Inducing Effect of Clofibric Acid on Stearoyl-CoA Desaturase in Intestinal Mucosa of Rats

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Abstract Fibrates have been reported to elevate the hepatic proportion of oleic acid (18:1n-9) through inducing stearoyl-CoA desaturase (SCD). Despite abundant studies on the regulation of SCD in the liver, little is known about this issue in the small intestine. The present study aimed to investigate the effect of clofibric acid on the fatty acid profile, particularly MUFA, and the SCD expression in intestinal mucosa. Treatment of rats with a diet containing 0.5 % (w/w) clofibric acid for 7 days changed the MUFA profile of total lipids in intestinal mucosa; the proportion of 18:1n-9 was significantly increased, whereas those of palmitoleic (16:1n-7) and *cis*-vaccenic (18:1n-7) acids were not changed. Upon the treatment with clofibric acid, SCD was induced and the gene expression of SCD1, SCD2 and fatty acid elongase (Elovl) 6 was up-regulated, but that of Elovl5 was unaffected. Fat-free diet feeding for 28 days increased the proportions of 16:1n-7 and 18:1n-7, but did not effectively change that of 18:1n-9, in intestinal mucosa. Fat-free diet feeding up-regulated the gene expression of SCD1, but not that of SCD2, Elovl6 or Elovl5. These results indicate that intestinal mucosa significantly changes its MUFA profile in response to challenges by clofibric acid and a fat-free diet and suggest that up-regulation of the gene expression of SCD along with Elovl6 is indispensable to elevate the proportion of 18:1n-9 in intestinal mucosa.

Keywords Stearoyl-CoA desaturase・Clofibric acid・Intestinal mucosa・Rat

Abbreviations

Acox	Peroxisomal acyl-CoA oxidase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
ChREBP	Carbohydrate responsive element-binding protein
Clofibric acid	2-(4-Chlorophenoxy)-2-methylpropionic acid
EDTA	Ethylenediaminetetraacetic acid
Elovl	Fatty acid elongase
ER	Endoplasmic reticulum
Fads	Fatty acid desaturase
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PPARα	Peroxisome proliferator-activated receptor α
SCD	Stearoyl-CoA desaturase
SREBP-1c	Sterol regulatory element-binding protein-1c
TAG	Triacylglycerol

Introduction

Stearoyl- CoA desaturase (SCD) is located in the endoplasmic reticulum (ER) membrane, and is involved in the *de novo* synthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids [1-3]. The major products of SCD are palmitoleoyl-CoA and oleoyl-CoA, which are formed by the desaturation of palmitoyl-CoA and stearoyl-CoA, respectively. Highly homologous isoforms of SCD have been shown to exist. Four isoforms of SCD (SCD1 to SCD4) have been identified in mice [4, 5], whereas two isoforms (SCD1 and SCD2) have been characterized in rats [6]. With regard to the regulation of SCD expression, hepatic SCD has been the most extensively studied; the activity and expression of hepatic SCD are affected by hormonal, nutritional, pathophysiological or pharmacological alterations [7].

2-(4-Chlorophenoxy)-2-methylpropionic acid (clofibric acid) is one of the fibrates, which lower triacylglycerol (TAG) and raise high-density lipoprotein in circulation. These beneficial effects were confirmed by several clinical trials, such as the Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) study [8] and Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study [9]. The finding of peroxisome proliferator-activated receptor α (PPARα) has helped understand mechanism behind the pharmacological action of fibrates. Fibrates have been demonstrated to exert its antiatherogenic effects on macrophages, smooth muscle cells, endothelial cells and leukocytes in artery wall through the activation of PPARα [10]. Moreover, fibrates augment the level of

high-density lipoprotein through the control of expression of aoplipoproteins A-I, A-II and A-IV. Triacylglycerol (TAG)-lowering effect of fibrates is generally considered to be attributable to suppression of apolipoprotein C-III expression and induction of lipoprotein lipase expression through the activation of PPARα [11]. In addition to beneficial effects of fibrates on lipoprotein metabolism, however, Karahashi et al. showed that fibrates up-regulate adipose triglyceride lipase along with fatty acid transporters and β-oxidation enzymes in the liver [12], suggesting the possibility that fibrates alleviate hepatic steatosis and hypertriglyceridemia. On the one hand, previous studies have revealed that clofibric acid has different modes of effects on fatty acid metabolism in the liver [12-15]. Namely, the drug has abilities to induce many enzymes related to fatty acid modification (desaturation and chain elongation) [13-15]. The administration of clofibric acid to rats increased the mass proportion of octadecenoic acid in hepatic lipids [13]. 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 SCD in the liver [13, 14] and that the induction of SCD by clofibric acid is due to elevation of the expression of SCD1 through the activation of PPARα [15, 16] and to the suppression of the degradation of SCD [16]. Thus, accelerated formation of oleic acid (18:1n-9) by inducing SCD in the liver of rats is considered to be one of the pharmacological effects of clofibric acid [17]. In a recent study, fibrates were shown to elevate the gene expression of SCD1 and SCD2 in the liver of rats [18]. Since SCD controls

formation of MUFAs and there is increasing evidence suggesting that 18:1n-9, palmitoleic (16:1n-7) and *cis*-vaccenic (18:1n-7) acids have individually distinct physiological significance [19-21], it is important to understand the regulation of MUFA profile in extrahepatic organs. However, different from the detailed studies on the regulation of SCD in the liver, little information is available about the effects of fibrates on SCD expression in extrahepatic tissues.

The small intestine is an organ for the absorption of nutrients and plays a central role in whole body lipid homeostasis. Most studies on the fatty acid metabolism in this organ have been focused on the uptake of fatty acids into mucosal cells from the lumen, their esterification to complex lipids and the subsequent formation of chylomicrons. On the other hand, little is known about the modification of fatty acids within this organ. Since the small intestine is the organ that is most susceptible to orally administered drugs, SCD expression in this organ is expected to be strongly influenced by the oral administration of fibrates. To our knowledge, however, the regulation of SCD by fibrates in intestinal mucosa has not been reported. The aim of this study is thus to clarify the effects of clofibric acid on the gene expression and enzyme activity of SCD and the subsequent change of the MUFA profile in the intestinal mucosa of rats.

Materials and Methods

Materials

[1-^{14}C] Stearic acid (18:0) (55 Ci/mol) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, USA). Clofibric acid, stearic acid and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibody against SCD1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NADH and CoA were purchased from Oriental Yeast Co. (Tokyo, Japan). Glutathione, polyoxyethylene (20) sorbitan monolaurate (Tween-20), polyoxyethylene (20) sorbitan monostearate (Tween-60) and peroxidase-conjugated secondary antibody were from Wako Pure Chemical (Osaka, Japan). ECL Prime Western Blotting Detection System was purchased from GE Healthcare (Chalfont St. Giles, UK). All other chemicals used were of analytical grade.

Animals and Treatments

All animal procedures were approved by Josai University's Institutional Animal Care Committee in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan). Five-week-old male Wistar rats were obtained from SLC (Hamamatsu, Japan). Animals were fed a standard diet (CE-2; Clea Japan Inc., Tokyo, Japan)

ad libitum and allowed free access to water. The fatty acid composition (in mol %) of the standard diet was as follows: palmitic acid (16:0), 23.2 %; 16:1n-7, 2.0 %; 18:0, 2.4 %; 18:1n-9, 19.7 %; 18:1n-7, 2.2 %; linoleic acid (18:2n-6), 42.1 %; α-linolenic acid (18:3n-3), 3.6 %; arachidonic acid (20:4n-6), 0.2 %; 5,8,11,14,17-eicosapentaenoic acid (20:5n-3), 2.9 %; 7,10,13,16,19-docosapentaenoic acid (22:5n-3), 0.3 %; and 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3), 1.3 %. After acclimatization for 1 week, the rats were divided into three groups. Group1 was fed a standard diet (CE-2) for 28 days; Group2 was fed a diet (CE-2) admixed with 0.5 % (w/w) clofibric acid for 7 days before sacrifice; Group3 was fed a fat-free diet for 28 days. The fat-free diet (fat-deprived AIN-93G) was purchased from Oriental Yeast (Tokyo, Japan) and contained (by weight): 46.7486 % cornstarch, 20.0000 % casein, 13.2000 % dextrinized cornstarch, 10.0000 % sucrose, 5.0000 % cellulose, 3.5000 % mineral mix AIN 93G, 1.0000 % vitamin mix AIN 93G, 0.3000 % L-cysteine, 0.2500 % choline bitartrate and 0.0014 % *tetra*-butylhydroquinone. The treatments (dose and period) with clofibric acid are known to cause conditions that effectively induce PPARα-mediated enzymes and proteins such as SCD [18], peroxisomal acyl-CoA oxidase [22-24], 1-acylglycerophosphocholine acyltransferase [22-24] and fatty acid-binding protein [25] in the liver, kidney or intestinal mucosa without any signs of untoward response. At the age of 10 weeks, under diethyl ether anesthesia, rats were killed and the small intestine was excised. After washing with ice-cold saline, the mucosa was scraped off. One part of the

intestinal mucosa was frozen in liquid nitrogen and stored at -80 °C for the determination of mRNA and fatty acids. The other part of the mucosa was used for preparing microsomes. After centrifugation at 800 × g for 5 min at 0 °C, collected mucosa was homogenized with 6 volumes of 0.25 M sucrose/1 mM ethylenediaminetetraacetic acid (EDTA)/10 mM Tris-HCl (pH 7.4) in a Potter glass-Teflon homogenizer. The homogenates were centrifuged at 10,000 × g for 20 min and supernatant was centrifuged at 105,000 × g for 60 min. The obtained pellet was resuspended in 0.25 M sucrose/0.1 mM EDTA/10 mM Tris-HCl (pH 7.4) and recentrifuged under the same conditions. The resulting pellets were resuspended in a small volume of 0.25 M sucrose/0.1 mM EDTA/10 mM Tris-HCl (pH 7.4). The suspension was frozen in liquid nitrogen and stored at -80 °C. The concentrations of protein were determined by the method of Lowry et al. [26] with BSA as a standard.

Fatty Acid Analyses

Total lipid was extracted from intestinal mucosa by the method of Bligh and Dyer [27], after the addition of nonadecanoic acid as an internal standard. The extracted lipids were saponified with 10 % methanolic KOH for 60 min at 80 °C under a nitrogen atmosphere. After removal of non-saponified materials with *n*-hexane three times, samples were acidified with 6 M HCl and free fatty acids were extracted with *n*-hexane three times. The extracts were heated with

15 % boron trifluoride in methanol at 100 °C for 10 min under a nitrogen atmosphere. All solvents employed for lipid analyses contained 0.005 % (w/v) butylated hydroxytoluene. The composition of the fatty acid methyl esters was determined by gas-liquid chromatography (Shimadzu GC-2014; Shimadzu, Kyoto, Japan), equipped with a flame-ionization detector using a fused silica capillary column (SLB-IL100, 30 m × 0.32 mm internal diameter, film thickness 0.26 μm) (Sigma-Aldrich) with helium gas as a carrier gas. Initial column temperature was 120 °C for 5 min, which was thereafter increased by 3 °C per min to a final temperature of 230 °C. The injection port temperature was 240 °C and a flame-ionization detector was used at 240 °C.

mRNA Expression

Total RNA was prepared from the intestinal mucosa using QIAzol reagent and RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 500 ng of total RNA with avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan). Real-time polymerase chain reaction (PCR) experiments were carried out using SYBR premix ExTaq (Takara). The amplification and detection were performed with Step One plus real-time PCR system (Life Technologies Corp., Carlsbad, CA, USA). The thermal cycling program was as follows: 10 sec denaturation step at 95 °C, followed by 50 cycles of 5 sec denaturation at 95 °C, and 34

sec annealing and extension at 60 °C. After the reaction, dissociation curve analyses were carried out to confirm the amplification of a single PCR product. Changes in gene expression were calculated by using the comparative threshold cycle (Ct) method. Ct values were first normalized by subtracting the Ct value obtained from β-actin (control). The sequences of primers used in this study are listed in Table 1.

Immunoblot Analyses

Microsomes (50 μg of protein) were separated by sodium dodecylsulfate/polyacrylamide gel electrophoresis on a 10 % gel and then transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (BioRad). The membrane was blocked in Tris-buffered saline (pH 7.6) containing 0.1 % Tween-20, 5 % skim milk and 1 % BSA for 60 min, and then incubated with primary antibodies (1:1000) overnight. After washing with Tris-buffered saline (pH 7.6) containing 0.1 % Tween-20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 60 min in Tris-buffered saline (pH 7.6) containing 0.1 % Tween-20, 5 % skim milk and 1 % BSA. After washing three times in Tris-buffered saline (pH 7.6) containing 0.1 % Tween-20, proteins were visualized using the ECL Prime reagent (GE Healthcare) and detected in a luminoimage analyzer, LAS-1000 (GE Healthcare).

Stearoyl-CoA Desaturase Assay

The stearoyl-CoA desaturation activity was measured essentially according to the method of Catalá [28] with some modifications. [1-^{14}C]18:0 and 18:0 dissolved in toluene were mixed, and the solution was taken to dryness under a stream of nitrogen. To the residue was added 0.1 M NH_4OH, and the solution was vortex-mixed to obtain a uniform milky suspension, as described by Ullman and Sprecher [29]. The [1-^{14}C]stearic acid solution was then diluted to a concentration of 250 nmol (0.5 μCi)/0.1 mL by the addition of a solution of 1 % Tween-60 in water. The reaction mixture contained 100 nmol [1-^{14}C] 18:0 (0.2 μCi), 0.4 μmol NADH, 0.1 μmol CoA, 4 μmol ATP, 4 μmol $MgCl_2$, 1.5 μmol glutathione, 1 mg of microsomal protein and 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. Incubation was performed at 37 °C for 20 min, and the reaction was stopped by adding 2 mL of 10 % methanolic KOH, followed by saponification at 85 °C for 60 min. After acidification with 6 M HCl, fatty acids were extracted with *n*-hexane three times, and methyl esters were prepared by heating at 100 °C for 10 min with boron trifluoride in methanol. The methyl esters were extracted with *n*-hexane and subjected to argentation-thin-layer chromatography in order to separate the monounsaturated from the saturated fatty acids. Silica gel G plates (0.25-mm-thick) (Merck, Darmstadt, Germany) were immersed in 15 % (w/v) $AgNO_3$ in

acetonitrile and then air-dried. The plate was heated at 115 °C for 120 min for activation before use. Plates were developed in *n*-hexane/diethyl ether (9:1, by vol.). The spots were visualized under ultraviolet light after spraying with 0.05 % (w/v) Rhodamine B in 95 % ethanol. The areas corresponding to authentic methyl stearate and methyl oleate were scraped off the plates, and methyl esters were extracted with toluene and then mixed with toluene-based scintillator. The radioactivity was measured using a liquid scintillation counter (Aloka LS-5100; Hitachi Aloka Medical Ltd., Tokyo, Japan).

Statistical Analyses

Homogeneity of variance was established using one-way analysis of variance. When a difference was significant ($p < 0.05$), Scheffé's multiple range test was used as a post hoc test. The statistical significance of the difference between two means was evaluated by Student's *t*-test or Welch's test after the F-test.

Results

Effects of Clofibric Acid on MUFA Profile of Lipids of Intestinal Mucosa

Table 2 shows the effects of clofibric acid on the fatty acid profile of total lipids of intestinal mucosa. By the treatment of rats with clofibric acid for 7 days, the proportion of 18:1n-9 was significantly increased to 148 % of that of the control. In contrast, the proportions of 16:1n-7 and 18:1n-7 were not significantly changed. Figure 1a shows the difference in mass (nmol/mg protein) of particular fatty acids between the two groups of rats. Among the MUFAs, the content of 18:1n-9 was significantly increased by treatment with clofibric acid. There were no considerable differences in the masses of 16:1n-7 and 18:1n-7 between control and clofibric acid-treated rats. Ratio 18:0/16:0 tended to be increased (0.72 *vs*. 0.87) by the treatment with clofibric acid.

Effects of Clofibric Acid on SCD Expression in Intestinal Mucosa

The effects of clofibric acid on the protein expression and activity of SCD in microsomes of intestinal mucosa were estimated (Fig. 2). SCD protein was measured by western blot analysis; SCD activity was determined using [^{14}C] 18:0 as a substrate. The treatment of rats with clofibric acid increased the protein level of SCD1 in microsomes by 2.1-fold (Fig. 2a). SCD activity in microsomes of intestinal mucosa was also elevated 2.4-fold by the administration of clofibric acid (Fig. 2b). To gain insight into the molecular basis of the effect of clofibric acid on the MUFA profile, the mRNA levels of key enzymes involved in the

metabolism of fatty acid modification were measured (Fig. 3). With regard to the expression of genes encoding SCD, the mRNA levels of SCD1 and 2 in the intestinal mucosa of the rats that were treated with clofibric acid were 24-fold and 8.2-fold, respectively, higher than those of the control. In addition, the mRNA level of fatty acid elongase (Elovl) 6 was 5.7-fold up-regulated by the treatment with clofibric acid, whereas the expression of Elovl5 was unchanged.

Effects of Clofibric Acid on Polyunsaturated Fatty Acid (PUFA) Profile in Intestinal Mucosa

As shown in Table 2, treatment of rats with clofibric acid increased the proportions of 8,11, 14-eicosatrienoic acid (20:3n-6) (212 %) and 5, 8, 11-eicosatrienoic acid (20:3n-9) (222 %) in total lipids of intestinal mucosa. On the other hand, the proportion of 18:2n-6 was decreased to about 83 % of the control by the treatment with clofibric acid. Figure 1a shows the increase in mass of 20:3n-6 and the decreases in those of 18:2n-6 and 20:4n-6. As a result, ratio of 20:3n-6 to 20:4n-6 was increased 2.4-fold by the treatment with clofibric acid. The mRNA levels of Δ6 fatty acid desaturase (Fads2) and Δ5 fatty acid desaturase (Fads1) were significantly increased by 7.9-fold and 5.8-fold, respectively, by the treatments of rats with clofibric acid (Fig. 3).

Comparison of Effects of Clofibric Acid with those of Fat-free Diet on Fatty Acid Profile in Intestinal Mucosa

Table 2 shows the effects of clofibric acid and fat-free diet feeding on the profile of fatty acid of total lipids in intestinal mucosa. The treatment of rats with fat-free diet for 28 days significantly increased the proportions of 16:1n-7 (450 %) and 18:1n-7 (238 %). In particular, the increase in mass of 16:1n-7 was the most significant and was 84 nmol/mg protein by fat deprivation from the diet (Table 2; Fig. 1b). Although clofibric acid treatment increased the proportion of 18:1n-9, fat-free diet feeding did not significantly change the proportion of 18:1n-9. Fat-free diet feeding caused a significant decrease in the proportion of 18:2n-6 (54 % of the control), whereas the proportion of 20:3n-9 was significantly increased by 4.2-fold. The effects of fat-free diet on gene expression of the enzymes involved in fatty acid modification were determined (Fig. 4). The mRNA level of SCD1 was increased by 3.6-fold, while that of SCD2 tended to increase, but not significantly. The gene expression of Elovl5, Elovl6, Fads1 and Fads2 was not significantly altered by feeding on the fat-free diet.

Effects of Clofibric Acid and Fat-free Diet on Expression of Nuclear Transcription Factors

As shown in Table 3, the administration of clofibric acid to rats increased the mRNA level of

acyl-CoA oxidase 1 (Acox1) by 3.5-fold and that of PPARα by 3.3-fold. The treatment of rats with clofibric acid also elevated the level of mRNA encoding sterol regulatory element-binding protein-1c (SREBP-1c) by 3.8-fold, whereas the mRNA level of carbohydrate regulatory element-binding protein (ChREBP) was lower (61 %) than that of the control. In contrast, fat-free diet feeding did not significantly affect the mRNA levels of PPARα, SREBP-1c, ChREBP and Acox1.

Discussion

Previous studies demonstrated that the treatment of rodents with fibrates markedly induced SCD activity, thereby increasing 18:1n-9 content in the liver [13-17]. However, the regulation of SCD in extrahepatic tissues, in particular the small intestine, has not been well investigated, and little is known about whether fibrates affect the MUFA profile in intestinal mucosa. Treatments with fibrates are known to affect the expression of various genes in the small intestine, such as Niemann-Pick C1-like 1 (NPCL1L) [30], uncoupling protein 2 (UCP2) [31], Acox1 [24, 32] and adipocyte triglyceride lipase [33], which are related to lipid metabolism. Fibrates alter TAG metabolism in enterocytes by increasing fatty acid oxidation [34, 35]. Our previous studies demonstrated that clofibric acid treatment of rats induced 1-acylglycerophosphocholine acyltransferase and fatty acid-binding protein in intestinal

mucosa [24, 36]. These findings, taken together, strongly suggest that the small intestine is susceptible to fibrates with regard to MUFA metabolism. As anticipated, the present results demonstrated that the treatment of rats with clofibric acid caused an increase in the proportion of 18:1n-9 in intestinal mucosa of rats. It seemed likely that the augmentation of the proportion and mass of 18:1n-9 was attributable to the induction of SCD1 and SCD2 along with Elovl6. On the other hand, the proportions of 16:1n-7 and 18:1n-7 were largely unchanged, in contrast to the pronounced rise in SCD expression. It is unclear why the proportions of 16:1n-7 and 18:1n-7 in the lipids were not significantly changed, in spite of the elevation of SCD activity in intestinal mucosa, by clofibric acid treatment. It is interesting to speculate that Elovl6, which was up-regulated by clofibric acid, actively elongated 16:0 to 18:0, and the increasingly formed 18:0 seemed to be desaturated to 18:1n-9 by the induced SCD. As a result, the pathway of 16:0 → 16:1n-7 → 18:1n-7 seemed to be overwhelmed by that of 16:0 → 18:0 → 18:1n-9 (Fig. 5). The conclusion that Elovl6 along with SCD1 plays a crucial role in the regulation of 18:1n-9 formation is the same as that of Karahashi et al. [37] and Marks et al. [38], who showed the role of Elovl6 in the liver of Goto-Kakizaki rats and female rats, respectively.

Previous studies demonstrated that fat-free diet feeding resulted in the up-regulation of SCD1 mRNA [39] and in the increase in the proportions of 16:1n-7 and 18:1n-9 in the liver [40]. Therefore, we attempted to compare the effect of clofibric acid treatment with that of

fat-free diet feeding on MUFA synthesis in intestinal mucosa. In contrast to the case of clofibric acid treatment, fat deprivation from the diet caused striking elevation of the proportions of 16:1n-7 and 18:1n-7, but not 18:1n-9, in the lipid of intestinal mucosa by the significant increase in expression of the SCD1 gene. It should be noted that the expression of Elovl6 was unchanged by feeding on a fat-free diet. It seems likely that the increased proportions of 16:1n-7 and 18:1n-7 were due to the predominant conversion of 16:0 to 16:1n-7, which was efficiently elongated to 18:1n-7 in the intestinal mucosa of rats fed a fat-free diet (Fig. 5).

The inducing effects of fibrates on SCD and Elovl are considered to be attributable to their actions as synthetic ligands for PPARα [41]; most of these conclusions are derived from experiments using knockout mice or cultured cells. PPARs are nuclear hormone receptors that function as transcriptional regulators of key metabolic pathways [42]. Our previous studies showed that treatment with clofibric acid elevated mRNA levels of SCD1, SCD2 and Elovl6 in the liver of rats [16, 18]. The present study evidently showed that the treatment of rats with clofibric acid augmented the expression of the PPARα gene and of genes encoding SCD1, SCD2, Elovl6 and Acox1 in intestinal mucosa. Acox1 is a typical gene of which the expression is up-regulated by peroxisome proliferators and this induction has been demonstrated to be mediated through the activation of PPARα [43]. Accordingly, the present results suggest that the increase in Acox1 gene expression by clofibric acid indicates that

PPARα was activated by the drug in intestinal mucosa. Thus, the clofibric acid-induced gene expression of SCD1, SCD2 and Elovl6 in the intestinal mucosa is likely to be mediated by PPARα, as reported in the liver. In addition to PPARα, the expression of SCD and Eovl6 is known to be regulated by SREBP-1c and ChREBP [44]. The present study showed that the treatment of rats with clofibric acid significantly increased expression of the SREBP-1c gene in intestinal mucosa. SREBP-1c is thought to be another regulator of SCD [45, 46], and the presence of PPARα has been shown to be required for proper functioning of SREBP-1c [47]. Overexpression of ChREBP in the liver leads to the induction of SCD1 and specific increases in the proportions of 16:1n-7 and 18:1n-9 [48]; in the present study, however, the treatment with clofibric acid significantly decreased the gene expression of ChREBP in intestinal mucosa. Consequently, it is the most likely that PPARα plays a central role in the regulation of the MUFA profile under the influence of clofibric acid in intestinal mucosa as well as hepatocytes. Different from the case of clofibric acid, fat-free diet feeding did not affect the expression of genes for PPARα, SREBP-1c and ChREBP in intestinal mucosa. However, the detailed mechanism for inductions of SCD1 by fat-free diet remains unclear.

The present study shows that the treatment of rats with clofibric acid considerably changed the PUFA profile in lipids of intestinal mucosa. The proportions of 20:3n-6, but not 20:4n-6, was significantly increased, while that of 18:2n-6 was evidently decreased, by the treatment. The process of 20:4n-6 formation requires Δ6 desaturation of 18:2n-6 to 18:3n-6,

chain elongation of 18:3n-6 to 20:3n-6 and Δ5 desaturation of 20:3n-6 to 20:4n-6. Metabolic alterations in this process have been extensively studied in the liver [4, 5], whereas less information is available on PUFA biosynthesis in intestinal mucosa. An early study by Garg et al. demonstrated that the rat small intestine also possesses Δ6-desaturase activity [49]. Moreover, Christiansen et al. showed that the activity of chain elongation of 18:3n-6 to 20:3n-6 in the small intestine is comparable to that in the liver [50]. The present study revealed that the treatment of rats with clofibric acid induced the expression of Fads2 (Δ6 desaturase) and Fads1 (Δ5 desaturase) in intestinal mucosa. Similarly, the rate of 20:4n-6 formation from 18:2n-6 has been reported to be increased in the liver of rats and mice treated with fibrates [51, 52]. Since a previous study demonstrated dual regulation of Fads1 and Fads2 gene expression by PPARα and SREBP-1c in the liver of mice [53], a similar mechanism is likely to operate for induction of the expression of these genes in intestinal mucosa. The up-regulation of Fads2 by clofibric acid may elevate the proportion of 20:3n-6 in lipids of intestinal mucosa (Fig. 6). Although the cause of unchanged proportion of 20:4n-6, despite the increase in mRNA level of Fads1, by clofibric acid still remains to be elucidated, ratio 20:3n-6/20:4n-6 was significantly altered. Eicosanoids are proposed as mediators involved in the regulation of epithelial structure and function [54]. Since 20:3n-6 and 20:4n-6 are precursors of eicosanoids, it is likely that alteration of the PUFA profile by clofibric acid in intestinal mucosa affects pathophysiological state of the small intestine. On the other hand,

fat-free diet feeding did not alter the expression of Fads1 and Fads2. Consequently, the levels of both 20:3n-6 and 20:4n-6 were not changed in intestinal mucosa (Fig. 6). Fat-free diet feeding caused an evident increase in 20:3n-9 content in intestinal mucosa. An increase in the mass of 20:3n-9 is generally observed in the liver of rodents when formation of 18:1n-9 is markedly increased, which is a condition found upon starved refeeding or the administration of PPARα agonist [55, 56]. The detailed mechanism involved in the elevation of 20:3n-9 proportion in intestinal mucosa has not been clarified.

A number of studies have demonstrated that the intestinal mucosa produces mucus phosphatidylcholine, lipid components of chylomicrons and phospholipids of cellular/organelle membranes utilizing fatty acids that originate from dietary fat. On the other hand, little is known about how extent the fatty acids that are produced endogenously by enterocytes themselves contribute to these processes. Of the fatty acids that could be provided by enterocytes themselves, 18:1n-9, 16:1n-7 and 18:1n-7 are of particularly interest, because recent evidence suggests that these MUFAs have individually distinct physiological significance. Namely, different from 18:1n-9, 16:1n-7 has been identified as an adipose tissue-derived lipid hormone capable of enhancing muscle insulin sensitivity [19], and 18:1n-7 is suggested to be linked to chronic kidney disease [21] and to gluconeogenesis [20]. These MUFAs seem to be delivered from the small intestine to various organs through circulation as the components of chylomicrons. Moreover, a saturation grade of fatty acid

residues in the mucus phosphatidylcholine was increased in patients with ulcerative colitis [57]. Interestingly, phosphatidylcholine, indispensable to the maintenance of an intact barrier function, in mucus in the intestine including colon originate from secretion by ileal and jejunal enterocytes [58]. These facts, taken together, imply the importance of modification (desaturation and elongation) of fatty acids in enterocytes of the ileum and jejunum, whereas little is known about the modification of fatty acids in the small intestine. The present study clearly showed that fatty acid modification system in the small intestine sensitively responds to clofibric acid and dietary alterations, so that MUFA profile significantly changed. It seems likely that the altered MUFA profile is reflected to fatty acid profile of mucus phosphatidylcholine and to that of other organs through the secretion of chylomicrons. Therefore, it is interesting to suggest that the small intestine could be one of the targets to manipulate fatty acid profile of mucus phosphatidylcholine in the intestine and to regulate MUFA profile on whole body level.

Finally, treatments of rats with clofibric acid elevated the proportion of 18:1n-9, but not that of either 16:1n-7 or 18:1n-7, and fat-free diet feeding increased the proportions of 16:1n-7 and 18:1n-7, but not that of 18:1n-9. SCD1 is markedly induced by clofibric acid or a fat-free diet in intestinal mucosa; moreover, clofibric acid, but not a fat-free diet, up-regulated the expression of Elovl6. Therefore, it seems likely that elevation of the gene expression of SCD1 along with Elovl6 is indispensable for the increases in mass and proportion of 18:1n-9.

This conclusion is the same as that from previous studies on 18:1n-9 formation in the liver of Goto-Kakizaki rats [37] and female rats [38].

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Figure Legends

Fig. 1 Difference in mass (nmol/mg protein) of fatty acids in total lipids of intestinal mucosa **(a)** between control rats and rats treated with clofibric acid, and **(b)** between control rats and rats fed on a fat-free diet. Rats were fed on a standard diet, a diet containing 0.5 % (w/w) clofibric acid for 7 days or a fat-free diet for 28 days. With regard to each fatty acid, differences in mass (nmol/mg protein) between the means of control rats and those of rats treated with clofibric acid or rats fed on a fat-free diet were calculated from the data in Table 2. Values are mean ± SD for 4 rats. Cont, control rats; Clo, clofibric acid-treated rats; Fat-free, rats fed a fat-free diet.

Fig. 2 Effects of clofibric acid on protein levels (a) and enzyme activities (b) of SCD in microsomes of intestinal mucosa of rats. Rats were fed on a standard diet or a diet containing 0.5 % (w/w) clofibric acid for 7 days. **(a)** The proteins (50 μg) of intestinal microsomes were separated by electrophoresis. Visible bands are SCD1, as indicated. Immunoblotting was carried out with anti-SCD1 antibody. **(b)** SCD activity in microsomes of intestinal mucosa was measured using [^{14}C] 18:0 as a substrate. Values are mean ± SD for 4 rats. Cont, control rats; Clo, clofibric acid-treated rats. Significantly different from the control, $^{**}p < 0.01$.

Fig. 3 Effects of clofibric acid on levels of mRNA encoding enzymes involved in fatty acid

modification in intestinal mucosa of rats. Rats were fed on a standard diet or a diet containing 0.5 % (w/w) clofibric acid for 7 days. The gene expression levels were analyzed by real-time PCR. Values are mean ± SD for 6-12 rats. Cont, control rats; Clo, clofibric acid-treated rats. Significantly different from the control, $*p < 0.05$, $**p < 0.01$.

Fig. 4 Effect of fat-free diet feeding on levels of mRNA encoding enzymes involved in fatty acid modification in intestinal mucosa of rats. Rats were fed on a standard diet or a fat-free diet for 28 days. The gene expression levels were analyzed by real-time PCR. Values are mean ± SD for 4 rats. Cont, control rats; FF, fat-free diet-fed rats. Significantly different from the control, $*p < 0.05$.

Fig. 5 Diagram of enzymes regulating the synthesis of 16:1n-7, 18:1n-7 and 18:1n-9

Fig. 6 Diagram of enzymes regulating the synthesis of 20:3n-6 and 20:4n-6

Table 1 Sequences of primers used for real-time PCR

	Primer sequence (5'-3')	Accession No.
SCD1	F : TCACCTTGAGAGAAGAATTAGCA	J02585
	R : TTCCCATTCCCTTCACTCTGA	
SCD2	F : TGCACCCCCAGACACTTGTAA	AB032243
	R : GGATGCATGGAAACGCCATA	
Fads1	F : TACAGGCAACCTGCAACGTTC	NM_053445
	R : GGTGCCACCTTGTGGTAGTTGT	
Fads2	F : GCCACTTAAAGGGTGCCTCC	BC081776
	R : TGCAGGCTCTTTATGTCGGG	
Elovl5	F : ACCACCATGCCACTATGCTCA	AB071985
	R : GGACGTGGATGAAGCTGTTG	
Elovl6	F : AGAACACGTAGCGACTCCGAA	AB071986
	R : CAAACGCGTAAGCCCAGAAT	
Acox1	F : TTCGTGCAGCCAGATTGGTAG	NM_017340
	R : CGGCTTTGTCTTGAATCTTGG	
PPARα	F : AATGCCCTCGAACTGGATGAC	NM_013196
	R : CACAATCCCCTCCTGCAACTT	
SREBP-1c	F : GGAGCCATGGATTGCACATT	AF286469
	R : AGGAAGGCTTCCAGAGAGGA	
ChREBP	F : AATAGAGGAGCTCAATGCT	AB074517
	R : CCCAGAACTTCCAGTTGTGC	
β-actin	F : TGCAGAAGGAGATTACTGCC	V01217
	R : CGCAGCTCAGTAACAGTCC	

Table 2 Effects of clofibric acid and fat free diet on fatty acid profile (mol%) of lipid of intestinal mucosa

Fatty acids	Control			Clofibric acid			Fat-free		
16:0	24.41	±	1.65	22.17	±	1.98	24.49	±	1.43
16:1n-7	1.43	±	0.19^{a}	1.77	±	0.35^{a}	6.43	±	0.56^{b}
18:0	17.55	±	1.81	19.22	±	0.55	18.54	±	0.86
18:1n-9	11.80	±	1.66^{a}	17.51	±	2.12^{b}	15.52	±	1.59^{ab}
18:1n-7	2.85	±	0.08^{a}	2.60	±	0.24^{a}	6.79	±	0.36^{b}
18:2n-6	25.77	±	1.08^{a}	21.51	±	1.49^{b}	13.81	±	1.18^{c}
18:3n-3	0.83	±	0.11^{a}	0.34	±	0.16^{b}	0.47	±	0.04^{b}
20:3n-9	0.37	±	0.05^{a}	0.82	±	0.11^{b}	1.54	±	0.18^{c}
20:3n-6	0.96	±	0.22^{a}	2.04	±	0.09^{b}	0.74	±	0.10^{a}
20:4n-6	10.66	±	1.26	9.35	±	1.26	9.65	±	2.90
20:5n-3	1.36	±	0.22^{a}	1.51	±	0.17^{a}	0.74	±	0.18^{b}
22:5n-3	0.92	±	0.11^{a}	0.68	±	0.18^{ab}	0.55	±	0.22^{b}
22:6n-3	1.11	±	0.17^{a}	0.49	±	0.06^{b}	0.76	±	0.37^{ab}
Total (nmol/mg protein)	1689.1	±	176.4	1490.3	±	103.6	1887.4	±	441.5

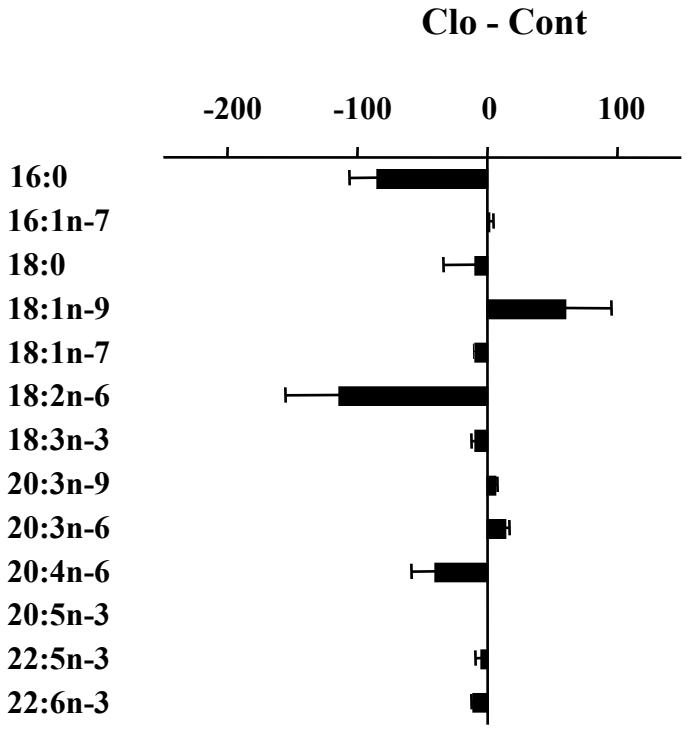
Rats were fed on a control diet, a diet containing 0.5 % (w/w) clofibric acid for 7 days or a fat-free diet for 28 days. Values represent mean ± SD for four rats. Differences in horizontal means without a common superscript (a, b, c) are significant ($p < 0.05$). In the absence of superscripts, differences in means are not significant ($p > 0.05$).

Table 3 Effects of clofibric acid and fat-free diet on gene expression of nuclear transcription factors in intestine of rats

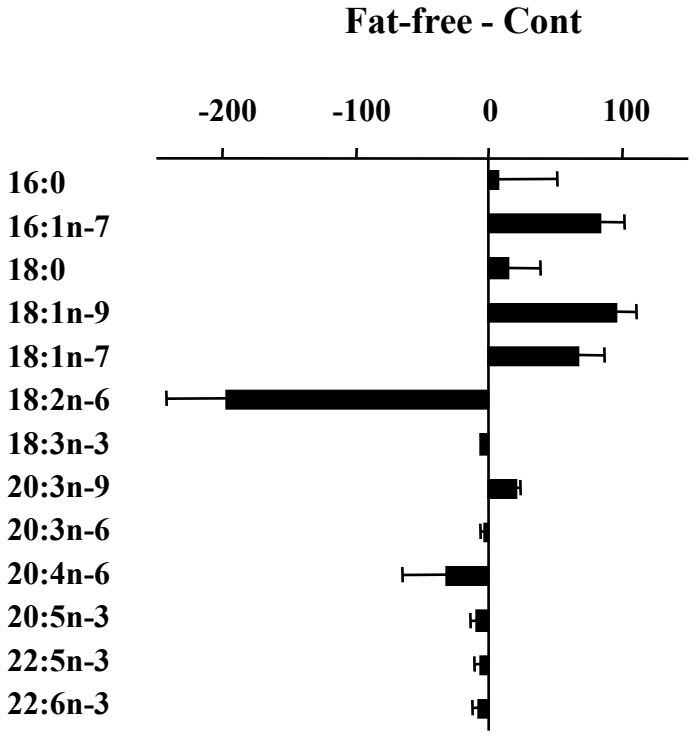
Genes	Control	Clofibric acid	Fat-free
Acox1	1.00 ± 0.21[a]	3.53 ± 0.71[b]	0.79 ± 0.16[a]
PPARα	1.00 ± 0.20[a]	3.25 ± 0.56[b]	0.55 ± 0.22[a]
SREBP-1c	1.00 ± 0.39[a]	3.81 ± 1.17[b]	0.61 ± 0.16[a]
ChREBP	1.00 ± 0.40[a]	0.61 ± 0.24[ab]	1.40 ± 0.67[ac]

Rats were fed on a control diet or a diet containing 0.5 % (w/w) clofibric acid for 7 days or a fat-free diet for 28 days. Values represent mean ± SD for 4-12 rats. Differences in horizontal means without a common superscript (a, b, c) are significant ($p < 0.05$). In the absence of superscripts, differences in means are not significant ($p > 0.05$).

(a)


Clo - Cont
-200
-100
0
100
16:0
16:1n-7
18:0
18:1n-9
18:1n-7
18:2n-6
18:3n-3
20:3n-9
20:3n-6
20:4n-6
20:5n-3
22:5n-3
22:6n-3

(b)


Fat-free - Cont
-200
-100
0
100
16:0
16:1n-7
18:0
18:1n-9
18:1n-7
18:2n-6
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20:4n-6
20:5n-3
22:5n-3
22:6n-3

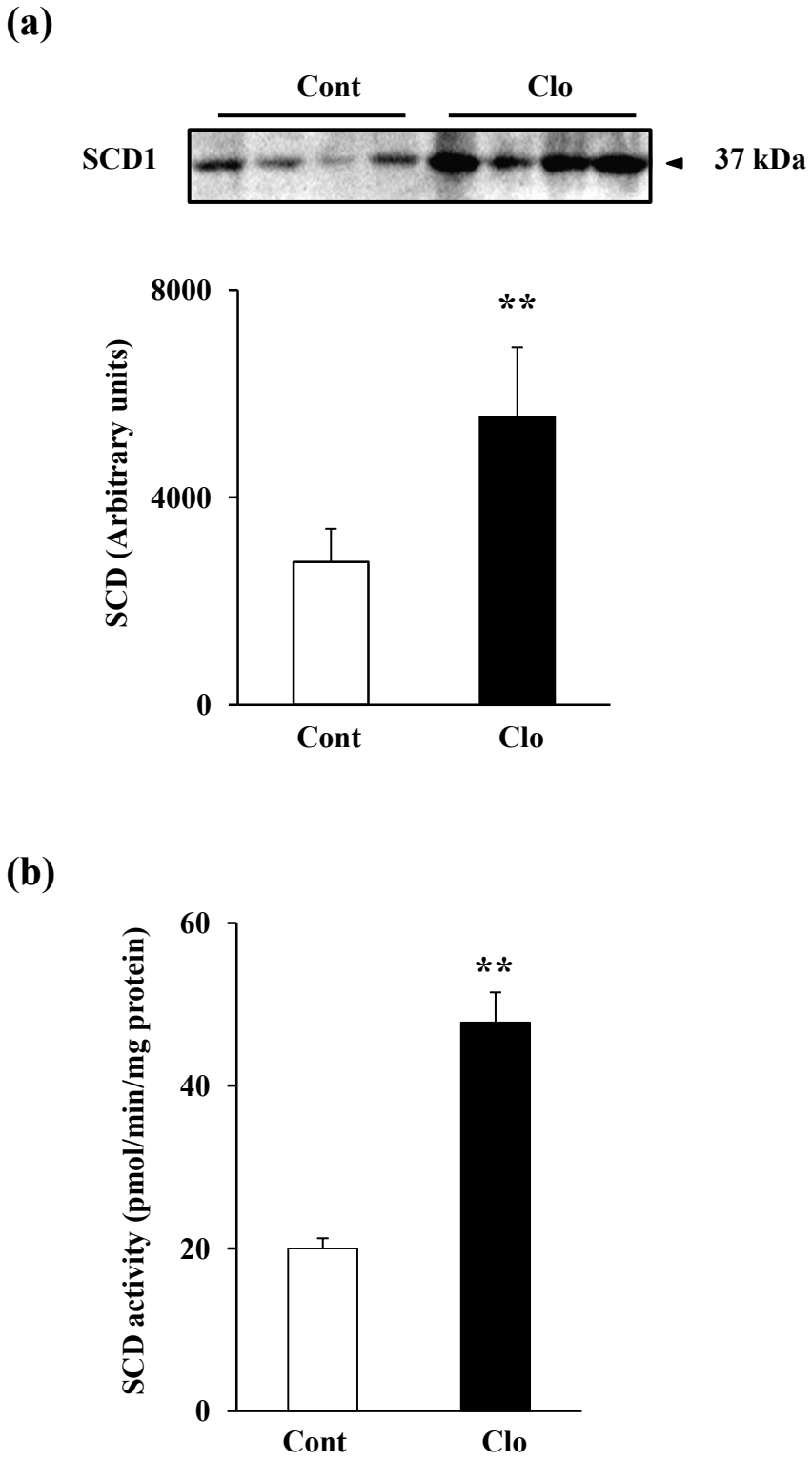
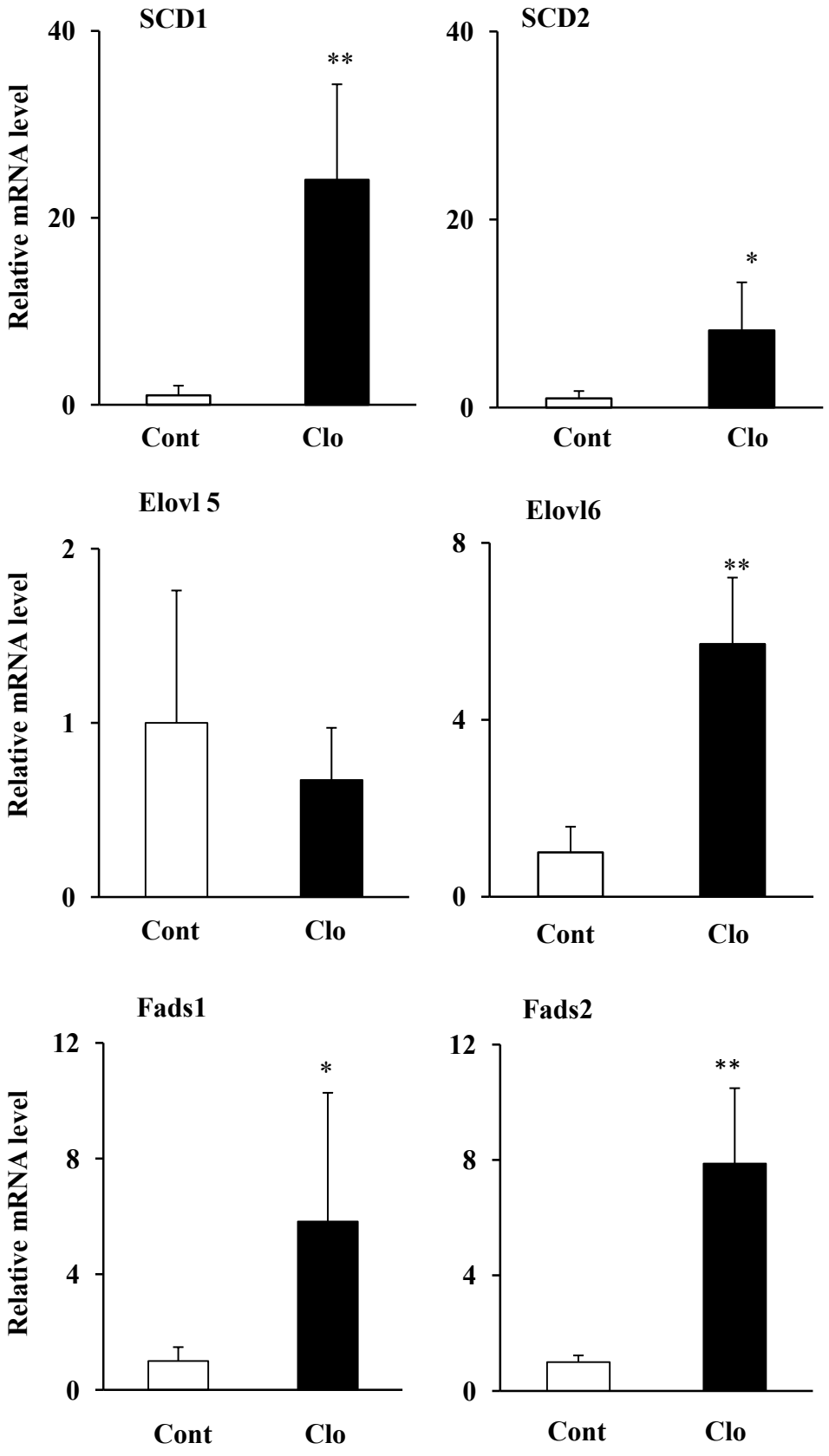

(a)
Cont
Clo
SCD1
37 kDa
8000
4000
0
SCD (Arbitrary units)
**
Cont
Clo
(b)
60
40
20
0
SCD activity (pmol/min/mg protein)
**
Cont
Clo

Fig. 3


SCD1
SCD2
Elovl 5
Elovl6
Fads1
Fads2
Relative mRNA level
40
20
0
12
8
4
2
1
**
*
Cont
Clo

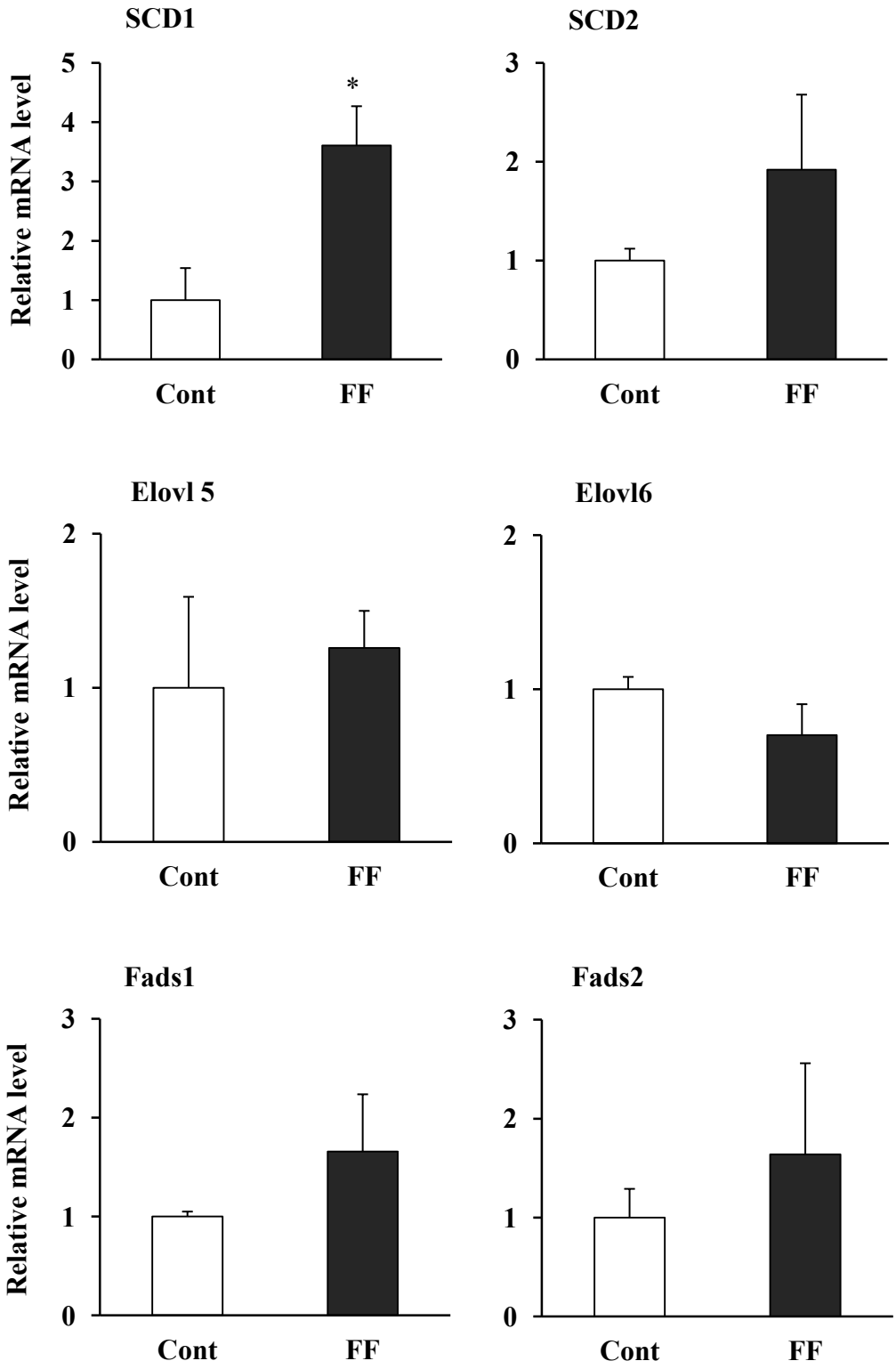
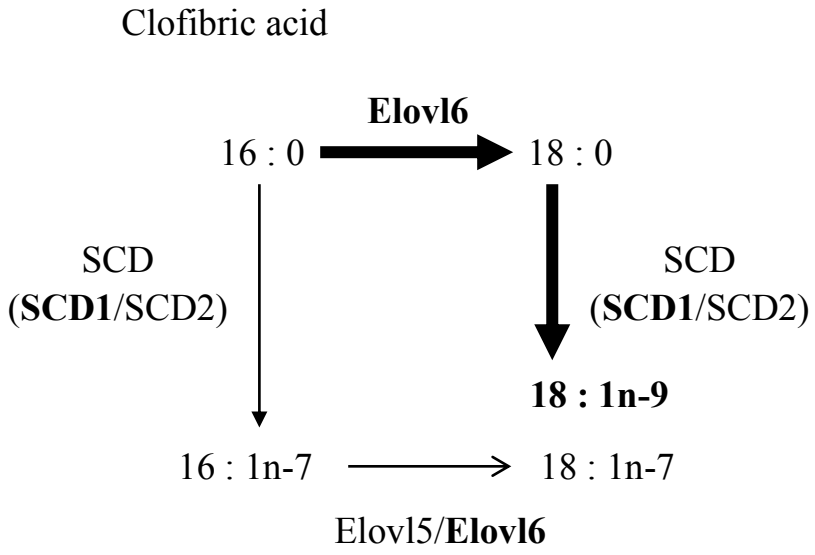

SCD1
Relative mRNA level
5
4
3
2
1
0
*
Cont
FF
SCD2
3
2
1
0
Cont
FF
Elovl 5
Relative mRNA level
2
1
0
Cont
FF
Elovl6
2
1
0
Cont
FF
Fads1
Relative mRNA level
3
2
1
0
Cont
FF
Fads2
3
2
1
0
Cont
FF

Fig. 5


Clofibric acid
Elovl6
16 : 0
18 : 0
SCD
(SCD1/SCD2)
SCD
(SCD1/SCD2)
18 : 1n-9
16 : 1n-7
18 : 1n-7
Elovl5/Elovl6

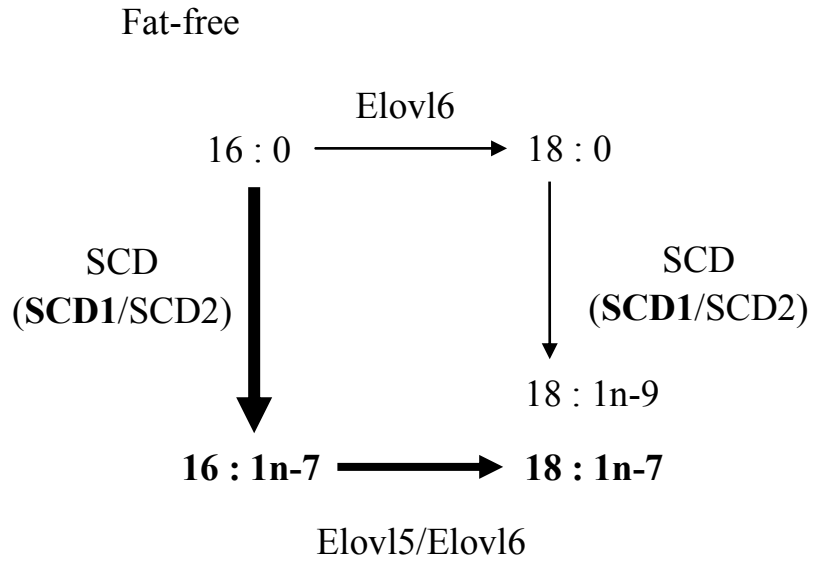
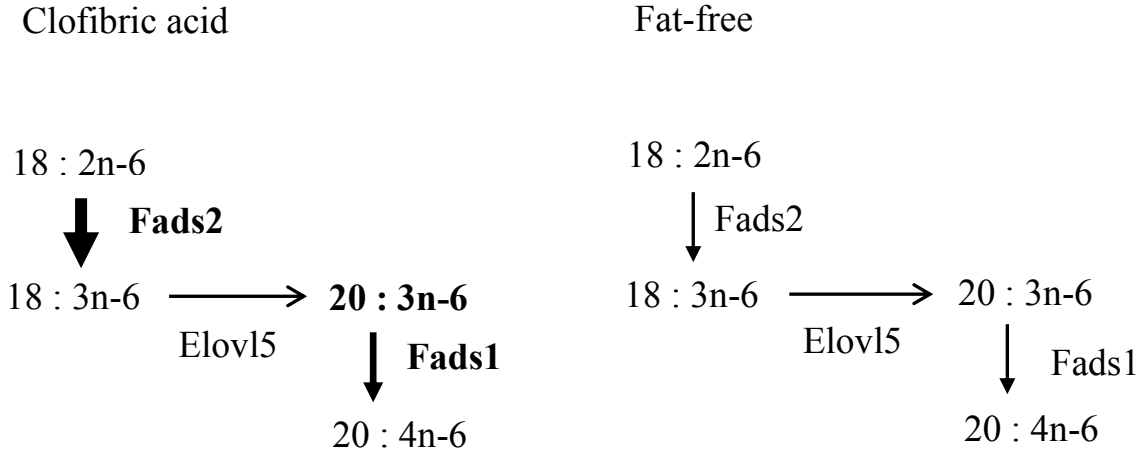

Fat-free
Elovl6
16 : 0
18 : 0
SCD
(SCD1/SCD2)
SCD
(SCD1/SCD2)
18 : 1n-9
16 : 1n-7
18 : 1n-7
Elovl5/Elovl6

Fig. 6


Clofibric acid
18 : 2n-6
Fads2
18 : 3n-6
Elovl5
20 : 3n-6
Fads1
20 : 4n-6
Fat-free
18 : 2n-6
Fads2
18 : 3n-6
Elovl5
20 : 3n-6
Fads1
20 : 4n-6