Full paper for ***Journal of Pharmacological Sciences*** (Category: Biochemical pharmacology)

Clofibric Acid Increases the Formation of Oleic Acid in Endoplasmic Reticulum of the Liver of Rats

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Abbreviations

clofibric acid, 2-(4-chlorophenoxy)-2-methylpropionic acid;
clofibrate, ethyl 2-(4-chlorophenoxy)-2-methylpropionic acid;
FABP, fatty acid binding protein;
LPC, 1-acylglycerophosphocholine;
LPCAT, 1-acylglycerophosphocholine acyltransferase;
PC, phosphatidylcholine;
PE, phosphatidylethanolamine;
PPARα, peroxisome proliferator-activated receptor α;
SCD, stearoyl-CoA desaturase;

Abstract. The effects of 2-(4-chlorophenoxy)-2-methylpropionic acid (clofibric acid) on the formation of oleic acid (18:1) from stearic acid (18:0) and utilization of the 18:1 formed for phosphatidylcholine (PC) formation in endoplasmic reticulum in the liver of rats were studied *in vivo*. [^{14}C]18:0 was intravenously injected into control Wistar male rats and rats that had been fed on a diet containing 0.5% (w/w) clofibric acid for 7 days, and the distribution of radiolabeled fatty acids among subcellular organelles, microsomes, peroxisomes and mitochondria, was estimated on the basis of correction utilizing the yields from homogenates of marker enzymes for these organelles. The radioactivity was mostly localized in microsomes and the radiolabeled fatty acids present in microsomes were significantly increased by the treatment of rats with clofibric acid. The formation of radiolabeled 18:1 in microsomes markedly increased and incorporations of the formed [^{14}C]18:1 into PC and phosphatidylethanolamine in microsomes were augmented in response to clofibric acid. The [^{14}C]18:1 incorporated into PC was mostly located at the C-2 position, but not the C-1 position, of PC, and the radioactivity in 18:1 at the C-2 position of PC was strikingly increased by clofibric acid. These results obtained from the in *vivo* experiments directly link the findings that clofibric acid treatment induces microsomal stearoyl-CoA desaturase and 1-acylglycerophosphocholine acyltransferase in the liver and the findings that the treatment with the drug elevated absolute mass and mass proportion of 18:1 at the C-2 position, but not the C-1 position, of PC in the liver together.

***Keywords*:** clofibric acid, stearoyl-CoA desaturation, oleic acid, phosphatidylcholine, endoplasmic reticulum

Introduction

The physiological role of fatty acids as the precursors of lipid mediators such as eicosanoids have been extensively studied with arachidonic acid, 8,11,14-eicosatrienoic acid and 5,8,11,14,17-eicosapentaenoic acid. In recent years, evidence supporting the crucial role of fatty acid species in maintaining systemic metabolic homeostasis has been increasingly reported. Polyunsaturated fatty acids have been shown to extend molecular effects to cellular homeostasis as lipid signaling molecules through nuclear receptors such as peroxisome proliferator-activated receptor (PPAR), sterol regulatory element-binding protein, retinoid X receptor, hepatic nuclear factor-4α and liver X receptor (1, 2). Recent studies showed that saturated fatty acids such as palmitic acid (16:0) and stearic acid (18:0) cause endoplasmic reticulum stress and that, as a result, insulin resistance, and apoptosis of liver cells and pancreatic β-cells are induced (3-5). With regard to monounsaturated fatty acid, especially oleic acid (18:1), most studies have been focused on the role of 18:1 in energy homeostasis, because 18:1 is a major component of triglyceride, accumulation of which causes fatty liver and obesity. Recently, however, evidence showing novel significance of monounsaturated fatty acids has increased. Namely, a particular molecular species of phosphatidylcholine (PC), palmitoyl-oleoyl-PC, has been demonstrated to be a physiologically relevant endogenous ligand for PPARα (6). A large amount of 18:1 was shown to induce hepatic endoplasmic reticulum stress, resulting in inhibition of VLDL secretion (7). Palmitoleic acid was shown to stimulate muscle insulin action and suppress hepatosteatosis (8). These findings, taken together, indicate that the nature of the fatty acid species, even if monounsaturated such as 18:1, is crucial for maintaining homeostasis in organs. In this context, therefore, it considered important to accumulate the information with regard to the effects of drugs on the formation and utilization of 18:1 in the liver.

2-(4-Chlorophenoxy)-2-methylpropionic acid (clofibric acid) is one of the fibrates, which are hypolipidemic drugs that reduce triglyceride in circulation. The mechanism behind the hypotriglyceridemic effects of fibrates has been extensively studied. On the other hand, however, a previous study showed that clofibric acid has a different type of effect on fatty acid metabolism in the liver; namely, the administration of clofibric acid to rats increased the mass proportion of 18:1 in hepatic lipids (9). To elucidate the physiological significance of this observation, a series of studies were performed and demonstrated that the administration of clofibric acid to rats induced stearoyl-CoA desaturase (SCD) (9, 10),

1-acylglycerophosphocholine acyltransferase (LCPAT) (11) and CTP:phosphocholine cytidylyltransferase (12), resulting in striking increases in the mass proportion and content of 18:1 at the C-2 position of PC (13) and of a particular molecular species of PC, palmitoyl-oleoyl-PC (14, 15), in the liver. Subsequent studies revealed that the induction of SCD by clofibric acid is due to the elevation of the expression of SCD1, one of the isoforms of SCD, through the activation of PPARα, and to the suppression of the degradation of SCD (16). Recently, the induction of LPCAT by clofibric acid has been shown to be due to the up-regulation of the expression of LPCAT3, one of the isoforms of LPCAT, through activation of PPARα (17). These enzymes mentioned above are believed to be localized mostly in endoplasmic reticulum (18,19). Thus, many detailed studies were conducted pharmacologically and biochemically to reveal the effects of clofibric acid on the expression of the genes, the regulation of the enzyme activities and the mass proportions of fatty acids and lipids. Nevertheless, to our knowledge, evidence is still lacking to directly link the findings of up-regulations of the enzymes and the findings of increase in 18:1 containing lipids together. Namely, with regard to metabolic alterations *in vivo* of fatty acids induced by clofibric acid, detailed information has remained unavailable.

The aim of the present study, therefore, is to ascertain by *in vivo* experiments (i) whether endoplasmic reticulum is the organelle that synthesizes 18:1 from 18:0 by desaturation in the liver, (ii) how much the formation of 18:1 in whole endoplasmic reticulum in the liver is enhanced by treatment of animals with clofibric acid, and (iii) how much the incorporation of 18:1, which is formed from 18:0 by desaturation, into PC is stimulated in whole endoplasmic reticulum in the liver.

Materials and Methods

Materials

[1-^{14}C]Stearoyl-CoA (55.0 Ci/mol), [1-^{14}C]stearic acid (55.0 Ci/mol) and [1-^{14}C]oleic acid (50.0 Ci/mol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). Oleoyl-CoA, stearoyl-CoA, clofibric acid, cytochrome *c*, phospholipase A_2 (from *Crotalus admanteus*), hydroxyalkoxypropyl-Dextran and bovine serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.); 1-acylglycerophosphocholine (LPC) from Avanti Polar Lipids Inc. (Pelham, AL, U.S.A); pentadecanoic acid from Nu-Chek-Prep Inc. (Elysian, MN, U.S.A.); NADH and NADPH from Oriental Yeast Co. (Tokyo, Japan); and Percoll and Sephadex G-50 from GE Healthcare (Buckinghamshire, England). All other

chemicals were of analytical grade.

Treatment of animals

All animal studies complied with the recommendations of the Institutional Board for Animal Studies, Josai University. Male Wistar rats aged 4 weeks were obtained from SLC (Hamamatsu, Japan). Animals were fed on a standard diet (CE-2, Clea Japan) (Tokyo, Japan) *ad libitum* and allowed free access to water. After acclimatization for 1 week, rats were divided into two groups and were fed on a standard diet or a diet containing 0.5% (w/w) clofibric acid for 7 days.

In vivo conversion of [^{14}C]18:0 to [^{14}C]18:1

The injection solution of [^{14}C]18:0 was prepared by dissolving in rat serum by the method as described previously (18, 20). Under light diethyl ether anesthesia, 0.2 ml of serum containing 1 μCi of [^{14}C]18:1 was injected into the tail vein of control and clofibric acid-treated rats. At 10 min after the injection, livers were isolated immediately. Blood remaining in the livers was washed out with ice-cold 0.9% NaCl. The livers were washed with cold 0.9% NaCl. Each liver was homogenized with 9 volumes of 0.25M sucrose containing 1 mM disodium EDTA (pH 7.3). The homogenates were used as the samples for lipid extraction as described below.

In vivo formation and metabolism of [^{14}C]18:1 from [^{14}C]18:0 in microsomes

Under light diethyl ether anesthesia, 0.2 ml of serum containing 30 μCi of [^{14}C]18:0 was injected into the exposed right jugular vein of control and clofibric acid-treated rats. At 5, 10, 15 and 30 min after the injection, livers were isolated immediately. Blood remaining in the livers was washed out with ice-cold 0.9% NaCl. Subcellular fractionations were carried out as described below.

Preparation of subcellular fractions

Subcellular fractionation of liver was conducted essentially according to de Duve *et al*. (21). Briefly, differential centrifugations were performed to remove the nuclear fraction from the homogenate and successively to obtain heavy mitochondrial, light mitochondrial, microsomal and cytosolic fractions. Peroxisomal fractions were isolated by isopycnic density gradient centrifugation of the light mitochondrial fraction in a self-generated Percoll gradient essentially according to Neat *et al*. (22), by using a vertical rotor RPV50T-107 (Hitachi,

Tokyo, Japan) . Similarly, mitochondrial factions were isolated by centrifugation of the heavy mitochondrial fraction in a self-generated Percoll gradient (22). The mitochondrial, peroxisomal and microsomal fractions obtained were resuspended in 0.25M sucrose. All fractions were stored at -80°C until use. The following marker enzymes were used: NADPH-cytochrome *c* reeducates (23), urate oxidase (24) and glutamate dehydrogenase (24). Protein was determined by the method of Lowry *et al*. (25) with bovine serum albumin as a standard.

Preparation of fatty acid binding protein-rich fraction

Livers were isolated from control and clofibric acid-treated rats under diethyl ether anesthesia, and were perfused with ice-cold 0.9% NaCl until traces of blood were washed out. The subsequent operations were performed at 0 - 4°C. Livers were homogenized with 1.5 volumes of 0.25M sucrose containing 1 mM disodium EDTA (pH 7.3). The homogenates were centrifuged at 20000 x g for 15 min and the resulting supernatant was recentrifuged at 105000 x g for 120 min. The supernatant fraction was separated from the pellet and surface lipid. 4 ml of the supernatant fraction was subjected to gel filtration on Sephadex G-50 column (2.5 x 40 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) at 4°C. Elution was performed with the same buffer at a flow rate of 10 ml /h, and fractions of 4 ml were collected. The capacities of the fractions to bind [^{14}C]18:1 were measured by the method with hydroxyalkoxypropyl-Dextran as described previously (26), and the fractions with a molecular weight is around 10000 and with the capacity to bind [^{14}C]18:1 were combined as fatty acid binding protein (FABP) -rich fraction.

Enzyme assays for SCD and LPCAT

Microsomal activity of stearoyl-CoA desaturation was assayed by measuring the conversion of [1-^{14}C]stearoyl-CoA to [^{14}C]18:1 as described previously (9). Microsomal SCD activity was assayed spectrophotometrically as the stearoyl-CoA-stimulated reoxidation of NADH-reduced cytochrome b_5, as described previously (27, 28). The first order constant for the re-oxidation of NADH-reduced cytochrome b_5 was calculated; the rate constant for the re-oxidation of NADH-reduced cytochrome b_5 was measured in the presence (k) and in the absence (k^-) of stearoyl-CoA. The rate constant for SCD was given by $k^+ = k - k^-$ (28). Microsomal LPCAT was assayed with oleoyl-CoA as substrate by the optical assays that are based on the reaction of liberated coenzyme A with 5,5'-dithiobis(2-nitrobenzoic acid) as described previously (11).

Lipid analyses

Total lipid was extracted from the preparations of subcellular fractionations by the method of Bligh and Dyer (29). To obtain total fatty acids, an aliquot of the total lipid extract was taken to dryness under a stream of nitrogen, and 1 ml of 5% (w/v) KOH/90% (v/v) methanol was added to the residue obtained. The mixture was heated under nitrogen atmosphere at 80°C for 60min for saponification. Non-saponified lipids were removed by extraction with *n*-hexane. After the addition of 6M HCl, free fatty acids were extracted with *n*-hexane. The extract was taken to dryness, and the fatty acids obtained were methylated with boron trifluoride in methanol as described previously (30).

PC and phosphatidylethanolamine (PE) were isolated from total lipid extract by TLC on silica gel G plates (Merck, Darmstadt, Germany), which were developed with chloroform:methanol:acetic acid:water (50:37.5:3.5:2, v/v) according to Holub and Skeaff (31). After visualizing by spraying 0.001% (w/v) primuline in acetone-water (4:1, v/v), regions on each plate that corresponded to PC and PE were scraped off and transferred to tubes. After the addition of a known amount of pentadecanoic acid as an internal standard, PC and PE were extracted as described previously (28). To analyze fatty acids at the C-1 and C-2 positions of PC, the isolated PC was hydrolyzed using phospholipase A_2 according to Lands and Merkl (32). Free fatty acid and LPC were separated by TLC on silica gel G plates, which were developed with chloroform:methanol:water (65:25:4, v/v). After the addition of pentadecanoic acid as an internal standard, free fatty acids and LPC were extracted from silica gel as described previously (28). Methyl esters of fatty acids were prepared from each extract with boron trifluoride in methanol.

The mass and proportion of the fatty acid methyl esters were determined by GLC as described previously (28, 30).

For the analyses of the conversion of [^{14}C]18:0 to [^{14}C]18:1, saturated and unsaturated fatty acid methyl esters were separated by TLC on $AgNO_3$-impregnated silica gel G, which was developed with *n*-hexane:diethyl ether (9:1, v/v). The spots were detected under UV light after spraying with 0.05% (w/v) Rhodamine B in 95% ethanol. The area corresponding to authentic methyl stearate and methyl oleate were scraped off the plate, and methyl esters were extracted with toluene and then mixed with toluene-based scintillator. The radioactivity was measured using a liquid scintillation counter.

Statistical analyses

Statistically significant difference between two means was evaluated by Student's *t*-test or Welch's test after *F*-test.

Results

Effects of clofibric acid on total activities of stearoyl-CoA desaturation in whole endoplasmic reticulum in the liver

One μCi of [^{14}C]18:0 was administered intravenously to control and clofibric acid-treated rats, and its conversion *in vivo* to labeled 18:1 for 10 min in the liver was measured (Fig. 1A). The conversion rate was increased 3.5 times by the treatment of rats with clofibric acid. The stearoyl-CoA desaturation system is composed of NADH-cytochrome b_5 reductase, cytochrome b_5 and SCD, and the activity of SCD can be measured as the rate constant for stearoyl-CoA-stimulated re-oxidation of NADH-reduced cytochrome b_5 (27). The treatment of rats with clofibric acid elevated the rate constant by 5.9 fold (Fig. 1B). The specific activity of stearoyl-CoA desaturation, which was assayed as the conversion *in vitro* of [^{14}C]stearoyl-CoA to [^{14}C]18:1 by microsomes, was increased by the treatment of rats with clofibric acid by 4.5-fold (Fig. 1C). The yields (% of total activity in 10% homogenates) of marker enzyme (NADPH-cytochrome *c* reductase) activity in microsomal preparations from the livers of control and clofibric acid-treated rats were 67.5 ± 10.6 and 36.2 ± 5.5 %, respectively. When correcting on the basis of the yields of the marker enzyme for microsomes, the total activity of stearoyl-CoA desaturation in whole microsomes in the liver of clofibric acid-treated rats was 11.8-fold that of the control (Fig. 1D).

Effects of clofibric acid on subcellular distribution of radiolabeled fatty acids

As the first step of our *in vivo* study, the effects of clofibric acid on the distribution of radiolabeled fatty acids among subcellular fractions of the liver homogenates were estimated in terms of the time-course after 30 μCi of [^{14}C]18:0 was intravenously injected into rats (Fig. 2). Since VLDL appears in circulation approximately 40 min after hepatic up-take of precursors for lipid components of the lipoprotein from circulation, the time-course experiments were designed to be completed within 30 min. The radioactivities that appeared in lipids, which were extracted from homogenates, microsomal, peroxisomal and mitochondrial fractions, were measured. Moreover, the activities of the marker enzymes were determined to correct contaminations by other subcellular fractions. The whole microsomes, peroxisomes and mitochondria in the liver were calculated on the basis of the activities of the

corresponding marker enzymes that were recovered in these subcellular fractions.

From the estimation by the activities of marker enzymes that were recovered in the subcellular fractions, microsomal fractions of control rats contained the contaminants that were from mitochondria (constituting 1.42 ± 0.62 % of protein) and peroxisomes (constituting 2.53 ± 0.65% of protein); microsomal fractions of clofibric acid-treated rats contained the contaminants that were from mitochondria (constituting 1.22 ± 0.64 % of protein) and peroxisomes (constituting 7.53 ± 2.46 % of protein). Similarly, mitochondrial fractions of control rats contained the contaminants that were from microsomes (constituting 4.14 ± 0.92% of protein) and peroxisomes (constituting 2.11 ± 0.81% of protein); mitochondrial fractions of clofibric acid-treated rats contained the contaminants that were from microsomes (constituting 4.68 ± 0.99 % of protein) and peroxisomes (constituting 1.31 ± 0.45 % of protein). Peroxisomal fractions of control rats contained the contaminants that were from microsomes (constituting 9.80 ± 4.97 % of protein) and mitochondria (constituting 26.0 ± 8.2% of protein); peroxisomal fractions of clofibric acid-treated rats contained the contaminants that were from microsomes (constituting 25.6 ± 9.0 % of protein) and mitochondria (constituting 34.7 ± 16.0 % of protein). Owing to the low levels of contaminations of mitochondrial fractions by microsomes, the values of radioactivity in mitochondrial fractions were not corrected. On the other hand, the values of radioactivity in peroxisomal fractions were corrected by subtracting radioactivity corresponding to microsomal and mitochondrial contamination, owing to higher levels of such contamination.

Five minutes after the administration of [^{14}C]18:0, 42.8% of the dosed radioactivity appeared in total lipid of the whole liver of control rats (Fig. 2A); the radioactivities recovered in microsomes, peroxisomes and mitochondria were 17.5, 12.6 and 2.4%, respectively, of the injected [^{14}C]18:0. The radioactivities that exist in microsomes and mitochondria persisted for 30 min, whereas peroxisomal radioactivity rapidly declined to the level of mitochondria. The levels of radioactivities found in microsomes, peroxisomes and mitochondria in the liver of clofibric acid-treated rats 5 min after administering [^{14}C]18:0 were 29.1, 18.3 and 4.5%, respectively, of the injected radioactivity of [^{14}C]18:0 (Fig. 2B). Similarly to the case of control rats, the levels of radioactivities in microsomes and mitochondria were sustained for 30 min, whereas peroxisomal radioactivity quickly decreased to the level of mitochondria. These results indicate that endoplasmic reticulum is the organelle in which radioactive fatty acids were retained the most among the organelles examined.

Effects of clofibric acid on 18:1 formation in endoplasmic reticulum

Before starting the experiments, the radioactivities that exist as [^{14}C]18:1 in microsomal, peroxisomal and mitochondrial fractions from the liver of control rats 15 min after administering [^{14}C]18:0, were preliminarily determined; the radioactivities of [^{14}C]18:1 found in these three fractions were approximately 4150 dpm/mg of microsomal protein, 500 dpm/mg of peroxisomal protein and 200 dpm/mg of mitochondrial protein. Moreover, the radioactivities in fatty acids that present in peroxisomal and mitochondrial fractions were considerably lower than those in microsomal fractions as shown in Fig. 2. Accordingly, the present study has focused on understanding the formation of 18:1 in microsomes. The conversion *in vivo* of [^{14}C]18:0 to [^{14}C]18:1 in microsomes increased in a time-dependent manners (Fig. 3A). The radioactivities in 18:1 in microsomes in the liver 30 min after administering [^{14}C]18:0 were 8.8 and 31.9%, respectively, in control and clofibric acid-treated rats (Fig. 3A). The radioactivities of [^{14}C]18:1 that were formed from [^{14}C]18:0 *in vivo* and retained in whole microsomes in the liver increased with time and reached a plateau 10 min after administering [^{14}C]18:0 in both control and clofibric acid-treated rats (Fig. 3B). The radioactivity in 18:1 present in whole microsomes in the liver of clofibric acid-treated rats 30 min after administering [^{14}C]18:0 was 7.27 x 10^6 dpm, which corresponds to 10.9% of the administered [^{14}C]18:0, and the value was 5.8-fold that of control rats.

Effects of clofibric acid on distribution of [^{14}C]18:1 to PC and PE in endoplasmic reticulum

The effects of clofibric acid on the incorporation *in vivo* of [^{14}C]18:1 formed from [^{14}C]18:0 into PC in whole microsomes were examined (Fig. 4A). The incorporation of [^{14}C]18:1 into PC increased with time and reached a plateau 10 min after administering [^{14}C]18:0 in control rats, whereas it continued to increase for 30 min in clofibric acid-treated rats. The radioactivity retained in PC of clofibric acid-treated rats was 16.5-fold higher than that of the control. [^{14}C]18:1 formed *in vivo* was incorporated into PE in microsomes as well as PC, but the radioactivities in 18:1 that exist in PE of whole microsomes 30 min after dosing [^{14}C]18:0 were 81.6% of PC in control rats and 35.2% of PC in clofibric acid-treated rats (Fig. 4B).

The absolute quantities of 18:1 and 18:0 in PC and PE that exist in whole microsomes in the liver were determined (Fig. 4E and F). The absolute mass of 18:1 in PC in whole microsomes was 11.1 μmol in the liver of control rats and was increased 7-fold by the treatment with clofibric acid. The absolute mass of 18:0 in PC in whole microsomes was 22.7 μmol in the liver of control rats and was increased by the treatment with clofibric acid by only 1.8-fold. With regard to PE, the absolute masses of 18:1 and 18:0 in whole microsomes were

3.9 and 11s.1 μmol, respectively, in the liver of control rats and were elevated 6.4- and 5.2-fold, respectively, by the treatment with clofibric acid.

With regard to PC, the distributions of radiolabeled fatty acids between C-1 and C-2 positions 30 min after administering [^{14}C]18:0 were determined (Fig. 5A and B). Most of the radioactive fatty acids incorporated into the C-1 position were [^{14}C]18:0 (Fig. 5A). The radioactivities of 18:0 at the C-1 position of control and clofibric acid-treated rats were 833- and 90-fold, respectively, higher than those of [^{14}C]18:1 (Fig. 5A). Conversely, most of the radioactive fatty acids incorporated into the C-2 position were [^{14}C]18:1, and the radioactivity of 18:1 of the C-2 position of clofibric acid-treated rats was 17 -fold that of control rats. The radioactivities in 18:1 at the C-2 position of control and clofibric acid-treated rats were 17- and 35-fold, respectively, higher than those of 18:1 at the C-1 position. The radioactivities of 18:0 present at the C-1 position of control and clofibric acid-treated rats were 133- and 77-fold, times, respectively, higher than those at the C-2 position.

The distributions of 18:1 and 18:0 in terms of absolute mass between the C-1 and C-2 positions of PC in whole microsomes of control and clofibric acid-treated rats were shown in Fig. 5C and D. The masses of 18:1 exist at the C-1 and C-2 positions of control rats were 5.6 and 5.5 μmol, respectively, in the liver of control rats. The treatments with clofibric acid increased the mass of 18:1 at the C-2 position by 11.5-fold, but that at the C-1 position by only 2.7-fold.

The specific activity of LPCAT in microsomes was increased 4.0-fold by the treatment of rats with clofibric acid (Fig. 6A). The total activity of LPCAT in whole microsomes in the liver of clofibric acid-treated rats, when calculated on the basis of the yields of the marker enzymes for microsomes, was 10.5-fold that of control rats (Fig. 6B).

Effects of clofibric acid on the concentrations of free 18:0 in the liver

Since the concentrations of free (non-esterified) 18:0 in the liver might affect the elucidation of the results of the present study, we examined the effects of the treatment of rats with clofibric acid on the concentrations of free 18:0 in homogenates, microsomes, cytosol and FABP-rich fraction (Fig. 7). The treatments with clofibric acid elevated the concentrations of free 18:0 in homogenates by 1.7-fold, microsomes by1.6-fold, cytosol by 2.0-fold and FABP-rich fraction by 2.2-fold.

Discussion

In previous studies, SCD was shown to be localized in endoplasmic reticulum in the liver (18). On the basis of this information, most of the subsequent studies determined the specific activity of SCD using microsomal fractions as an enzyme source, but the microsomal fractions were conventionally prepared without determining its yield from liver homogenates. Accordingly, the information concerning the whole activity of SCD in the liver was not described in these studies. Moreover, to our knowledge, evidence directly showing that 18:1 is formed from 18:0 in endoplasmic reticulum *in vivo* has not been reported yet. It was difficult, therefore, to estimate how much18:1 is synthesized in whole endoplasmic reticulum in the liver when SCD is induced by clofibric acid. Our results shown in Fig. 1 largely confirm the earlier findings, but some new facts have come to light. Namely, in the present study, we assumed the total activity of stearoyl-CoA desaturation in whole endoplasmic reticulum in the livers of control and clofibric acid-treated rats on the basis of the yields of microsomal marker enzyme, the whole activity being increased 4.5-fold by the treatment of rats with clofibric acid. Moreover, it should be noted that, to our knowledge, the present investigation is the first study showing that the conversion *in vivo* of 18:0 to 18:1 is brought about mostly in endoplasmic reticulum in the liver and that the formation *in vivo* of 18:1 in endoplasmic reticulum is enhanced 5.8-fold by clofibric acid at 30 min after administering [^{14}C]18:0. These results are roughly consistent with the previous findings obtained from the *in vitro* studies that clofibric acid increased the specific activity of SCD in microsomes in the liver (9, 10, 16). It should be considered here that it is impossible to calculate the absolute amounts of 18:1 formed in endoplasmic reticulum, because [^{14}C]18:0, which was intravenously injected into rats, entered the liver from circulation and then was mixed with endogenous free 18:0 that exists in 18:0 pool(s) before being utilized by SCD in endoplasmic reticulum. The 18:0 that enters a hepatocyte may be carried to endoplasmic reticulum by FABP, which is a cytosolic protein that transports free fatty acids to various organelles in the cell and is induced by clofibric acid (33, 34). No information, however, is available with regard to the number and size of 18:0 pools in the liver. Accordingly, we did not assume the absolute amounts of 18:1 formed from 18:0 and presented the results as values relative to those of control rats. Moreover, in the present experiments *in vivo*, [^{14}C]18:0 was used as the substrate for desaturation. There is, however, palmitoyl-CoA chain elongase, which converts 16:0 to 18:0, in endoplasmic reticulum (35), and this enzyme is induced by the treatment of rats with clofibric acid as well as SCD (36, 37). It seems plausible, therefore, that the 18:0 formed from 16:0 by chain elongation constructs a pool distinct from the pool(s) that consists of the18:0 derived from the circulation. In the present study, we determined the absolute amounts of free

18:0 in homogenates, microsomes, cytosol and FABP-rich fraction; the amounts of free 18:0 in all the four preparations were significantly greater in clofibric acid-treated rats than in control rats by 1.6- to 2.2-fold. Accordingly, the radiospecific activity of the injected [^{14}C]18:0 may be reduced by the endogenous free 18:0 in the liver of clofibric acid-treated rats 1.6- to 2.2-fold more than that of control rats. As a result, the absolute amounts of 18:1 formed from 18:0 in whole endoplasmic reticulum in the liver of clofibric acid-treated rats are assumed to be 9.3- to 12.8-fold higher than that of control rats at 30 min after administering [^{14}C]18:0. The role of the pool(s) of free 18:0 remains controversial.

18:1 formed in endoplasmic reticulum is considered to be incorporated into esterfied lipids such as glycerolipids and cholesterol ester, which are utilized as components of biological membranes and of VLDL. Our previous study showed that the treatment of rats with clofibric acid increased absolute contents of PC and PE and mass proportions of 18:1 in PC and PE in the liver (12). Moreover, the mass proportion of 18:1 at the C-2 position, but not the C-1 position, was strikingly increased by clofibric acid (13). This differential increase in the mass proportion of 18:1 at the C-2 position of PC is considered, in part, to be due to selective induction of microsomal LPCAT, but not 2-acylglycerophospholcholine acyltransferase, by clofibric acid (11, 38). Consistent with these previous findings, the present study showed that [^{14}C]18:1 formed in endoplasmic reticulum was incorporated into PC and PE at a ratio similar to that of the mass proportion of 18:1 in these phospholipids. Almost all the [^{14}C]18:1 incorporated into PC was found at the C-2 position while [^{14}C]18:1 at the C-1 position exhibited very low radioactivity, whereas mass analyses revealed considerable amounts of 18:1 at the C-1 position of PC. These results strongly suggest the possibility that the pool of 18:1 available for C-1 position of PC is different from that for the C-2 position.

It is known that clofibrate (ethyl 2-(4-chlorophenoxy)-2-methylpropionic acid) stimulates biogenesis of not only peroxisomes, but also mitochondria and endoplasmic reticulum (39-41). It seems likely that the rapid biogenesis of these organelles requires the quick supply of considerable amounts of PC and PE containing 18:1 because 18:1 is a major unsaturated fatty acid that is synthesized in the liver by itself. However, the precise physiological significance of the marked increase by clofibric acid of PC and PE containing 18:1 in the endoplasmic reticulum remains to be elucidated.

In summary, the present study has shown that clofibric acid markedly increases the formation *in vivo* of 18:1 in endoplasmic reticulum and facilitates highly selective incorporation of the *in vivo* formed 18:1 into the C-2 position of PC in endoplasmic reticulum. These results are the direct evidence that bridges the gap between the previous findings of

clofibric acid-mediated inductions of SCD and LCPAT and the previous findings regarding the clofibric acid-mediated increase in absolute mass of PC containing 18:1 at the C-2 position in the liver.

Acknowledgments

This work was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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Figure legends

Fig. 1. Effects of clofibric acid on conversion *in vivo* of 18:0 to 18:1 in the liver and on microsomal activity of SCD in the liver. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. A, Conversion of 18:0 to 18:1 in the liver; rats were injected with 1 μCi of [^{14}C]18:0 into the tail vein, and the livers were isolated 10 min after the injection. B, Stearoyl-CoA desaturase (SCD). C, Stearoyl-CoA desaturation, which was assayed as the conversion of [^{14}C]stearoyl-CoA to [^{14}C]18:1 by microsomes *in vitro*. D, The values in C were corrected to total microsomes in the liver on the basis of the yield of microsomes from liver homogenates. C (open columns, □), control rats; T (closed columns, ■), clofibric acid-treated rats. Each value represents the mean ± SD for three or four rats. Significantly different from control: ***P*<0.01, ****P*<0.001.

Fig. 2. Effects of clofibric acid on subcellular distribution *in vivo* of radiolabeled fatty acids after administering [^{14}C]18:0. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were injected with 30 μCi of [^{14}C]18:0 into jugular vein. At 5 (two rats each), 10 (two rats each), 15 (two rats each) and 30 min (four rats each) after the injection, livers were isolated. Homogenates, mitochondrial, peroxisomal and microsomal fractions were isolated. The yields of the subcellular fractions were determined by assaying marker enzymes. Total lipid was extracted from the subcellular fractions and radioactivities in the total lipid were measured, and the values obtained were corrected to total value in the liver on the basis of the yields of the marker enzymes. A, control rats; B, clofibric acid-treated rats. Δ▲, homogenates; ♦◊, microsomes; ○●, peroxisomes; □■, mitochondria. Value represents mean and individual value (vertical bar) of two rats at the time points of 5, 10 and 15 min. At 30 min, value represents mean ± SD for four rats.

Fig. 3. Effects of clofibric acid on 18:1 formation *in vivo* in endoplasmic reticulum. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were injected with 30 μCi of [^{14}C]18:0 into jugular vein. At 5, 10, 15 and 30 min after the injection, microsomal fractions were isolated, and radioactivities in 18:1 and 18:0 in microsomal fractions were determined, and the values obtained were corrected to total value in the liver on the basis of the yields of the marker enzymes. A, % of radioactivity in 18:1 in total radiolabeled fatty acids. Δ, control rats; ▲, clofibric acid-treated rats. B, [^{14}C]18:1

formation in microsomes: ○, 18:1 in control rats; ●, 18:1 in clofibric acid-treated rats; □, 18:0 in control rats; ■, 18:1 in clofibric acid-treated rats. Value represents mean and individual value (vertical bar) of two rats at the time points of 5, 10 and 15 min. At 30 min, value represents mean ± SD for four rats. Significantly different from control: ***P<0.001.

Fig. 4. Effects of clofibric acid on distribution *in vivo* of [^{14}C]18:1 to PC and PE in endoplasmic reticulum. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Rats were injected with 30 μCi of [^{14}C]18:0 into jugular vein. At 5, 10, 15 and 30 min after the injection, microsomal fractions were isolated, and radioactivities in 18:1 and 18:0 in PC and PE of microsomal fractions were determined; masses of 18:1 and 18:0 in PC and PE in the microsomes in the liver of rats were determined. The values obtained were corrected to total value in the liver on the basis of the yields of the marker enzymes. A, Radioactive 18:1 in PC; B, Radioactive 18:1 in PE; C, Radioactive 18:0 in PC; D, Radioactive 18:0 in PE; E, Masses of 18:1 and 18:0 in PC; F, Masses of 18:1 and 18:0 in PE. In A and B: ○, 18:1 in control rats; ●, 18:1 in clofibric acid-treated rats. In C and D: □, 18:0 in control rats; ■, 18:1 in clofibric acid-treated rats. In E and F: open column (□), control rats; closed column (■), clofibric acid-treated rats. Value represents mean and individual value (vertical bar) of two rats at the time points of 5, 10 and 15 min. At 30 min, value represents mean ± SD for four rats. Significantly different from control: *P<0.05, ***P<0.001.

Fig. 5. Effects of clofibric acid on incorporation *in vivo* of [^{14}C]18:1 formed into C-1 and C-2 positions of PC in endoplasmic reticulum. PC, which was obtained from microsomal fractions of the liver of the rats 30 min after administering [^{14}C]18:0 as shown in Fig. 4, was hydrolyzed by phospholipase A_2, and radioactivities and masses of 18:1 and 18:0 at C-1 and C-2 positions were separately determined. The values obtained were corrected to total value in the liver on the basis of the yields of the marker enzymes. A and C, C-1 position; B and C, C-2 position. In A and B: C (open columns, □), control rats; T (closed columns, ■), clofibric acid-treated rats. In C and D: open columns (□), control rats; closed columns (■), clofibric acid-treated rats. Value represents mean ± SD for four rats. Significantly different from control:**P<0.01, ***P<0.001.

Fig. 6. Effects of clofibric acid on microsomal activity of LPCAT in the liver. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. A, Specific activity of LPCAT that was assayed *in vitro* utilizing microsomes. B, The values in A

were corrected to total microsomes in the liver on the basis of the yield of microsomes from liver homogenates. Each value represents the mean ± SD for four rats. Significantly different from control: ***P<0.001. C (open columns, □), control rats; T (closed columns, ■), clofibric acid-treated rats.

Fig. 7. Effects of clofibric acid on the concentration of free 18:0 in the liver. Rats were fed on either a control diet or a diet containing 0.5% (w/w) clofibric acid for 7 days. Absolute masses of 18:0 in homogenates, microsomal fraction, cytosol and FABP-rich fractions were determined. Each value represents the mean ± SD for four rats. Significantly different from control: *P<0.05, **P<0.01, **P<0.001. Open columns (□), control rats; closed columns (■), clofibric acid-treated rats.

Fig. 1

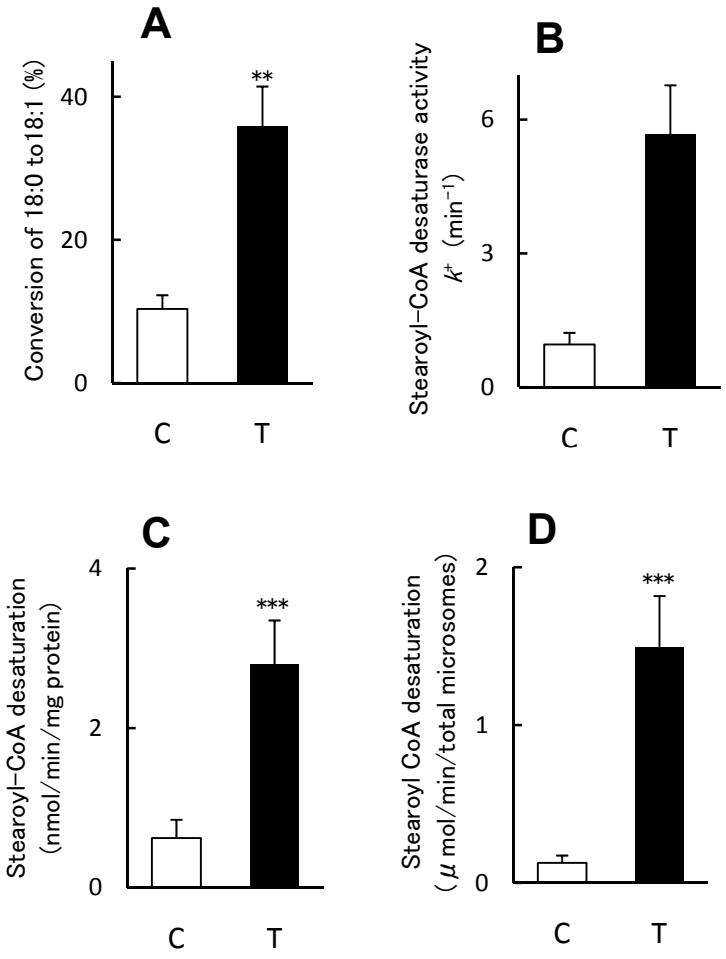

A
Conversion of 18:0 to18:1 (%)
40
20
0
**
C
T
B
Stearoyl-CoA desaturase activity
k+ (min-1)
6
3
0
C
T
C
Stearoyl-CoA desaturation
(nmol/min/mg protein)
4
2
0
***
C
T
D
Stearoyl CoA desaturation
(μ mol/min/total microsomes)
2
1
0
***
C
T

Fig. 2

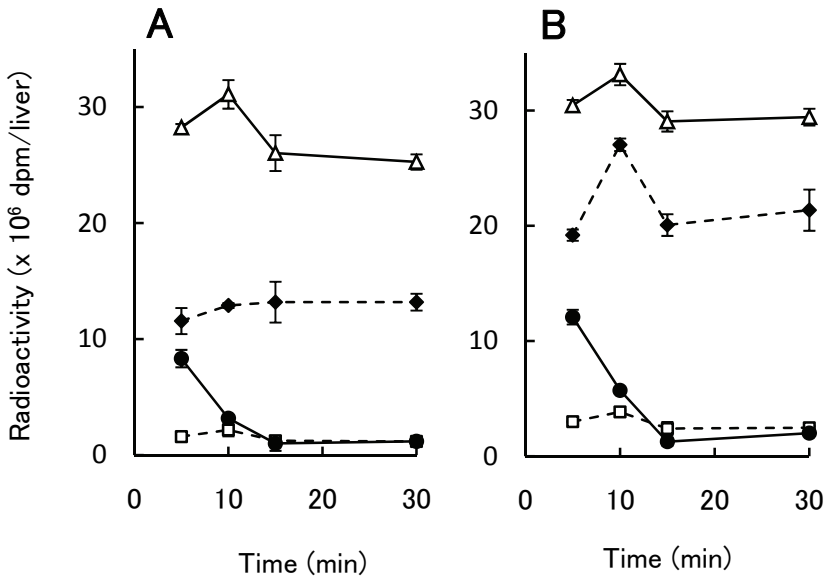


Fig. 3

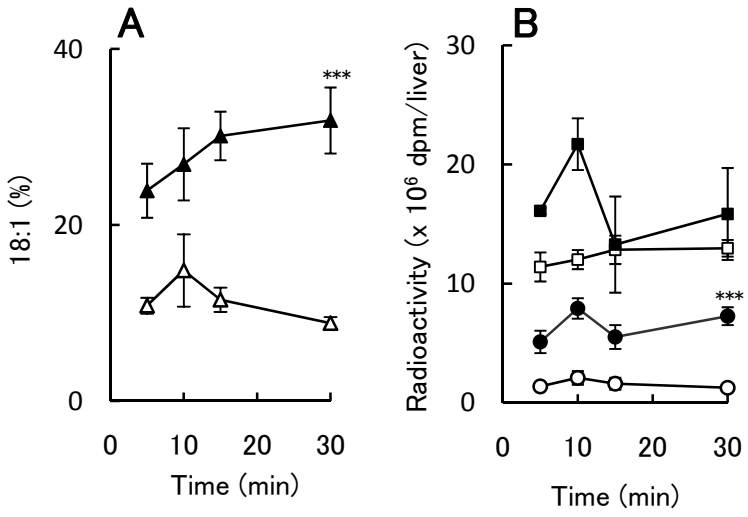

A
B
18:1 (%)
Radioactivity (x 10^6 dpm/liver)
Time (min)
Time (min)
***
***
0
20
40
0
10
20
30
0
10
20
30

Fig. 4

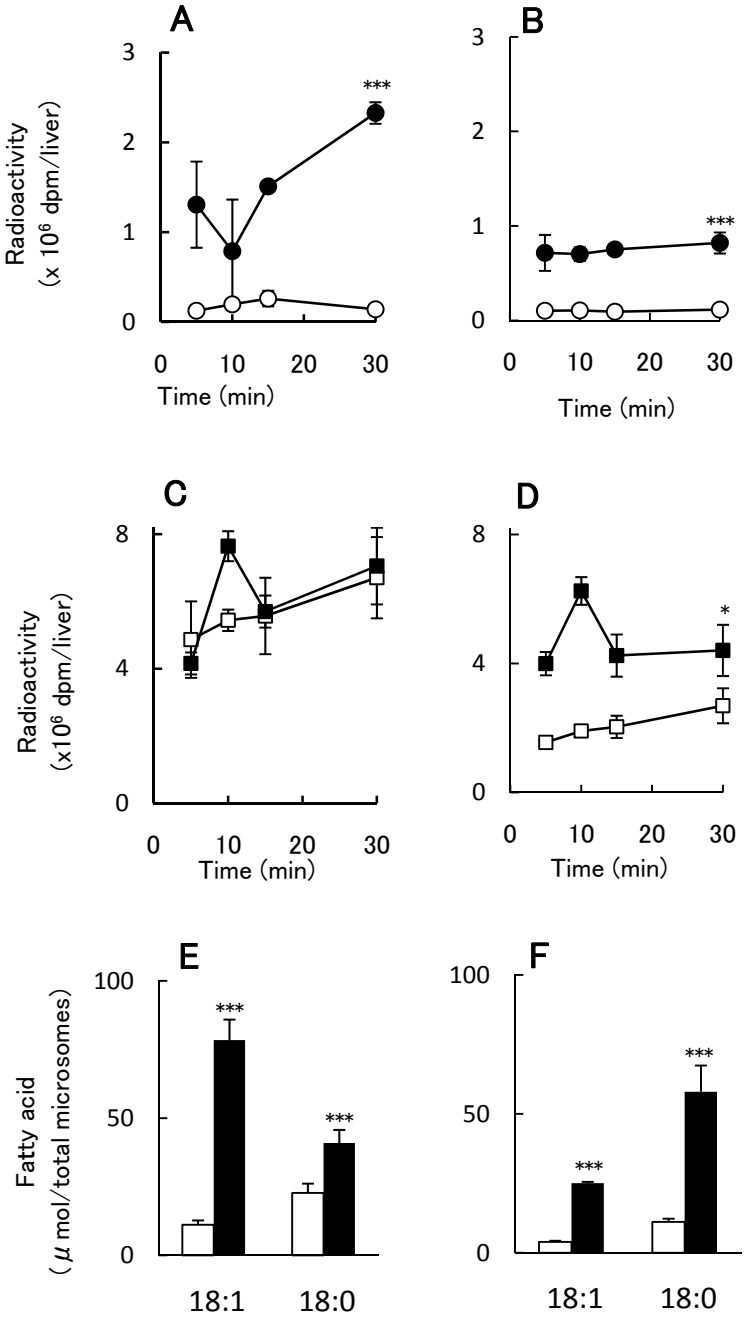

A
B
C
D
E
F
Radioactivity
(x 10⁶ dpm/liver)
Time (min)
Radioactivity
(x10⁶ dpm/liver)
Fatty acid
(μ mol/total microsomes)
***
*
18:1
18:0

Fig. 5

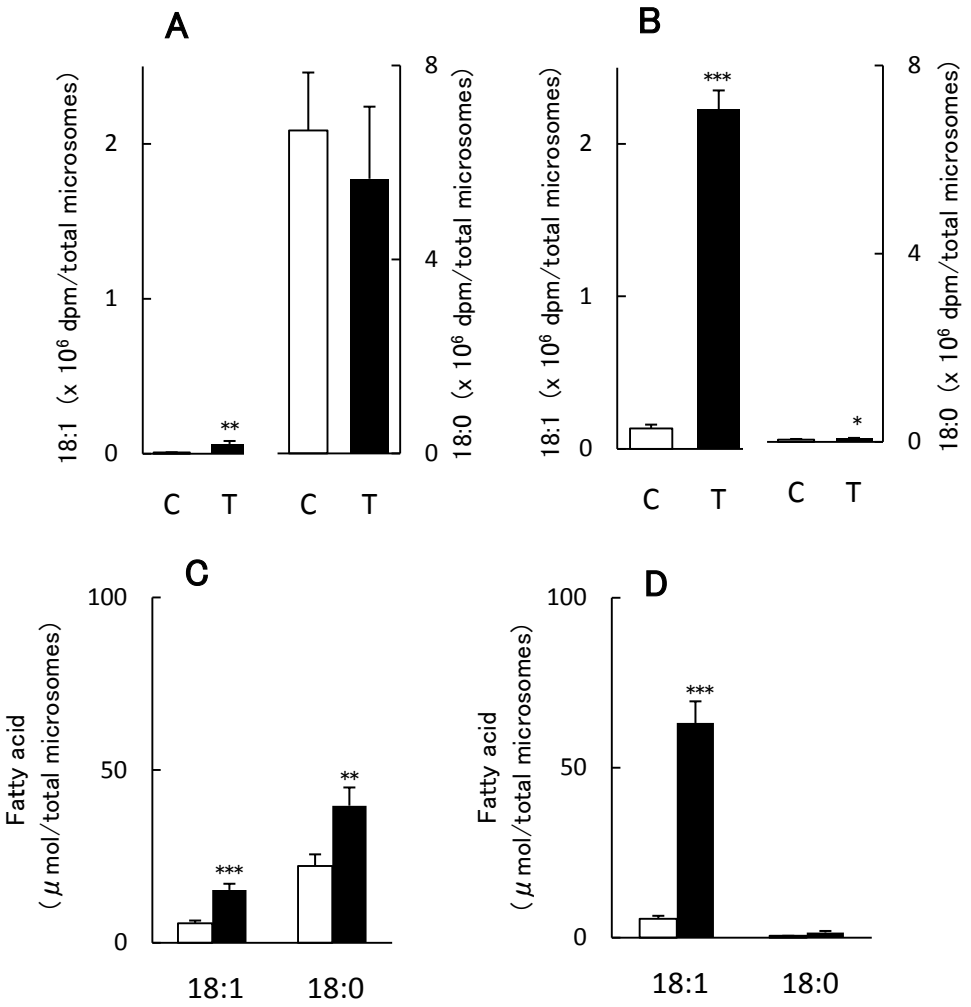

A
B
C
D
18:1 (x 10^6 dpm/total microsomes)
18:0 (x 10^6 dpm/total microsomes)
Fatty acid
(μmol/total microsomes)
C
T
18:1
18:0
0
1
2
4
8
50
100
*
**
***

Fig. 6

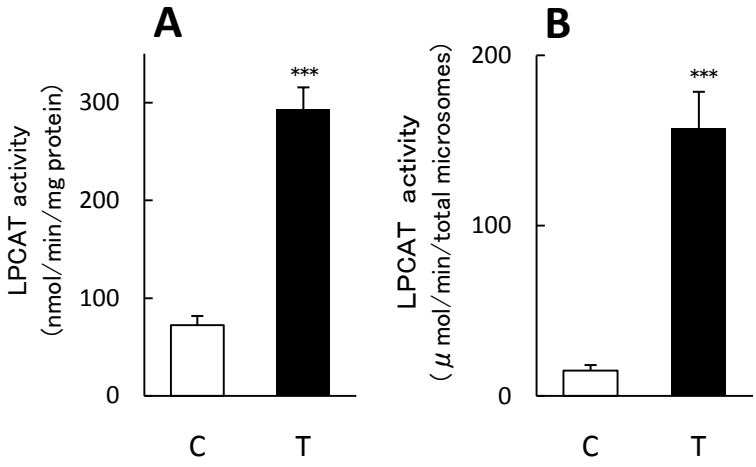
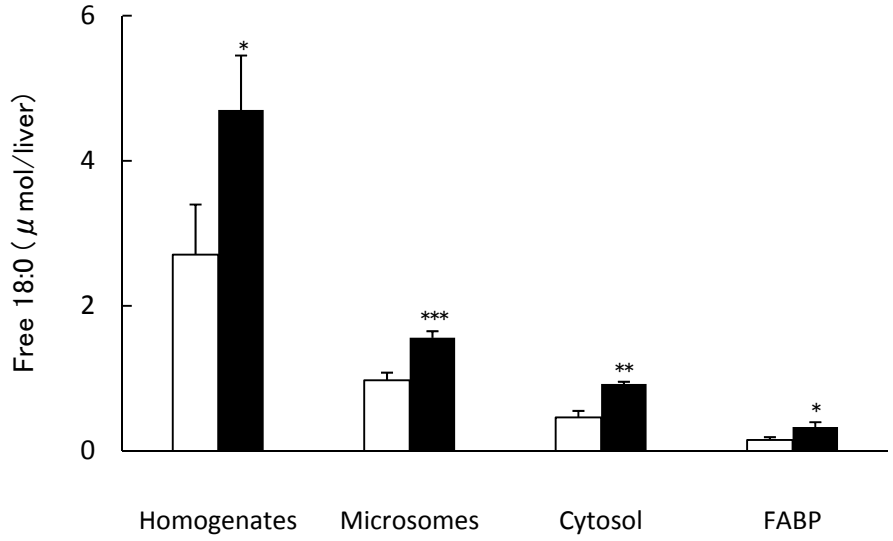

A
B
LPCAT activity
(nmol/min/mg protein)
LPCAT activity
(μmol/min/total microsomes)
300
200
100
0
200
100
0
***
***
C
T
C
T

Fig. 7


6
4
2
0
Free 18:0 (μmol/liver)
*
***
**
*
Homogenates
Microsomes
Cytosol
FABP