0.001% (w/v) primuline in acetone:water (4:1, v/v), the regions that corresponded to the standard PC or PE, which were run simultaneously on each plate, were scraped off and transferred to tubes. A volume of 10 mL of chloroform:methanol:0.1 M HCl (4:4:1, by vol.) was added to the tubes. After being kept at 4° overnight under nitrogen, the tubes were sonicated for 15 min with a bath-type sonicator. The lipid was extracted from the silica gel, and the extract was washed with 3 mL of 0.1 M HCl. Lipid phosphorus was determined according to Rouser *et al.* [19]. To determine the hepatic content of DG, neutral lipids were separated by TLC, which was developed with *n*-hexane:diethyl ether:acetic acid (80:30:1, by vol.). DG was detected and extracted from silica gel as described above. After adding pentadecanoic acid as an internal standard, fatty acids in DG were converted to methyl esters and were analyzed by GLC as described previously [20].

2.6. Analyses of molecular species

The analyses of diacyl-forms of choline glycerophospholipid and molecular species formed from [^{3}H]glycerol were carried out according to Blank *et al.* [21] with some modifications as described previously [2]. In brief, those lipids were converted to DG benzoates, and the molecular species formed were separated by reverse-phase HPLC using an octadecylsilyl column (4.6 × 250 mm; Lichrosorb RP-18) and an isocratic elution with acetonitrile:2-propanol (70:30, v/v). Fractions under the peaks corresponding to more than two molecular species were separated by reverse-phase HPLC with methanol:2-propanol (95:5, v/v). To measure radioactivity, the solvent was evaporated, the residue was dissolved in a toluene-based scintillator, and the radioactivity was determined by a scintillation counter.

DG and [^{3}H]DG formed from [^{3}H]glycerol were separated from other lipids by TLC on silica gel G plates, which were developed with *n*-hexane:diethyl ether:acetic acid (80:30:1, by vol.). DG was extracted from silica gels with 10 mL of chloroform:methanol:0.1 M HCl, and the extracts were washed with 3 mL of water. DG was converted to benzoyl derivatives, and the molecular species formed were analyzed by HPLC as described above.

The molecular species of PC formed from 1–16:0-[^{3}H]GPC were isolated by the methods of Patton *et al.* [14]. In short, the molecular species were separated by reverse-phase HPLC using an octadecylsilyl column (4.6 × 250 mm; Wakosil 5C18–200) and an isocratic elution with methanol:acetonitrile:water (70:24:6, by vol.) containing 20 mM choline chloride. To measure radioactivity, an aliquot of the eluate was taken to dryness, and the residue was dissolved in a toluene:Triton X-100 (2:1, v/v)-based scintillator. To determine specific radioactivity of 1–16:0-GPC in the liver, lipid was extracted by the method of Bligh and Dyer [17]. Lyso-PC was separated by TLC on silica gel G, which was developed with chloroform:methanol:water (65:25:4, by vol.). The region of the plate that corresponded to lyso-PC was scraped off and transferred to tubes. Lyso-PC was extracted from silica gels with chloroform:methanol:0.1 M HCl. To measure radioactivity, a part of the lyso-PC extract was taken to dryness, and the residue was dissolved in a toluene:Triton X-100 (2:1, v/v)-based scintillator. The other part of the lyso-PC extracts was methylated by sodium methoxide at 50° for 10 min after the addition of methyl heptadecanoate as an internal standard, and the content of palmitic acid was determined by GLC as described previously [20]. The amounts of the individual molecular species of PC formed from 1–16:0-[^{3}H]GPC were calculated by dividing the radioactive content (dpm/g liver) by the specific radioactivity of 1–16:0-[^{3}H]GPC in the livers (dpm/μmol).

The molecular species of PC and PE formed from [^{14}C]ethanolamine were separated by the methods of Patton *et al.* [14] as described above. With respect to the molecular species of PE formed from [^{14}C]ethanolamine, fractions under the peaks corresponding to more than two molecular species were separated by reverse-phase HPLC with methanol:acetonitrile:water (90.5:2.5:7, by vol.) containing 20 mM choline chloride. To measure radioactivity, an aliquot of the eluate was taken to dryness, and the residue was dissolved in a toluene:Triton X-100 (2:1, v/v)-based scintillator. Lipid phosphorus of the separated molecular species was determined by the method of Rouser *et al.* [19].

2.7. Statistical analyses

The statistical significance between two means such as control and clofibric acid-treated groups was determined by Student's *t*-test.

3. Results

3.1. Changes in the hepatic content and proportion of the molecular species of PC

The composition of the molecular species of hepatic PC was compared between control rats and rats that had been

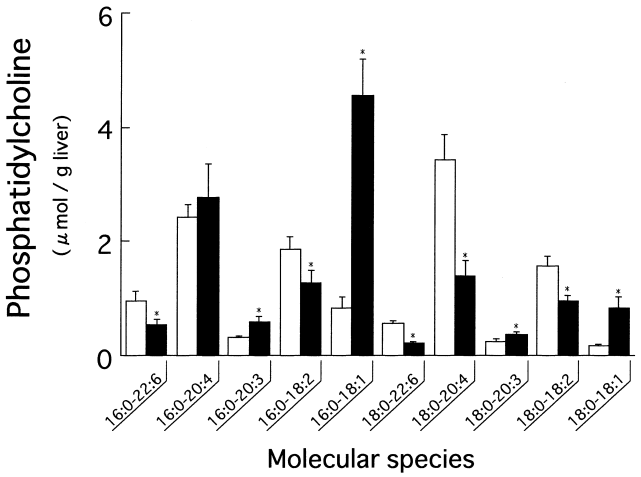

Fig. 1. Changes induced by clofibric acid in the mass proportion of the molecular species of PC in the liver. Rats were fed a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Livers were isolated, and lipid was extracted; PC was separated by TLC. The molecular species of PC were analyzed by HPLC. Values are means ± SD of six rats. Key: (□) control; and (■) clofibric acid-fed rats. Significant differences from controls: (*) $P < 0.001$.

fed a diet containing 0.5% (w/w) clofibric acid for 7 days. The absolute quantity and relative abundance of the molecular species of PC were changed markedly by the treatment of rats with clofibric acid (Fig. 1). The content of 16:0–18:1 PC increased 5.5 times compared with the control (4.56 ± 0.63 vs 0.83 ± 0.19 μmol/g liver). As a result, 16:0–18:1 PC became the most abundant molecular species of PC in the liver of clofibric acid-fed rats. By contrast, the contents of 16:0–22:6, 18:0–20:4, and 18:0–18:2 PC decreased to 57, 41, and 61%, respectively, of the controls. Clofibric acid treatment of rats did not alter the content of 16:0–20:4 PC. The contents of 16:0–18:1 PC of control and clofibric acid-fed rats were 7.1 and 64.5 μmol/whole liver, respectively, because the drug caused hepatomegaly (8.57 ± 1.16 vs 14.05 ± 2.16 g) and increased the hepatic content of PC (14.45 ± 1.02 vs 17.59 ± 1.16 μmol/g liver).

3.2. In vivo formation of molecular species of PC

To estimate the contribution of *de novo* synthesis in the observed quantitative changes in the expression of the PCs, [^{3}H]glycerol was injected intravenously into control and clofibric acid-fed rats, and the incorporation of [^{3}H]glycerol into each molecular species of PC was measured in a time-course up to 120 min (Fig. 2). At 5 min after the injection, the radioactivity that appeared in 16:0–18:1 PC in clofibric acid-fed rats was approximately seven times higher than in controls. By contrast, the radioactivity that was incorporated into 16:0–18:2 PC decreased markedly. In clofibric acid-fed rats, the incorporation of [^{3}H]glycerol into 16:0–18:1 PC increased gradually up to 30 min at which time the incorporation was 5-fold that of the control. The incorporation of [^{3}H]glycerol into either 16:0–22:6 or 18:0–20:4 PC was not changed considerably by the treatment of rats with clofibric acid. The incorporation of [^{3}H]glycerol into 16:0–20:4 PC increased gradually up to 120 min in both control and clofibric acid-fed rats. The rate of incorporation of [^{3}H]glycerol into 16:0–20:4 PC, which was calculated from the slope between 5 and 120 min, was 1.7 times higher in

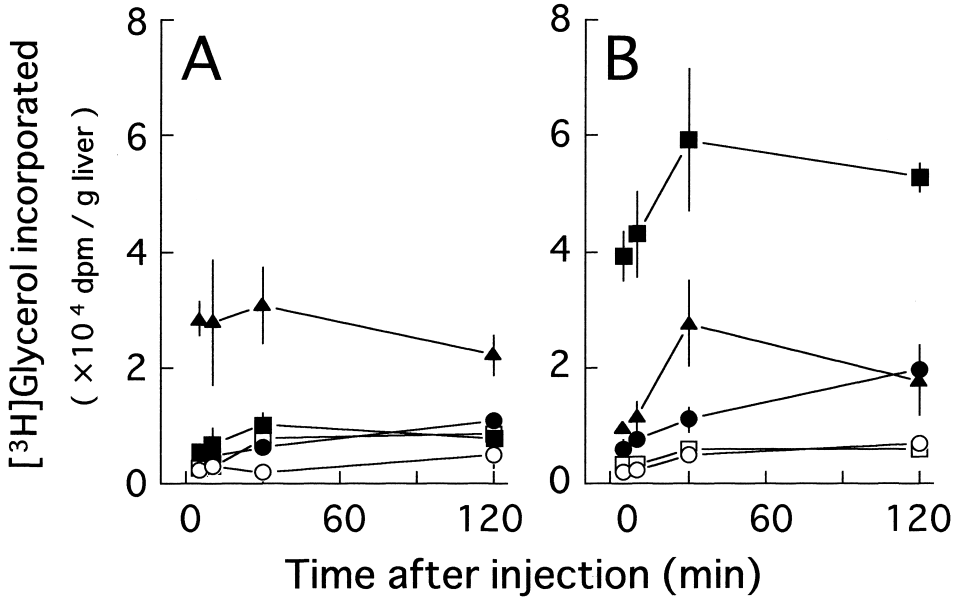

Fig. 2. Effects of clofibric acid on the *in vivo* formation of molecular species of PC from [^{3}H]glycerol in the liver. [^{3}H]Glycerol was injected intravenously into control rats or rats that had been fed a diet containing 0.5% (w/w) clofibric acid for 7 days. Livers were isolated, and lipid was extracted; PC was separated by TLC. The individual molecular species of PC were analyzed by HPLC. Values are means ± SD of three or four rats. (A) control; (B) clofibric acid-fed rats. Key: (■) 16:0–18:1; (▲) 16:0–18:2; (●) 16:0–20:4; (□) 16:0–22:6; and (○) 18:0–20:4.

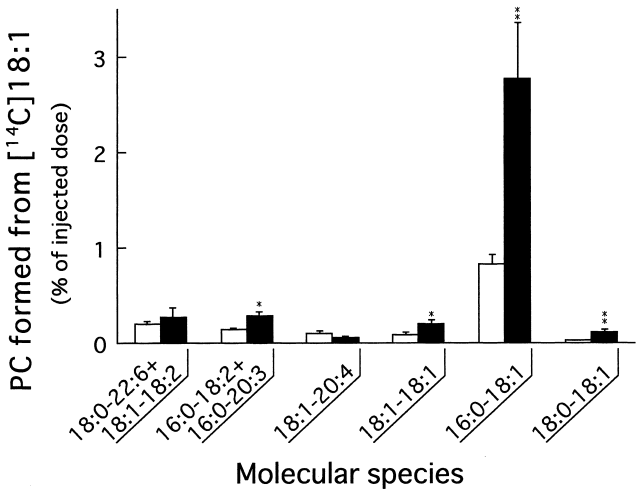

Fig. 3. Effects of clofibric acid on the *in vivo* incorporation of [^{14}C]18:1 into the molecular species of PC in the liver. [^{14}C]18:1 was injected intravenously into control rats or rats that had been fed a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were killed 10 min after the injection. Livers were isolated, and lipid was extracted; PC was separated by TLC. The molecular species of PC were separated by HPLC. Values are means ± SD of three or four rats. Key: (□) control; and (■) clofibric acid-fed rats. Significant differences from controls: (*) $P < 0.05$; and (**) $P < 0.01$.

clofibric acid-fed rats (155 dpm/min/g liver) than in controls (91 dpm/min/g liver).

To estimate further the effect of clofibric acid on the synthesis of 18:1-containing PCs, [^{14}C]oleic acid was injected intravenously into control and clofibric acid-fed rats, and the incorporation of [^{14}C]18:1 was measured at 10 min after injection (Fig. 3). In control rats, 52.5 ± 4.3% of the radiolabeled PC (0.83 ± 0.10% of injected [^{14}C]18:1) was the 16:0–18:1 species. In clofibric acid-fed rats, 68.6 ± 1.9% of the radiolabeled PC (2.77 ± 0.59% of injected [^{14}C]18:1) was the 16:0–18:1 species. It is noteworthy that [^{14}C]18:1 was hardly incorporated into 18:0–18:1 PC (in either control or clofibric acid-treated rats).

3.3. Increased formation of 16:0–18:1 PC by de novo synthesis

[^{3}H]Glycerol was incorporated into 16:0–18:1 PC more rapidly in clofibric acid-fed rats than in control rats, when compared at 5 min after the injection of the label (Fig. 2), suggesting that the increase in mass of this particular molecular species is due to the enhancement of *de novo* synthesis. Two possibilities can be considered for the increased *de novo* formation of 16:0–18:1 PC. The first is that clofibric acid changes the substrate specificity of CDP-choline:DG cholinephosphotransferase; the second is that clofibric acid stimulates the formation of 16:0–18:1 DG, which is utilized for *de novo* synthesis of PC.

To examine the first possibility, liver microsomes isolated from rats treated with clofibric acid were assayed for CDP-choline:DG cholinephosphotransferase substrate specificity using mixtures of 16:0–18:1 and 16:0–18:2 DGs as substrates in various ratios (Table 1). Treatment of rats with clofibric acid did not affect the ratio of the two molecular species of PC formed. Next, the effects of clofibric acid on the incorporation of [^{3}H]glycerol into molecular species of DG and on the absolute quantity and relative abundance of pre-existing DG were investigated (Table 2). The relative

Table 1
Effect of clofibric acid on the substrate specificity of CDP-choline: DG cholinephosphotransferase

Experiment	Substrates	CDP-choline: DG cholinephosphotransferase (nmol/min/mg protein)	
		Control	Clofibric acid
1	16:0–18:1 (100.0%)	41.09 (100.0%)	32.95 (100.0%)
	16:0–18:2 (0.0%)	0.00 (0.0%)	0.00 (0.0%)
2	16:0–18:1 (66.7%)	20.61 (55.5%)	17.43 (53.9%)
	16:0–18:2 (33.3%)	16.54 (44.5%)	14.90 (46.1%)
3	16:0–18:1 (50.0%)	12.54 (36.0%)	11.69 (36.7%)
	16:0–18:2 (50.0%)	22.18 (64.0%)	20.15 (63.3%)
4	16:0–18:1 (33.3%)	8.73 (23.4%)	7.76 (24.4%)
	16:0–18:2 (66.7%)	28.62 (76.6%)	24.10 (75.6%)
5	16:0–18:1 (0.0%)	0.00 (0.0%)	0.00 (0.0%)
	16:0–18:2 (100.0%)	35.43 (100.0%)	33.21 (100.0%)

Rats were fed a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. CDP-choline: DG cholinephosphotransferase was assayed using CDP-[^{14}C]choline and a mixture of 16:0–18:1 and 16:0–18:2 DG in various ratios. The molecular species of PC formed were separated by HPLC. Values represent the means of two separate experiments.

Table 2
Effect of clofibric acid on the contents and *in vivo* formation of the molecular species of DG in the liver

Molecular species	Mass proportion (nmol/g liver)		[^{3}H]Glycerol incorporation (% of radioactivity)	
	Control	Clofibric acid	Control	Clofibric acid
16:0–22:6	39 ± 3	27 ± 3**	3.41 ± 2.22	0.74 ± 0.08
18:2–18:2	118 ± 15	46 ± 3**	6.03 ± 0.57	0.64 ± 0.45***
16:0–20:4	46 ± 9	56 ± 10	3.06 ± 1.29	1.21 ± 0.40
18:1–18:2	21 ± 7	114 ± 7***	10.00 ± 2.83	4.23 ± 1.23*
16:0–18:2	207 ± 38	64 ± 13**	35.84 ± 2.81	4.97 ± 1.68***
18:0–20:4	103 ± 22	67 ± 28	0.35 ± 0.13	0.16 ± 0.05
18:1–18:1	59 ± 15	137 ± 74	4.49 ± 1.18	7.47 ± 0.68*
16:0–18:1	133 ± 22	347 ± 26***	18.25 ± 1.71	60.79 ± 3.72***
18:0–18:2	66 ± 16	32 ± 3*	1.49 ± 0.28	1.53 ± 1.36
18:0–18:1	12 ± 5	23 ± 3*	0.55 ± 0.12	0.89 ± 0.39
Others	296 ± 56	245 ± 27	16.53 ± 2.34	17.38 ± 0.87
Total	1099 ± 114	1157 ± 56	100.00	100.00

Rats were fed a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were injected intravenously with [^{3}H]glycerol and killed 5 min after the injection. DG was isolated by TLC, and the molecular species of DG were separated by HPLC. The radioactivities found in DG in the livers of control and clofibric acid-fed rats were: 32,521 ± 3,758 and 42,824 ± 14,439 dpm/g liver, respectively. Values represent the means ± SD of three separate experiments.

*–*** Significant differences from controls: * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$.

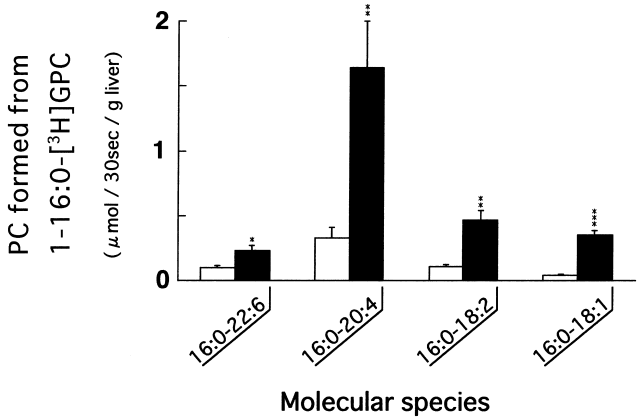

Fig. 4. Effects of clofibric acid on the *in vivo* formation of molecular species of PC from 1–16:0-[^{3}H]GPC in the liver. 1–16:0-[^{3}H]GPC was injected into the portal vein in anesthetized control rats or rats that had been fed a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were killed 30 sec after the injection. Livers were isolated, and lipid was extracted. PC and lyso-PC were isolated by TLC, and radioactivities were measured. The amount of 1–16:0-GPC was determined by GLC. The molecular species of PC were separated by HPLC, and radioactivity was measured. The absolute amounts of individual molecular species of PC formed were calculated by dividing the radioactive content (dpm/g liver) by the specific radioactivity of 1–16:0-[^{3}H]GPC in the liver (dpm/μmol). Values are means ± SD of three rats. Key: (□) control; and (■) clofibric acid-fed rats. Significant differences from controls: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$.

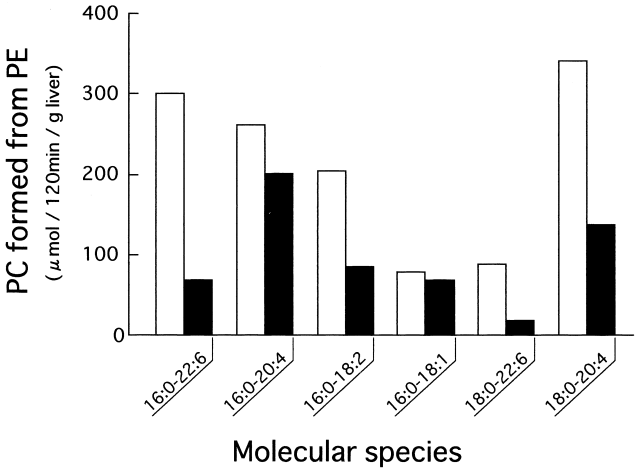

Fig. 5. Effects of clofibric acid on the *in vivo* formation of molecular species of PC from PE in the liver. [^{14}C]Ethanolamine was injected intravenously into control rats or rats that had been fed a diet containing 0.5% (w/w) clofibric acid for 7 days. At 120 min after the injection, livers were isolated. Hepatic lipid was extracted; PC and PE were separated by TLC. The molecular species of PC and PE were separated by HPLC, and radioactivity and lipid phosphorus were determined. The amounts of PC formed from PE were calculated by dividing the radioactive content of PC (dpm/g liver) by the specific radioactivity of PE at 120 min (dpm/μmol). Values represent the means of two separate experiments. Key: (□) control; and (■) clofibric acid-fed rats.

incorporation of [^{3}H]glycerol into 16:0–18:1 DG was increased 3.3 times by the administration of clofibric acid to rats, and 61% of the newly synthesized radioactive DG was the 16:0–18:1 species in the clofibric acid-fed rats. By contrast, the relative proportion of radioactive 16:0–18:2 DG decreased from 36 to 5%. The mass of pre-existing 16:0–18:1 DG increased 2.5 times (from 12.1 to 30.0%) following the administration of clofibric acid; the content of 16:0–18:2 DG decreased from 18.8 to 5.5%. Clofibric acid did not change the total content of DG in the liver.

3.4. Increased formation of 16:0–18:1 PC by reacylation

Figure 4 shows the effect of clofibric acid on *in vivo* formation of molecular species of PC from 1–16:0-[^{3}H]GPC at 30 sec after the injection of the label into the portal vein. The formation of molecular species of PC was calculated using the specific radioactivity of 1–16:0-[^{3}H]GPC in the liver. The hepatic contents of 1–16:0-GPC in control and clofibric acid-fed rats were 296 ± 89 and 305 ± 20 nmol/g liver, respectively, and the specific radioactivities of 1–16:0-[^{3}H]GPC in livers of control and clofibric acid-fed rats were 12.65 ± 3.20 and 6.69 ± 2.66 dpm/nmol, respectively. In control rats, 1–16:0-[^{3}H]GPC was preferentially incorporated into 16:0–20:4 PC, and the amount of 16:0–18:1 PC formed was less than one-ninth of 16:0–20:4 PC. Although the administration of clofibric acid to rats increased the formation of all four molecular species from 1–16:0-[^{3}H]GPC, the extent of the increase was the greatest in the formation of 16:0–18:1 PC. The value was approximately 9.3 times that of control. The formation of 16:0–20:4 and 16:0–18:2 PC from 1–16:0-[^{3}H]GPC increased 5.0 and 4.4 times, respectively.

3.5. Decreased formation of 16:0–22:6 and 18:0–20:4 PC through the PE methylation pathway

To investigate the effect of clofibric acid on the formation of each molecular species of PC through methylation of PE, [^{14}C]ethanolamine was injected intravenously into control and clofibric acid-fed rats, and the incorporation of the label into several molecular species of PC was measured. The formation of molecular species of PC from PE was calculated using the specific radioactivity of PE at 120 min after the injection of [^{14}C]ethanolamine (Fig. 5). The formation of molecular species of PC containing 22:6 or 18:2 was decreased by the treatment of rats with clofibric acid. The formation of 16:0–20:4 PC was decreased by 23%, and the formation of 18:0–20:4 PC was reduced to 40% of control. Only the formation of 16:0–18:1 PC remained unaltered by clofibric acid through the PE methylation pathway.

4. Discussion

4.1. Increased formation of 16:0–18:1 PC

The most interesting finding in this study was the marked increase in the proportion and content of 16:0–18:1 PC by

Table 3
Alterations by clofibric acid in activities of enzymes that participate in the biosynthesis of PC

Enzymes	Enzyme activities (nmol/min/mg protein)		References
	Control	Clofibric acid	
Palmitoyl-CoA chain elongase	0.32 ± 0.09	0.82 ± 0.15	23
Stearoyl-CoA desaturase	0.66 ± 0.02	2.39 ± 0.53	24
1-Acylglycerophosphate acyltransferase	93.2 ± 14.2	147.6 ± 20.2	23
Glycerol-3-phosphate acyltransferase	9.44 ± 1.00	27.67 ± 1.49	7
Choline kinase	2.74 ± 0.23	3.06 ± 0.28	7
CTP:phosphocholine cytidylyltransferase	3.57 ± 0.73	4.97 ± 1.44	7
CDP-choline:DG cholinephosphotransferase	36.61 ± 3.30	30.73 ± 1.29	7
1-Acyl-GPC acyltransferase	54.6 ± 6.3	206.3 ± 25.0	6
PE *N*-methyltransferase	9.88 ± 0.57	8.01 ± 0.36	7,8

Values represent means ± SD of at least four separate experiments.

clofibric acid. Our previous study showed that administration of clofibric acid to rats increased the hepatic contents of phospholipids and the size of the liver [7]. Namely, hepatic contents of diacyl glycerophospholipid in control and clofibric acid-fed rats were 123 and 248 μmol/whole liver, respectively [7]. When calculated from these data and the data presented in Fig. 1, the proportion of 16:0–18:1 PC in diacyl glycerophospholipid in the liver was increased from 5.8 to 26.0% by the administration of clofibric acid. It should be noted, therefore, that 16:0–18:1 PC represents one-fourth of the phospholipid molecules in the liver of clofibric acid-fed rats, because the liver contains extremely small amounts of alkyl-acyl and alkenyl-acyl glycerophospholipids [22].

Previous studies showed that clofibric acid changes the activities of several enzymes participating in the biosynthesis of PC, as summarized in Table 3. Since this drug induced stearoyl-CoA desaturase and 1-acyl-GPC acyltransferase (Table 3), our previous study concluded that these two enzymes cooperatively facilitated the remodeling of PC, resulting in the striking increase in the proportion of 16:0–18:1 PC [25]. In hepatocytes in physiologically normal conditions, however, 16:0–18:1 PC is synthesized from the CDP-choline pathway [26–28]. Moreover, clofibric acid increases the activity of CTP:phosphocholine cytidylyltransferase (Table 3). These findings suggest the possibility that clofibric acid increases the mass and proportion of 16:0–18:1 PC by enhancing *de novo* synthesis. The present study examined this possibility, first. The *in vivo* formation of 16:0–18:1 PC from [^{3}H]glycerol was increased markedly by clofibric acid at 5 min after the injection of the label, suggesting the enhancement of *de novo* synthesis (Fig. 2). There seem to be two mechanisms responsible for the increased formation of 16:0–18:1 PC through the CDP-choline pathway. The first possibility is that clofibric acid changes the substrate specificity of CDP-choline:DG cholinephosphotransferase, which is altered to utilize 16:0–18:1 DG in preference to 16:0–18:2 DG in clofibric acid-fed rats. The second possibility is that the proportion of 16:0–18:1 species in DG is increased by clofibric acid. It is known that, in physiologically normal conditions, CDP-choline:DG cholinephosphotransferase unselectively utilizes species of DG differing in the degree of unsaturation [29]. The present study confirmed that clofibric acid did not alter substrate specificity (Table 1). On the other hand, clofibric acid increased the generation of 16:0–18:1 DG and decreased the formation of 16:0–18:2 DG (Table 2). As a result, the proportion of 16:0–18:1 DG was increased markedly. Since clofibric acid induces palmitoyl-CoA chain elongase and stearoyl-CoA desaturase (Table 3), the supply of oleoyl-CoA seems to be increased. Moreover, this drug induces glycerol-3-phosphate acyltransferase and 1-acylglycerophosphate acyltransferase (Table 3). These metabolic changes, taken together, lead to an increased proportion of 16:0–18:1 DG. Since clofibric acid increases the activity of CTP:phosphocholine cytidylyltransferase (Table 3), 16:0–18:1 PC appears to be preferentially synthesized through the CDP-choline pathway.

Since clofibric acid induces 1-acyl-GPC acyltransferase (Table 3), there is a possibility that the induced 1-acyl-GPC acyltransferase facilitates remodeling of PC by utilizing abundantly formed oleoyl-CoA. In fact, the *in vivo* formation of 16:0–18:1 PC from [^{3}H]glycerol increased gradually in the initial phase of the time course after the injection of the label, and its formation was stimulated by clofibric acid (Fig. 2). To examine whether clofibric acid facilitates the formation of 16:0–18:1 PC by reacylation, the effect of clofibric acid on the *in vivo* formation of 16:0–18:1 PC from 1–16:0-[^{3}H]GPC at 30 sec after an injection of the label into the portal vein was studied. The *in vivo* formation of 16:0–18:1 PC from 1–16:0-[^{3}H]GPC was stimulated by clofibric acid, and the extent of its stimulation was greater than that of other molecular species (Fig. 4). These results strongly indicate the involvement of remodeling in the increased formation of 16:0–18:1 PC.

Accordingly, it can be concluded that clofibric acid stimulated the formation of 16:0–18:1 PC through both the CDP-choline pathway and the deacylation–reacylation pathway.

4.2. Formation of more long chain, polyunsaturated species

The major PC molecules formed through the CDP-choline pathway in physiologically normal conditions are the 16:0–18:2 and 16:0–18:1 species, which appear to be remodeled by deacylation–reacylation to 16:0–20:4 and 18:0–20:4 PC [27,28]. In agreement with these previous findings, the present data on the *in vivo* formation of PC species from [^{3}H]glycerol suggest that 16:0–20:4 and 18:0–20:4 species are derived from 16:0–18:2 and 16:0–18:1 PC by

deacylation–reacylation, because the former two molecular species were increased gradually up to 120 min in concert with the decline of radioactivity in 16:0–18:2 and 16:0–18:1 PC (Fig. 2A). The formation of 16:0–20:4, but not 18:0–20:4 PC, from 16:0–18:2 and 16:0–18:1 PC by remodeling appears to be stimulated markedly by the feeding of clofibric acid (Fig. 2B). The facilitated formation of 16:0–20:4 PC by reacylation was confirmed by the experiment on the *in vivo* synthesis of 16:0–20:4 from 1–16:0-[^{3}H]GPC (Fig. 4). It should be noted here that the present results are the first evidence that the 1-acyl-GPC acyltransferase induced by clofibric acid operates *in vivo* to enhance the remodeling of PC.

In physiologically normal hepatocytes, 70% of PC is formed through the CDP-choline pathway and 30% is generated through the PE methylation pathway; moreover, PC derived from the methylation pathway contains more long chain polyunsaturated PC species [28]. In particular, 16:0–22:6 PE appears to be preferentially methylated to PC [30]. In agreement with these previous findings, the present study showed that the major species of PC formed from PE by methylation *in vivo* are 16:0–22:6, 18:0–20:4, and 16:0–20:4 (Fig. 5). Our previous studies demonstrated that clofibric acid suppresses the activity of PE *N*-methyltransferase (Table 3) and inhibits the *in vivo* formation of PC from PE [8]. In the liver of rats treated with clofibric acid, individual PC species that were synthesized from [^{14}C]ethanolamine through PE were not always suppressed to the same extent by clofibric acid. Namely, formation of 16:0–22:6, 18:0–22:6, and 18:0–20:4 PC was strikingly reduced (Fig. 5), resulting in a decrease in the proportion and content of these three molecular species of PC (Fig. 1). On the other hand, although the formation of 16:0–20:4 PC from PE through the methylation pathway was suppressed to a lesser extent (Fig. 5), the formation of this species by deacylation–reacylation was simulated markedly by clofibric acid (Fig. 4). Consequently, the proportion and content of 16:0–20:4 PC in the liver were not changed, in spite of the striking reduction of the content of the 18:0–20:4 species (Fig. 1).

4.3. Other conclusions

The present study demonstrated the metabolic basis for the elucidation of the changes induced by clofibric acid in the formation of individual molecular species of PC. 16:0–18:1 DG, the proportion of which in the liver was increased by clofibric acid, is the molecular species that is preferentially available for the synthesis of both PC and triacylglycerol. Sorting of this particular molecular species of DG between PC and triacylglycerol syntheses is an important point to be studied. A previous study demonstrated that administration of clofibric acid to rats increases hepatic PC by 4.29 μmol/g liver (from 14.91 to 19.20 μmol/g liver), but decreases hepatic triacylglycerol by 2.66 μmol/g liver (from 5.18 to 2.52 μmol/g liver) [3]. These findings suggest that 16:0–18:1 DG is preferentially utilized for the synthesis of PC rather than triacylglycerol during treatment with clofibric acid. A detailed mechanism responsible for the metabolic alterations in this sorting of 16:0–18:1 DG, however, has not been clarified yet. In the present study, we have not dealt with the effects of PC excretion (either as lipoprotein or as biliary PC) and of fatty acid uptake from circulation on the proportion and content of molecular species of hepatic PC during treatment with clofibric acid. Our previous studies demonstrated that the administration of clofibric acid to rats reduces the serum concentration of PC by 54% [3] and the rate of PC secretion into circulation by 40% [7]. The preferred PC molecules secreted as a component of lipoprotein are the 16:0–18:2 and 18:0–18:2 species [2], and clofibric acid seems to accomplish the reduction of serum PC by lowering, in particular, PC molecular species containing 18:2 without changing the serum concentration of 16:0–18:1 PC [3]. The reduced secretion of PC into the blood circulation by clofibric acid, therefore, does not seem to be responsible for the increase in the proportion and content of 16:0–18:1 PC in the liver. In contrast to the effect on lipoprotein secretion, clofibric acid stimulated biliary secretion of PC 1.7-fold by increasing levels of multidrug-resistance gene mRNA in the liver of mice [31]. Moreover, the molecular species of PC that appears to be preferentially secreted into bile is 16:0–18:2 PC [32]. However, since no information is available as to the effects of clofibric acid on the proportion of molecular species of PC in the bile, the quantitative relationship between the clofibric acid-induced alteration in the proportion of molecular species of hepatic PC and the increased biliary excretion of PC remains to be elucidated. Moreover, since clofibric acid is considered to induce lipoprotein lipase [33], the uptake of fatty acids from blood circulation may be enhanced by the drug. To our knowledge, however, little information is available as to the alterations caused by clofibric acid in either the species of fatty acid derived from the circulation or its utilization by the liver for the synthesis of PC.

The precise physiological significance of the striking increase in the content of 16:0–18:1 PC has not been clarified yet. Clofibric acid increases the size of organelles, including peroxisomes, mitochondria, and endoplasmic reticulum, and enlarges the liver itself [34,35], which must require an abundant supply of PC with appropriate unsaturation to maintain the fluidity of biological membranes. Since clofibric acid induces peroxisomal β-oxidation [36] and polyunsaturated fatty acids are preferentially broken down by peroxisomal β-oxidation [37], polyunsaturated fatty acids seem to be depleted in the livers of animals treated with clofibric acid. In response to essential fatty acid deficiency, stearoyl-CoA desaturase is induced in the liver [38,39], and the enzyme catalyzes the synthesis of the only monounsaturated fatty acids, especially 18:1, that mammals can synthesize *de novo*. That is, the 16:0–18:1 species is the unsaturated PC that animals can produce by their own enzyme systems. The physiological significance of the re-

duction of the content of the 16:0–22:6 and 18:0–20:4 species remains to be elucidated.

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