Fibrates Reduce Triacylglycerol Content by Up-regulating Adipose Triglyceride Lipase in the Liver of Rats

Minako Karahashi[1], Miki Hoshina[1], Tohru Yamazaki[1], Takeshi Sakamoto[1], Atsushi Mitsumoto[2], Yoichi Kawashima[1] and Naomi Kudo[1,*]

[1] *Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan*

[2] *Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chiba 283-8555, Japan*

Running title: Fibrate and Adipose Triglyceride Lipase

Corresponding author: Naomi Kudo

E-mail: naokudo@josai.ac.jp

Tel.: +81-49-271-7031

Fax: +81-49-271-7984

Abbreviations

Acot1	Acyl-CoA thioesterase 1
Acox1	Acyl-CoA oxidase 1
ACSL	Long-chain acyl-CoA synthetase
ATGL	Adipose triglyceride lipase
BrMDMC	4-Bromomethyl-6,7-dimethoxycoumarin
BSA	Bovine serum albumin
CGI-58	Comparative gene identification-58
CPT1a	Carnitine palmitoyltransferase 1a
DGAT	Acyl-CoA:diacylglycerol acyltransferase
FABP1	Fatty acid binding protein 1
FABPpm	Plasma membrane-associated fatty acid binding protein
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
GPAT	Glycerol-3-phosphate acyltransferase
LCAD	Long-chain acyl-CoA dehydrogenase
MCAD	Medium-chain acyl-CoA dehydrogenase
NAFLD	Nonalcoholic fatty liver disease
PPARα	Peroxisome proliferator-activated receptor α
TAG	Triacylglycerol
TLC	Thin-layer chromatography
VLDL	Very-low-density lipoprotein

Abstract. Hepatic triacylglycerol (TAG) homeostasis is maintained by carefully regulated balance between its synthesis and disposal. Impairment in this balance causes steatosis. The aims of this study were (i) to uncover whether fibrates control TAG concentration through the action of adipose triglyceride lipase (ATGL) and (ii) to compare the potency of the effects on ATGL expression and TAG concentration among fenofibrate, bezafibrate and clofibric acid, in the liver of rats. Treatments of rats with the three fibrates induced ATGL and concomitantly decreased hepatic TAG concentration. The up-regulation of ATGL was likely mediated through the activation of peroxisome proliferator-activated receptor α. Fibrates also expanded capacity of fatty acid β-oxidation. Importantly, three fibric acids (fenofibric, bezafibric and clofibric acids) that are active metabolites formed in the liver exhibited almost the same potency to elevate ATGL expression *in vivo*, despite the fact that there were considerable differences in this regard among fenofibrate, bezafibrate and clofibric acid when compared on the basis of their dosage. These results suggest that ATGL represents a potential therapeutic target for ameliorating hepatic steatosis and that fibric acids are promising agents to ameliorate and/or protect against hepatic steatosis.

***Keywords*:** fibrates, fibric acid, adipose triglyceride lipase, triacylglycerol, rat liver

Introduction

Fibrates are well known as a class of drugs used to treat dyslipidemia, in particular hypertriglyceridemia. The primary clinical outcomes are decreased plasma triacylglycerol (TAG), increased low-density lipoprotein size, increased high-density lipoprotein synthesis and increased reverse cholesterol transport, thus reducing the risk of cardiovascular disease (1 – 5). Moreover, there is increasing evidence with regard to the amelioration of insulin sensitivity, and vascular and systemic inflammation by fibrates (1, 3, 6). In hepatocytes, excessive accumulation of TAG is the common denominator of a wide range of pathophysiological entities known as hepatic steatosis or nonalcoholic fatty liver disease (NAFLD). Tight regulation between TAG synthesis, hydrolysis, secretion and fatty acid oxidation is required to prevent TAG accumulation in the liver. Therefore, understanding the pathway that regulates hepatic TAG metabolism in the liver is critical for the development of therapies to ameliorate pathophysiological conditions associated with excessive hepatic TAG accumulation, such as hepatic steatosis and NAFLD. However, it remains yet to be established whether fibrates ameliorate NAFLD (7, 8). This may be due to the fact that biochemical mechanism behind the effects of fibrates on the regulation and physiological function of adipose triglyceride lipase (ATGL) in TAG metabolism is not fully understood in the liver.

Catabolism of TAG is believed to be initiated by the action of ATGL, which is known to catalyze the initial step in lipolysis by hydrolyzing TAG into diacylglycerol and free fatty acid (9). Consequently, it is most likely that functional deficiency of ATGL results in TAG accumulation in essentially all tissues and cells. ATGL has been demonstrated to be expressed in most tissues with the highest expression in adipose tissues but very low expression in the liver (9). Subsequent studies have identified ATGL as a major functional lipase in adipose tissue (10), whereas its role in the liver has been poorly understood. However, there is

evidence demonstrating that ATGL ablation leads to pronounced hepatic steatosis, whereas increasing ATGL production in the liver reduces hepatic steatosis in rodents (11 – 14). These findings suggest that ATGL plays a pivotal role in TAG metabolism in the liver and that ATGL represents a pharmacological therapeutic target for NAFLD. In this regard, it is of interest to understand in detail the effects of fibrates on ATGL in the liver. As far as we know, little study has been carried out *in vivo* on whether fibrates cause modification of ATGL function in the liver. There are several kinds of fibrates. Clofibrate (ethyl ester of clofibric acid) is a prototype of fibrates, but has been gradually replaced by more efficient drugs, like fenofibrate (isopropyl ester of fenofibric acid) and bezafibrate (also referred to as bezafibric acid). On the other hand, it is now considered that there are differences in the mode of actions among fibrates (15 – 17). However, fundamental information is lacking about the action of fibrates on hepatic ATGL, despite the fact that these drugs are the most promising agents for the therapy of hepatic steatosis. In this context, the present study aimed (i) to uncover whether fibrates regulate TAG concentration through the action of ATGL and (ii) to compare the potency of the effects on ATGL expression among fenofibrate, bezafibrate and clofibric acid in the liver of rats.

Materials and Methods

Materials

Bezafibrate, clofibric acid, fenofibrate, phosphatidylcholine (from egg yolk), phosphatidylinositol (from soybean), triolein, 4-bromomethyl-6,7-dimethoxycoumarin (BrMDMC) and bovine serum albumin (BSA) were obtained from (Sigma-Aldrich Co., St. Louis, MO, USA); 18-crown-6-ether from Wako Chemicals (Osaka); anti ATGL (F-7) mouse monoclonal IgG from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA); anti mouse IgG HRP-linked antibody from Cell Signaling Technology, Inc., (Danvers, MA, USA). Triolein

[carboxyl-^{14}C] (108.8 mCi/mmol) was purchased from PerkinElmer (Waltham, MA, USA), and was purified by thin-layer chromatography (TLC) on silica gel G plates, which were developed with *n*-hexane/diethyl ether/acetic acid (80:30:1, by vol.). The regions that corresponded to TAG were scraped off and transferred to a tube. [^{14}C]TAG was extracted from silica gel as described previously (18).

Animals

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). Male Wistar rats were obtained from SLC (Hamamatsu). The animals were fed on a standard diet (CE-2; Clea Japan Inc., Tokyo) *ad libitum* and allowed free access to water. After acclimatization for at least 1 week, rats aged 8 weeks were fed on a diet admixed with fenofibrate, bezafibrate or clofibric acid. To examine the dose-response effects, the rats were fed on a diet containing various concentrations of fenofibrate (0, 0.025, 0.05, 0.1 or 0.2% (w/w)), bezafibrate (0, 0.025, 0.05, 0.1 or 0.2% (w/w)) or clofibric acid (0, 0.05. 0.1, 0.15, 0.2 or 0.3% (w/w)) for 14 days. Food consumption by each rat was measured and the amounts of fibrates taken in by each rat were calculated. In time-course experiments, the rats were fed on a diet containing 0.1% (w/w) fenofibrate, 0.1% (w/w) bezafibrate or 0.3% (w/w) clofibric acid for 0, 2, 7 or 14 days. Under diethyl ether anesthesia, blood was withdrawn from the inferior vena cava and serum was prepared. The liver was rapidly removed, washed with saline and weighed. It was then divided into small portions, frozen in liquid nitrogen and stored at -80°C for biochemical and analytical determinations.

Determination of TAG

Total lipid was extracted from frozen liver tissues by the method of Bligh and Dyer (19). TAG was separated by TLC on silica gel G plates, which were developed with

n-hexane/diethyl ether/acetic acid (80:30:1, by vol.). After visualization by spraying 0.001% (w/v) primuline in 80 % acetone, the regions that corresponded to TAG were scraped off and transferred to tubes. TAG was extracted from silica gel as described previously (18). The TAG extract was dried under a flow of nitrogen and re-dissolved in isopropanol–chloroform (9:1, v/v) containing 9% (v/v) Triton X-100, and then assayed for TAG concentration using a commercially available kit (Triglyceride E-Test Wako; Wako Chemicals, Osaka). TAG in the serum was directly determined using the kit (Triglyceride E-Test Wako).

Determination of fibric acids

One part of the liver was homogenized in nine volumes of 0.25 M sucrose–1 mM EDTA–10 mM potassium phosphate buffer (pH 7.4) in a Potter glass-Teflon homogenizer. Fibric acids were extracted and analyzed as described previously (20). In brief, fibric acids were extracted into *n*-hexane–ethyl acetate (85:15, by vol.) from liver homogenates (10 mg of liver) after acidification with 0.5 M HCl, back-extracted with 0.1 M disodium hydrogen phosphate, and then re-extracted with *n*-hexane–ethyl acetate after acidification with 0.5 M HCl. The obtained fibric acids were derivatized with BrMDMC and 18-crown-6-ether, and were separated isocratically on a reversed phase with acetonitrile–water (43:57, v/v) for derivatives of clofibric acid and bezafibric acid and with acetonitrile–water (46:54, v/v) for derivatives of clofibric acid and fenofibric acid by high-performance liquid chromatography with fluorescence detection (excitation at 340 nm; emission at 425 nm).

Quantitation of mRNA

For the determination of mRNA, one portion of the liver was frozen in liquid nitrogen and then stored at –80°C. Total RNA was isolated from the liver tissues using QIAzol reagent and RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 500 ng of total RNA, as described previously (21). PCR amplification was carried out using SYBR Premix

EX Taq (2x, Perfect Real Time; Takara Bio Inc., Otsu). The amplification and detection were performed with Applied Biosystems Step One Plus™ real-time PCR system (Life Technologies Corp., Carlsbad, CA, USA). The thermal cycling program was as follows: 10 s denaturation step at 95°C followed by 50 cycles of 5 s denaturation at 95°C, and 34 s annealing at 60°C. Melting curve analysis was performed to confirm the real-time quantitative RT-PCR products. Changes in gene expression were calculated 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.

Western blot analysis

Western bolt analyses were performed essentially according to Reid *et al*. (14). Briefly, one portion of the liver was homogenized in RIPA lysis buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate supplemented with protease inhibitors (aprotinin at 2 μg/ml, bestatin at 13.8 μg/ml, leupeptin at 10 μg/ml, pepstatin at 5 μg/ml and AEBSF at 250 μg/ml)) using a Polytrone RT2100 homogenizer (Kinematica, Luzern, Switzerland) at 4°C, incubated on ice for 30 min and centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were recentifuged at 10,000 x g for 10 min, and the resulting supernatants were collected for determination of protein concentration using BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) using BSA as a standard. Total tissue lysates containing 10 μg of protein were boiled in 0.063 M Tris–HCl (pH 6.8) containing 5% (v/v) 2-mercaptoethanol, 2% sodium dodecyl sulfate, 6% (v/v) glycerol and 0.02% bromophenacylblue. The denatured samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis with 8% acrylamine. Proteins were then transferred onto a polyvinylidene difluoride membrane (Amersham Hybond-P PVDF Transfer membrane; GE Healthcare, Buckinghamshire, England) using a Trans-Blot

Semi-Dry Transfer Cell (Bio-Rad Inc., Hercules, CA, USA). Membranes were incubated at room temperature for 30 min with blocking buffer (Tris-buffered saline (pH 7.6) containing 0.1% Tween 20, 5% skim milk and 1% BSA). Membranes were incubated with anti-ATGL (F-7) mouse monoclonal IgG. After 12 h of incubation, membranes were washed three times in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. Membranes were then incubated with a secondary antibody conjugated to horseradish peroxidase (anti-mouse IgG HRP-linked antibody) for 2 h. Membranes were washed three times with Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. Proteins were visualized using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected in a LAS-1000 (GE Healthcare).

Measurement of TAG hydrolase activity

Cytoplasmic fraction was prepared as previously described (12, 22). About 1 g of the liver was homogenized in four volumes of homogenate buffer (0.5 M sucrose, 1 mM EDTA, 20 mM Tris-HCl (pH 7.4), 1 mM dithioerythritol, leupeptin at 20 μg/ml, antipain at 2 μg/ml and pepstatine at 1 μg/ml) using a Dounce homogenizer. The homogenates were centrifuged at 1,000 g for 10 min. The resulting supernatant was applied to a discontinuous gradient of 0.25 M, 0.5 M (the supernatant of homogenate) and 1.5 M sucrose. The tubes were centrifuged at 100,000 g in a swinging bucket rotor for 3 h. The 0.5 M sucrose fraction was collected and used as cytoplasmic fraction. TAG hydrolase activity was measured essentially as previously described (12) with some modifications. Substrate was prepared as described previously (23). Briefly, 2.96 mg (3 μCi) of [^{14}C]triolein (final raidospecific activity, 0.9 μCi/μmol) and 300 μg of phosphatidylcholine/phosphatidylinositol (3:1) were mixed. After drying under nitrogen and then in vacuo, the mixture was emulsified by sonication with a sonicator (Astrason ultrasonic processor XL2020; Misonix Inc.; Farmingdale, NY, USA) in 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0). After completed sonication, to the solution was added 0.5 ml of 20% (w/v) BSA (defatted) in 0.1 M potassium phosphate buffer

(pH 7.0), and the mixture was gently vortex-mixed. For each assay, 100 μl substrate solution with 50 μl cytoplasmic fraction and 50 μl buffer (20 mM potassium phosphate, 1 mM EDTA, 1mM dithioerythritol and 0.02% (w/v) defatted BSA, pH 7.0) were incubated for 60 min at 37°C. The incubation mixture without cytoplasmic fraction was run simultaneously. Reaction was stopped by adding 6 ml of chloroform/methanol (1:2, v/v), and then oleic acid (56.4 μg as ethanol solution) and diolein (124.2 μg as ethanol solution) were added as carriers. After mixing, 1.4 ml of 0.1M HCl was added to the mixture. Lipids were extracted as previously described (19). The extracts were taken to dryness under a flow of nitrogen, and the dried lipids were dissolved in a known volume of chloroform/methanol (1:1, v/v). An aliquot of the solution was used to measure total radioactivity in the extract. Another aliquot of the solution was subjected to TLC on silica gel G plates, which were developed with *n*-hexane/diethyl ether/acetic acid (80:30:1, by vol.). After visualization by spraying primuline solution, the regions that corresponded to fatty acid, diacylglycerol and monoacylglycerol were scraped off and transferred to tubes. These lipids were extracted from silica gel as described previously (18), and the extracts obtained were transferred to vials. After being dried under a flow of nitrogen, the residues obtained were dissolved in scintillator, and radioactivities were counted to calculate the amounts of TAG hydrolyzed. The control value, which was obtained from the incubation without cytoplasmic fraction, was subtracted to give the net TAG hydrolyzed by cytoplasmic fraction.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Scheffé's multiple range test as a *post hoc* test. Differences were considered significant at P-value < 0.05. Linear regression analysis was performed to evaluate the correlation between two variables.

Results

Body weight and daily food intake of the rat were measured throughout the treatment. The body weights of treated rats were similar to that of control (Fig. 1A). Daily food intake was not significantly changed by the treatment (Fig. 1B). On the basis of these data, the amounts of fibrate taken in by each rat were calculated (Fig. 1C, D and E). There were very strong linear correlations between the % of fibrate in diet and the calculated dose. These results indicated that the dietary concentrations of fenofibrate at 0.025, 0.05, 0.1 and 0.2% (w/w) correspond to, respectively, 15.2 ± 2.7, 34.3 ± 5.0, 67.7 ± 10.4 and 133.4 ± 22.1 mg/kg body weight per day, that those of bezafibrate at 0.025, 0.05, 0.1 and 0.2% (w/w) correspond to, respectively, 17.0 ± 3.2, 34.0 ± 5.3, 66.6 ± 11.7 and 140.6 ± 25.5 mg/kg body weight per day, and that those of clofibric acid at 0.05, 0.1, 0.15, 0.2 and 0.3% (w/w) correspond to, respectively, 34.1 ± 4.9, 66.4 ± 16.4, 98.8 ± 20.2, 134.5 ± 30.8 and 202.1 ± 40.2 mg/kg body weight per day. Thus, substantially, there was no difference in the calculated dose among the animal groups treated with the three fibrates. These results may allow us to compare the effects of three fibrates on TAG levels in the liver.

Dose- and time-dependent reduction of hepatic TAG by fibrates

Contents of TAG in the liver of rats that were treated with fenofibrate, bezafibrate and clofibric acid at various doses for 14 days were estimated; the treatments with the three fibrates significantly decreased hepatic contents of TAG in dose-dependent manners (Figs. 2A, C, E). At a dietary concentration of 0.1% (w/w), TAG contents in the liver of rats that were treated with fenofibrate and bezafibrate were 48 and 58%, respectively, of those of the control. Although the treatments of rats with 0.1% (w/w) clofibric acid did not significantly reduce hepatic TAG contents, TAG contents in the liver of rats treated with 0.3% (w/w) clofibric acid were 40% of those of the control. The treatments of rats with 0.1% (w/w) fenofibrate, 0.1% (w/w) bezafibrate or 0.3% (w/w) clofibric acid reduced hepatic contents of TAG in

time-dependent manners (Figs. 2B, D, F). The treatments of rats with fenofibrate and bezafibrate significantly decreased serum concentrations of TAG; clofibric acid tended to reduce it, effects that were not significant (Fig. 3).

To gain insight into the molecular basis of TAG reduction by fibrates, mRNA levels of key enzymes involved in the metabolism of fatty acids and TAG in the liver of rats that were fed on a diet containing 0.1% (w/w) fenofibrate, 0.1% (w/w) bezafibrate or 0.3% (w/w) clofibric acid for 14 days were estimated (Table 2). The treatments with the fibrates up-regulated the expression of genes encoding enzymes and proteins participating in the degradation of TAG and fatty acids (adipose triglyceride lipase (ATGL), comparative gene identification-58 (CGI-58), carnitine palmitoyltransferase 1a (CPT1a), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD) and acyl-CoA oxidase 1 (Acox1)). With regard to proteins and enzymes involved in transport/trafficking of fatty acids, treatments with the fibrates caused significant increases in mRNA levels of fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP) 2, fatty acid binding protein1 (FABP1), long-chain acyl-CoA synthetase (ACSL) 1 and ACSL3. By contrast, FATP5 was down-regulated by fibrates. Fibrates did not change the mRNA levels for either FATP4, plasma membrane-associated fatty acid binding protein (FABPpm) or ACSL5, except for slight up-regulation of ACSL5 expression by fenofibrate. With regard to fatty acid synthesis, the levels of mRNAs encoding fatty acid elongase 6 (Elovl6) and stearoyl-CoA desaturase 1 (SCD1) were elevated by the three fibrates, but that of fatty acid synthease (FAS) was increased by only fenofibrate. As for enzymes involved in glycerolipid synthesis, fenofibrate and bezafibrate slightly augmented mRNA level of GPAT4 while that was decreased by clofibric acid. Gene expression of DGAT2 was slightly down-regulated by the three fibrates; on the other hand, fenofibrate alone increased mRNA level of DGAT1.

Up-regulation of ATGL by fibrates

Levels of mRNA encoding ATGL in the liver of rats that were treated with fenofibrate, bezafibrate and clofibric acid at various doses for 14 days were estimated (Figs. 4A, C, E). At relatively low doses, fenofibrate and bezafibrate elevated the mRNA level of ATGL more potently than clofibric acid did. The mRNA level of ATGL rose with an increasing dose of fibrates, and almost reached a plateau at a dose of fenofibrate and bezafibrate of 0.1% (w/w) and at a dose of clofibric acid of 0.15% (w/w). When comparing the potency at a dietary concentration of 0.1% (w/w), fenofibrate, bezafibrate and clofibric acid increased the mRNA level of ATGL by 4.5-, 4.3- and 1.1-fold, respectively. Moreover, the treatments of rats with the three fibrates increased the level of mRNA encoding ATGL in time-dependent manners (Figs. 4B, D, F). The level of mRNA for CGI-58 in the liver was also increased by the treatments of rats with the three fibrates in dose- and time-dependent manners (Fig. 5). To estimate the relationship between the level of ATGL mRNA and the concentration of TAG in the liver, linear regression analysis was performed (Fig. 6). There was a fairly strong inverse correlation between the mRNA level of ATGL and the content of TAG ($r^2 = 0.6808$).

We next measured TAG hydrolase activity in cytoplasmic fraction of livers of the rats, that were treated with three fibrates, with [^{14}C]triolein as a substrate. The treatment with 0.1% (w/w) fenofibrate and 0.3% (w/w) clofibric acid for 14 days increased TAG hydrolase activity by 1.8- and 1.6-fold, respectively, compared with that of the control, and the treatment with 0.1% (w/w) bezafibrate tended to elevate the activity by1.4-fold (Fig. 7). The effects of the three fibrates on the ATGL protein level in the liver were also evaluated by Western blot analysis (Fig. 8). The ATGL protein levels in the liver of rats, which were fed on a diet containing one of 0.1% (w/w) fenofibrate, 0.1% (w/w) bezafibrate and 0.3% (w/w) clofibric acid for 14 days, were 2.7-, 3.8- and 3.5-fold, respectively, greater than that of the control.

To understand possible involvement of peroxisome proliferator-activated receptor α (PPARα) in the induction of ATGL, the relationship between level of the mRNA encoding acyl-CoA thioesterase 1 (Acot1), a typical PPARα target gene, and that encoding ATGL was

examined (Fig. 9). Upon the treatment of rats with various doses of fenofibrate, bezafibrate and clofibric acid, the level of the mRNA for Acot1 in the liver rose with increasing dose (Fig. 9A). Linear regression analysis revealed a strong correlation between the mRNA level of Acot1 and that of ATGL (r^2 = 0.9383) (Fig. 9B)

Potency of fibric acids to induce ATGL and to reduce TAG in the liver

To estimate the potency of fibric acids to up-regulate ATGL and reduce TAG in the liver, hepatic concentrations of fibric acids, active forms of fibrates, in the liver of rats treated with various doses of fibrates for 14 days were measured (Fig. 10A). There was a large difference in hepatic concentration between clofibric acid and fenofibric acid or bezafibric acid. However, the concentration of clofibric acid in the liver of rats treated with 0.3% (w/w) clofibric acid was almost the same as those of fenofibric acid and bezafibric acid in the livers of rats fed on a diet containing 0.1% (w/w) fenofibrate or 0.1% (w/w) bezafibrate. Figure 10B shows the relationship between concentration of fibric acids and the mRNA level of ATGL in the liver; there was a strong correlation between these two variables (r^2 = 0.9025). Moreover, a fairly good inverse correlation was observed between fibric acid concentration and TAG content in the liver (r^2 = 0.8008) (Fig. 10C).

Discussion

The present study clearly showed that three fibrates, fenofibrate, bezafibrate and clofibric acid, reduced the hepatic concentration of TAG in dose- and time-dependent manners, although conflicting results appear in the literature concerning the effects of fenofibrate on hepatic TAG content (11, 24). In the current study, fenofibrate behaved as the most powerful hepatic TAG suppressor, while clofibric acid was the least potent. The order of potency in decreasing hepatic TAG (fenofibrate ≥ bezafibrate > clofibric acid) did not always agree with

the reported manifestation of PPARα activation (25). To date, the TAG-reducing activity of fibrates has been considered to be mainly mediated by the elevation of peroxisomal and mitochondrial fatty acid degradation. Nevertheless, the detailed mechanism underlying the action of fibrates to reduce hepatic TAG is not fully understood. Hepatic TAG concentration is maintained by a carefully regulated balance of the two predominant metabolic pathways of synthesis and disposal in the liver. Namely, hepatic TAG content could be controlled by (i) *de novo* fatty acid synthesis, (ii) delivery of fatty acids from extra-hepatic sources, (iii) TAG synthesis, (iv) fatty acid β-oxidation, and (v) secretion of very-low-density lipoprotein (VLDL). To deal with these possibilities, we measured the expression of genes encoding enzymes and proteins for lipid degradation, fatty acid translocation/trafficking and lipogenesis. The expression of genes for enzymes involved in oxidation and utilization of fatty acids, CPT1a, MCAD, LCAD, Acox1 and ACSL1, was significantly up-regulated by all the three fibrates. These results strongly suggest the increased degradation of fatty acids in hepatocytes, which is generally considered to be the cause of the reduction of hepatic TAG by fibrates (26, 27). As for the contributions of mitochondrial and peroxisomal β-oxidation to total fatty acid β-oxidation in the liver, Mannaerts *et al*. (28) has estimated the contribution of peroxisomal β-oxidation to total fatty acid oxidation to be less than 10% in the living hepatocytes from both control and clofibrate-treated rats, despite the fact that activity of peroxisomal β-oxidation in liver homogenates is strikingly increased by the treatment of rats with clofibrate. The same authors demonstrated that the activity of mitochondrial β-oxidation is increased several fold in both the liver homogenates and the living hepatocytes by the treatment of rats with clofibrate. Our current results that fibrates up-regulated the expression of the gene for CPT1a are in good agreement with the results of Mannaerts *et al*. (28) because CPT1a is a critical rate-determining regulator of mitochondrial β-oxidation in the liver (29). A previous study demonstrated that a moderate up-regulation of CPT1a is sufficient to substantially reduce hepatic TAG (30). Therefore, it is likely that mitochondrial β-oxidation,

in particular CPT1a, plays a greater role in fatty acid degradation in the liver of fibrate-treated rats, whereas the up-regulation of Acox1, a rate-limiting enzyme of peroxisomal β-oxidation, by fibrates plays a physiologically less dominant role in fatty acid catabolism. Collectively, it might be conceivable that up-regulation of CPT1a by fibrates may be a cause for reduction of hepatic concentration of TAG by the drugs. However, it should be pointed out two facts here. One is the finding of Lankester *et al*. (31) who suggested that rat hepatocytes preferentially oxidized fatty acid derived from TAG hydrolysis *versus* those derived from exogenous uptake. The other is that TAG is required to be hydrolyzed to fatty acids before the fatty acids undergo β-oxidation. Until recently, the first step of TAG degradation in the liver was not fully understood. Growing evidence has been emerged indicating that ATGL plays a pivotal role in regulating TAG content in the liver (11 - 14). Recent studies demonstrated that, despite the fact that ATGL expression in the liver is relatively low compared with that in other tissues, hepatic absence of ATGL causes massive accumulation of TAG in the liver (11 - 13) and that the overexpression of ATGL in the liver abolishes hepatic TAG stores (13, 14). Namely, fatty acids released from TAG by ATGL are channeled to oxidation rather than to other metabolic pathways (14). Thus, the existing evidence strongly suggest that ATGL dominates the process of reducing hepatic TAG and that fatty acid oxidation by mitochondria and peroxisomes is secondary to TAG hydrolysis by ATGL with regard to TAG catabolism. In this context, among the biochemical manifestations observed in the current study following the administration of fibrates, we consider the importance of the modifications in ATGL caused by fibrates. The present results clearly revealed that the fibrates augmented the expression of ATGL concomitantly with elevation of the gene expression of CGI-58, a cofactor of ATGL (10). The manifestation was dose- and time-dependent and commonly exhibited by the three fibrates; the order of potency in inducing hepatic ATGL was fenofibrate ≥ bezafibrate > clofibric acid *in vivo*. Moreover, the present study confirmed that the levels of activity and protein of ATGL were also increased by the treatment of rats with the three fibrates. Importantly, there was a

fairly strong inverse correlation between ATGL mRNA level and TAG content in the liver. These results, taken together, lead to the conclusion that ATGL is a key player that triggers TAG degradation by fibrates. On the one hand, it is also more likely that fibrates affected TAG synthesis. There are two sources of fatty acids as substrates for TAG synthesis. One is the fatty acids synthesized *de novo* in the liver, and the other is the fatty acids taken up from circulation. The present study confirmed that expression of the FAS gene (*de novo* fatty acid synthesis) was largely unchanged; accordingly, the supply of *de novo* synthesis of palmitic acid may not be affected by fibrate treatments. On the other hand, supply of fatty acids from circulation to hepatocytes is most likely to be increased by fibrates because the expression of FAT/CD36 gene, a main fatty acid transporter in hepatocytes (32), was strikingly up-regulated and that of FATP2 was significantly augmented by fibrates. The current study showed that ACSL3 was significantly up-regulated by fibrates. Interestingly, a recent study using lined cells showed that ACSL3 is localized to the endoplasmic reticulum and cytosolic lipid droplets and that ACSL3 not only esterifies fatty acids with CoA but also is involved in the cellular uptake of fatty acids, presumably by indirect metabolic trapping, despite the fact that ACSL3 is not a fatty acid transporter (33). Therefore, it is likely that ACSL3 up-regulated by fibrates activates and channels fatty acids toward TAG synthesis pathway in the liver. Moreover, in accordance with previous findings (34, 35), fibrates markedly elevated the expression of both Elovl6 and SCD1 participating in oleic acid synthesis from palmitic acid and stearic acid, results that suggest increased formation of oleic acid not only from palmitic acid synthesized *de novo* but also from palmitic acid and stearic acid taken up from circulation. The latter had been demonstrated by our previous study that the *in vivo* conversion of [^{14}C] stearic acid to [^{14}C] oleic acid in the liver is stimulated by the treatment of rats with clofibric acid (36, 37). Therefore, despite slight down-regulation of DGAT2 expression by fibrates, it is most likely that a massive amount of oleic acid is formed and available for the formation of TAG in the liver of rats treated with fibrates because oleic acid

is known to be incorporated into TAG more preferentially than other fatty acids (38 - 40). Since a previous study showed that fatty acids released from TAG by ATGL are more readily oxidized than those supplied as fatty acids in media for cultured cells (31), it is more likely that fatty acids derived from TAG hydrolysis mediated by ATGL underwent β-oxidation, the activity of which was markedly increased by the treatment with fibrates. As for the secretion of VLDL-TAG, the present study showed that fenofibrate and bezafibrate decreased serum concentration of TAG and that clofibric acid tended to reduce it, but not significantly. These results suggest that the changes in VLDL-TAG secretion into circulation are not a cause for the fibrate-mediated decrease in hepatic TAG levels.

The ATGL gene is considered to be a PPARα target (41) and fibrates are known to be PPARα agonists (25), implying the possibility that fibrates induced ATGL in the liver through the activation of PPARα. However, little information is available with respect to potency of fibrates to activate PPARα in the liver *in vivo* because the potency as a PPARα agonist is generally estimated by reporter assay employing cultured cells (25). Since it is difficult to provide definite experimental proof that the induction of ATGL by fibrates working in the liver is quantitatively mediated through the activation of PPARα *in vivo*, the present study employed an approach to estimate the *in vivo* potency of the three fibrates by the determination of the increase in mRNA abundance of the Acot1 gene. Acot1 encodes long-chain acyl-CoA thioesterase 1 (42, 43), which was found to be one of two different peroxisome proliferator-inducible palmitoyl-CoA hydrolases in rat liver cytosol and is not a constitutive enzyme in the liver (44). Moreover, Acot1 has been demonstrated to be regulated by fibrate treatment through the action of PPARα *in vivo* (43). The finding may indicate that Acot1 can be used as a likely outcome of activated PPARα working functionally *in vivo* in the liver. The current results demonstrated a strong correlation between the mRNA levels of Acot1 and ATGL in the liver of rats treated with the three fibrates. This result is not direct proof, but suggests the possible involvement of PPARα activation in ATGL induction by

fibrates in the liver.

ATGL overexpression enhances TAG hydrolysis, and the consequential release of fatty acids drives PPARα activity in rat hepatocytes (11, 14, 45); conversely, ATGL deficiency leads to down-regulation of PPARα target genes in the liver (11). These findings strongly suggest that ATGL is critically involved in the hepatocellular mobilization of fatty acids and fatty acid signaling (45). ATGL resides predominantly on cytosolic lipid droplets and on intracellular membranes (22). How fatty acids traffic from the lipid droplets to the nucleus to activate PPARα remains unclear, but it is plausible that the process involves FABP (46). The present study showed that fibrates increased expression of the FABP1 gene, a result that is in good agreement with our previous finding that the treatment of rats with clofibric acid induced FABP protein in the liver (47). Various types of fatty acids have been shown to activate PPARα (48, 49). Therefore, it is logical to speculate that fatty acids released from cytosolic lipid droplets by ATGL are channeled to two metabolic pathways. One is that the fatty acids are transported by FABP to mitochondria, where they are exclusively utilized for β-oxidation. The other is that the released fatty acids are similarly transported to the nucleus, where they activate PPARα as its ligand (45), activation of the transcription factor that relays further stimulation of fatty acid uptake (FAT/CD36), fatty acid trafficking (FABP1), fatty acid activation (ACSL1, 3), mitochondrial β-oxidation (CPT1a, MCAD, LCAD) and peroxisomal β-oxidation (Acox1), and ATGL as well. Thus, ATGL may further expand this positive feedback loop interplaying among PPARα, ATGL and β-oxidation system in hepatocytes. In doing so, hepatic TAG decreases.

The present study showed that there are differences in the *in vivo* potency to induce ATGL and to reduce hepatic TAG among fenofibrate, bezafibrate (also referred to as bezafibric acid) and clofibric acid. However, our present study has uncovered strong correlations not only between the hepatic concentrations of fibric acids irrespective of their chemical structures and mRNA levels of ATGL, but also between these and hepatic TAG

concentrations. Fibrates, ester forms of fibric acids, are prodrugs, and it is known that they are hydrolyzed by hepatic esterase(s) to form active metabolites, fibric acids. Importantly, the current results suggest that the three fibric acids, fenofibric, bezafibric and clofibric acids, have almost the same potency to induce ATGL, possibly through activation of PPARα *in vivo*, *in vivo* inside the liver. However, it should be noted here that the three fibrates also displayed considerable differences in the mode of action on the expression of genes, which was investigated in the present study (as shown in Table 2), despite the fact that intra-hepatic concentrations of fenofibric acid, bezafibric acid and clofibric acid were largely the same (as shown in Fig. 10A). These results imply that there is some difference in mode of action among the three fibrates.

Finally, the present study evidently revealed that the three fibric acids exhibit ATGL induction and TAG reduction in the liver with largely the same potency. Turpin *et al*. demonstrated that hepatic ATGL production is reduced in several models of rodent obesity and that ATGL ablation leads to pronounced steatosis, which is characterized by an impairment in fatty acid oxidation, whereas increasing ATGL production in the liver reduces hepatic steatosis and mildly enhances liver insulin sensitivity (13). Collectively, these findings suggest the possibility that ATGL represents a pharmacological therapeutic target for ameliorating NAFLD, and that fibric acids are the likely drugs to ameliorate and/or protect against hepatic steatosis associated with insulin resistance.

Acknowledgements

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

References

1 Berger J, Wagner JA. Physiological and therapeutic roles of peroxisome proliferator-activated receptors. Diabetes Technol. Ther. 2002;4:163-174.

2 Kersten S. Peroxisome proliferator activated receptors and lipoprotein metabolism. PPAR Res. 2008; 2008:132960.

3 Barter PJ, Rye K-A. Is there a role for fibrates in the management of dyslipidemia in the metabolic syndrome? Arterioscler Thromb Vasc Biol. 2008;28:39-46.

4 Jun M, Foote C, Lv J, Neal B, Patel A, Nicholls SJ, Grobbee DE, Cass A, Chalmer J, Perkovic V. Effects of fibrates on cardiovascular outcomes: a systematic review and meta-analysis. Lancet 2010;375:1875-1884.

5 Wilding JPH. PPAR agonists for the treatment of cardiovascular disease in patients with diabetes. Diabetes Obese Metab. 2012;14:973-982.

6 Guerre-Millo M, Gervois P, Raspé E, Madsen L, Poulain P, Derudas B, Herbert J-M, Winegar DA, Willson TM, Fruchart J-C, Berge RK, Staels B. Peroxisome proliferator-activated receptor α activators improve insulin sensitivity and reduce adiposity. J Biol Chem. 2000;275:16638-16642.

7 Chatrath H, Vuppalanchi R, Chalasani N. Dislipidemia in patients with nonalcoholic fatty liver disease. Semin Liver Dis. 2012;32:22-9.

8 Nseir W, Mograbi J, Ghali M. Lipid-lowering agents in nonalcoholic fatty liver disease and steatohepatitis: Human studies. Dig Dis Sci. 2012;57:1773-1781.

9 Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science. 2004;306:1383-1386.

10 Zimmermann R, Lass A, Haemmerle G, Zechner R. Fate of fat: The role of adipose triglyceride lipase in lipolysis. Biochim Biophys Acta. 2009;1791:494-500.

11 Ong KT, Mashek MT, Bu SY, Greenberg AS, Mashek DG. Adipose triglyceride lipase is a major hepatic lipase that regulates triacylglycerol turnover and fatty acid signaling and

partitioning. Hepatology. 2011;53:116-126.

12 Wu JW, Wang SP, Alvarez F, Casavant S, Gauthier N, Abed L, Soni KG, Yang G, Mitchell GA. Deficiency of liver adipose triglyceride lipase in mice causes progressive hepatic steatosis. Hepatology. 2011; 54:122-132.

13 Turpin SM, Hoy AJ, Brown RD, Rudaz CG, Honeyman J, Matzaris M, Watt MJ. Adipose triacylglycerol lipase is a major regulator of hepatic lipid metabolism but not insulin sensitivity in mice. Diabetologia. 2011; 54:146‑156.

14 Reid NB, Ables GP, Otlivanchik OA, Schoiswohl G, Zechner R, Blaner WS, Goldberg IJ, Schwabe RF, Chua, SC. Huang L-S. Hepatic overexpression of hormone-sensitive lipase and adipose triglyceride lipase promotes fatty acid oxidation, stimulates direct release of free fatty acids, and ameliorates steatosis. J Biol Chem. 2008; 283:13087-13099.

15 Fruchart JC, Duriez P. Mode of action of fibrates in the regulation of triglyceride and HDL-cholesterol metabolism. Drugs Today. 2006;42:39-64.

16 Heald M, Cawthorne MA. Dual acting and pan-PPAR activators as potential anti-diabetic therapies. In: Schwanstecher M, editor. Diabets - Perspectives in drug therapy. Handbook Exp Pharmacol. Heidelberg: Springer; 2011;203:35-51.

17 Goldenberg I, Benderly M, Goldbourt U. Update on the use of fibrates: focus on bezafibrate. Vasc Health Risk Manag. 2008;4:131–141.

18 Imai K, Koyama M, Kudo N, Shirahata A, Kawashima Y. Increase in hepatic content of oleic acid induced by dehydroepiandrosterone in the rat. Biochem Pharmacol. 1999;58:925–933.

19 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37:911-917.

20 Karahashi M, Fukuhara H, Hoshina M, Sakamoto T, Yamazaki T, Mitsumoto A, Kawashima Y, Kudo N. A simple and sensitive method for the determination of fibric acids in the liver by liquid chromatography. submitted to Biol Pharm Bull.

21 Karahashi M, Ishii F, Yamazaki T, Imai K, Mitsumoto A, Kawashima Y, Kudo N. Up-regulation of stearoyl-CoA desaturase 1 increases liver MUFA content in obese Zucker but not Goto-Kakizaki rats. Lipids. 2013;48:457-467.

22 Soni KG, Mardones GA, Sougrat R, Smirnova E, Jackson CL, Bonifacino JS. Coatomer-dependent protein delivery to lipid droplets. J Cell Sci. 2009;122:1834-1841.

23 Holm C, Østerlund T. Hormone-sensitive lipase and neutral cholestery ester lipase. Methods Mol Biol. 1999;109:109-121.

24 Chen W-L, Chen Y-L, Chiang Y-M, Wang S-G, Lee H-M. Fenofibrate lowers lipid accumulation in myotubes by modulating the PPARα/AMPK/FoxO1/ATGL pathway. Biochem Pharmacol. 2012;84:522‑531.

25 Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: From orphan receptors to drug discovery. J Med Chem. 2000;43:527–550.

26 Cornwell PD, De Souza AT, Ulrich RG. Profiling of hepatic gene expression in rats treated with fibric acid analogs. Muat Res. 2004;549:131-145.

27 Akkaoui M, Cohen I, Esnous C, Lenoir V, Sournac M, Girard J, Prip-Buus C. Modulation of the hepatic malonyl-CoA–carnitine palmitoyltransferase 1A partnership creates a metabolic switch allowing oxidation of *de novo* fatty acids. Biochem J. 2009;420:429-438.

28 Mannaerts GP, Debeer LJ, Thomas J, De Schepper PJ. Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. J Biol Chem. 1979;254:4585-4595.

29 McGarry JD, Brown NF. The Mitochondrial carnitine palmitoyltransferase system — from concept to molecular analysis. Eur J Biochem. 1997;244:1-14.

30 Stefanovic-Racic M, Perdomo G, Mantell BS, Sipula IJ, Brown NF, O'Doherty RM. A moderate increase in carnitine palmitoyltransferase 1a activity is sufficient to substantially reduce hepatic triglyceride levels. Am J Physiol Endocrinol Metab. 2008;294:E969-E977.

31 Lankester DL, Brown AM, Zammit VA. Use of cytosolic triacylglycerol hydrolysis

products and of exogenous fatty acid for the synthesis of triacylglycerol secreted by cultured rat hepatocytes. J Lipid Res. 1998;39:1889-1895.

32 Glatz JFC, Luiken JJFP, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol Rev. 2010;90:367-417.

33 Poppelreuther M, Rudolph B, Du C, Großmann R, Becker M, Thiele C, Ehehalt R, Füllekrug J. The N-terminal region of acyl-CoA synthetase 3 is essential for both the localization on lipid droplets and the function in fatty acid uptake. J Lipid Res. 2012; 53:888-900.

34 Yamazaki T, Okada H, Sakamoto T, Sunaga K, Tsuda T, Mitsumoto A, Kudo N, Kawashima Y. Differential induction of stearoyl-CoA desaturase 1 and 2 genes by fibrates in the liver of rats. Biol Pharm Bull. 2012;35:116-120.

35 Kawashima Y, Kozuka H. Regulation of palmitoyl-CoA chain elongation and linoleoyl-CoA elongation in rat liver microsomes and the differential effects of peroxisome proliferators, insulin and thyroid hormone. Biochim Biophys Acta. 1985;834:118-123.

36 Kawashima Y, Kozuka H. Increased activity of stearoyl-CoA desaturation in liver from rat fed clofibric acid. Biochim Biophys Acta. 1982;713:622-628.

37 Hirose A, Yamazaki T, Sakamoto T, Sunaga K, Tsuda T, Mitsumoto A, Kudo N, Kawashima Y. Clofibric acid increases the formation of oleic acid in endoplasmic reticulum of the liver of rats. J Pharmacol Sci. 2011;116:362-372.

38 Man WC, Miyazaki M, Chu K, Ntambi J. Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis. J Lipid Res. 2006;47:1928-1939.

39 Miyazak M, Kim Y-C, Ntambi JM. A lipogenic diet in mice with a disruption of the stearoyl-CoA desaturase 1 gene reveals a stringent requirement of endogenous monounsaturated fatty acids for triglyceride synthesis. J Lipid Res. 2001;42:1018-1024.

40 Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Ory DS, Schaffer JE.

Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci USA. 2003;100:3077-3082.

41 Rakhshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, de Groot PJ, Müller M, Kersten S. Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling. PPAR Res. 2007;2007:26839.

42 Huhtinen K, O'Byrne J, Lindquist PJG, Contreras JA, Alexson SEH. The peroxisome proliferator-induced cytosolic type I acyl-CoA thioesterase (CTE-I) is a serine-histidine-aspartic acid α/β hydrolase. J Biol Chem. 2002;277:3424-3432.

43 Dongol B, Shah Y, Kim I, Gonzalez FJ, Hunt MC. The acyl-CoA thioesterase I is regulated by PPARα and HNF4α via a distal response element in the promoter. J Lipid Res. 2007; 48:1781-1791.

44 Kawashima Y, Katoh H, Kozuka H. Sex-related difference in the effect of clofibric acid on induction of two novel long-chain acyl-CoA hydrolases in rat liver. Biochim Biophys Acta. 1982;712:48-56.

45 Sapiro JM, Mashek MT, Greenberg AS, Mashek DG. Hepatic triacylglycerol hydrolysis regulates peroxisome proliferator-activated receptor α activity. J. Lipid Res. 2009;50:1621-1629.

46 Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. Nat Rev Drug Discov. 2008;7:489-503.

47 Nakagawa S, Kawashima Y, Hirose A, Kozuka H. Regulation of hepatic level of fatty-acid-binding protein by hormones and clofibric acid in the rat. Biochem J. 1994;297:581-584.

48 Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc Natl Acad Sci USA. 1997;94:4312-4317.

49 Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand R,

Wahli W, Willson TM, Lenhard JM, Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci USA. 1997;94:4318-4323.

(Figure legends)

Fig. 1. Effects of fibrates on body weight, food consumption and calculated dose. A: Body weight of rats. B: Food consumption of rats. C, D, E: Calculated dose. Rats were fed on a standard diet or a diet admixed with fenofibrate at a dietary concentration of 0.025, 0.05, 0.1 or 0.2% (w/w), bezafibrate at a dietary concentration of 0.025, 0.05, 0.1 or 0.2% (w/w) or clofibric acid at a dietary concentration of 0.05, 0.1, 0.15, 0.2 or 0.3% (w/w) for 14 days. Values are means ± S.D. (n = 4). The amounts of fibrate taken in by a rat were calculated using the regression lines. C, The relationship between the dietary dose and the calculated dose of fenofibrate, y = 670.0x - 0.1 (r^2 = 0.9997); D, The relationship between the dietary dose and the calculated dose of bezafibrate, y = 701.6x - 1.0 (r^2 = 0.9992); E, The relationship between the dietary dose and the calculated dose of clofibric acid, y = 672.8x - 0.4 (r^2 = 0.9998).

Fig. 2. Effects of fibrates on TAG concentration in the liver of rats. A, C, E: The dose-dependent reduction of TAG; rats were fed on a standard diet or a diet admixed with fenofibrate (A) at a dietary concentration of 0.025 (15.2 ± 2.7 mg/kg body weight per day), 0.05 (34.3 ± 5.0 mg/kg body weight per day), 0.1 (67.7 ± 10.4 mg/kg body weight per day) or 0.2% (w/w) (133.4 ± 22.1 mg/kg body weight per day), bezafibrate (C) at a dietary concentration of 0.025 (17.0 ± 3.2 mg/kg body weight per day), 0.05 (34.0 ± 5.3 mg/kg body weight per day), 0.1 (66.6 ± 11.7 mg/kg body weight per day) or 0.2% (w/w) (140.6 ± 25.5 mg/kg body weight per day) or clofibric acid (E) at a dietary concentration of 0.05 (34.1 ± 4.9), 0.1 (66.4 ± 16.4 mg/kg body weight per day), 0.15 (98.8 ± 20.2mg/kg body weight per day), 0.2 (134.5 ± 30.8 mg/kg body weight per day) or 0.3% (w/w) (202.1 ± 40.2 mg/kg body weight per day) for 14 days. B, D, F: The time-dependent reduction of TAG; rats were fed on a diet containing (B) 0.1% (w/w) fenofibrate (67.7 ± 10.4 mg/kg body weight per day), (D)

0.1% (w/w) bezafibrate (66.6 ± 11.7 mg/kg body weight per day) or (F) 0.3% (w/w) clofibric acid (202.1 ± 40.2 mg/kg body weight per day) for 0, 2, 7 or 14 days. Values are means ± S.D. (n = 4). [a, b, c] Differences in the mean without a common superscript (a, b or c) are statistically significant ($P < 0.05$).

Fig. 3. Effects of fibrates on TAG concentration in serum. Rats were treated with fenofibrate, bezafibrate or clofibric acid at various doses for 14 days. The treatments were the same as those described in the legend to Fig. 2A, C and E. A, Fenofibrate; B, bezafibrate; C, clofibric acid.

Fig. 4. Effects of fibrates on mRNA encoding ATGL in the liver of rats. The treatments of rats were the same as described in the legend to Fig. 2. A, C, E: The dose-dependent increase in ATGL mRNA; A, Fenofibrate; C, bezafibrate; E, clofibric acid. B, D, F: The time-dependent increase in ATGL mRNA; B, fenofibrate; D, bezafibrate; F, clofibric acid. Values are means ± S.D. (n = 4). [a, b, c] Differences in the mean without a common superscript (a, b or c) are statistically significant ($P < 0.05$).

Fig. 5. Effects of fibrates on mRNA encoding CGI-58 in the liver of rats. The treatments of rats were the same as described in the legend to Fig. 2. A, C, E: The dose-dependent increase in CGI-58 mRNA; A, Fenofibrate; C, bezafibrate; E, clofibric acid. B, D, F: The time-dependent increase in CGI-58 mRNA; B, fenofibrate; D, bezafibrate; F, clofibric acid. Values are means ± S.D. (n = 4). [a, b, c] Differences in the mean without a common superscript (a, b or c) are statistically significant ($P < 0.05$). In the absence of a superscript, the differences in the means are not significant ($P > 0.05$).

Fig. 6. Relationship between ATGL mRNA level and TAG concentration in the liver of rats.

○, Control; ●, fenofibrate; □, bezafibrate; ▲, clofibric acid. The relationship between TAG concentration (data from Fig. 2A, C and E) and the ATGL mRNA level (data from Fig. 4A, C and E) was determined as Y = -1.0629X + 7.8595 (r^2 = 0.6808).

Fig. 7. Effects of fibrates on TAG hydrolase activity in the liver of rats. Rats were fed on a standard diet or a diet containing 0.1% (w/w) fenofibrate (67.7 ± 10.4 mg/kg body weight per day), 0.1% (w/w) bezafibrate (66.6 ± 11.7 mg/kg body weight per day) or 0.3% (w/w) clofibric acid (202.1 ± 40.2 mg/kg body weight per day) for 14 days. Values are means ± S.D. (n = 4). [a, b] Differences in the mean without a common superscript (a or b) are statistically significant ($P < 0.05$).

Fig. 8. Effects of fibrates on protein expression of ATGL in the liver of rats. Rats were fed on a standard diet or a diet containing 0.1% (w/w) fenofibrate (67.7 ± 10.4 mg/kg body weight per day), 0.1% (w/w) bezafibrate (66.6 ± 11.7 mg/kg body weight per day) or 0.3% (w/w) clofibric acid (202.1 ± 40.2 mg/kg body weight per day) for 14 days. Immunoblot was carried out on liver extracts. Liver extracts (10 μg of protein each) were loaded and separated by electrophoresis. Visible bands represent ATGL and β-actin as indicated. Quantitation of the amount of ATGL was carried out using Multi gauge software (GE Healthcare) and normalized by loaded β-actin. Values represent means ± S.D. (n = 4). [a, b, c] Differences without a common superscript (a, b or c) are statistically significant ($P < 0.05$).

Fig. 9. Up-regulation of the expression of ATGL gene and Acot1 gene by fibrates in the liver. Rats were treated with fenofibrate, bezafibrate or clofibric acid at various doses for 14 days. The treatments were the same as those described in the legend to Fig. 2A, C and E. ○, Control; ●, fenofibrate; □, bezafibrate; ▲, clofibric acid. A: Fibrate-induced elevation of Acot1 mRNA level. Values are means ± S.D. (n = 4). [*] The means (fenofibrate-treated) are

significantly different from the control ($P < 0.05$). [§] The means (bezafibrate-treated) are significantly different from the control ($P < 0.05$). [#] The means (clofibric acid-treated) are significantly different from the control ($P < 0.05$). B: The relationship between the ATGL mRNA level (data from Fig. 4A, C and E) and the Acot1 mRNA level (data from Fig. 9A) was determined as $Y = 0.0434X + 0.4045$ ($r^2 = 0.9383$).

Fig. 10. Hepatic concentrations of fibric acids and their potency with respect to up-regulation of ATGL mRNA expression and reduction of TAG concentration. Rats were treated with fenofibrate, bezafibrate or clofibric acid at various doses for 14 days. The treatments were the same as those described in the legend to Fig. 2A, C and E. ○, Control; ●, fenofibrate; □, bezafibrate; ▲, clofibric acid. A: The concentrations of active metabolites, fibric acids, in the liver. B: The relationship between ATGL mRNA level (data from Fig. 4A, C and E) and fibric acid concentration (data from Fig. 10A) was determined as $Y = 0.0088X + 0.6454$ ($r^2 = 0.9025$). C: The relationship between hepatic ATG concentration (data from Fig. 2A, C and E) and fibric acid concentration (data from Fig. 10A) was determined as $Y = -0.0107X + 7.4985$ ($r^2 = 0.8008$).

Table 1. Sequences of primers used for real-time PCR

	Primer (5' - 3')	Accession No.
ATGL	F: TCACCAACACCAGCATCCAA	NM_001108509
	R: TCCATCTCGGTAGCCCTGTTT	
CGI-58	F: TGCATAGATGGCAACTCTGGC	NM_212524
	R: ATACACATAATGCCCTGCCCC	
CPT1a	F: AAGGCAGCGTTCTTCGTGA	NM_031559
	R: GTCAAAGCATCTTCCATGC	
MCAD	F: CTTTGCCTCTATTGCGAAGGC	J02791
	R: TCCGAAAATCTGCACAGCATC	
LCAD	F: TGTATTGGTGCCATAGCCATGA	L11276
	R: CCCAGACCTTTTGGCATTTGT	
Acox1	F: ACTACGACGACCTCCCCAAGA	NM_031315
	R: TGGCCACGCAGGTAGTTCA	
FAT/CD36	F: CGAAGGCTTGAATCCTACCG	NM_031561
	R: TGTTGACCTGCAGTCGTTT	
FATP2	F: TTCAACAGTGGCGATCTCCTG	NM_031736
	R: ACCGGAAGGTGTCTCCAACT	
FATP4	F: CCTGGTGTACTATGGATTCCGC	NM_001100706
	R: GCTGAAAACTTCTTCCGGATCA	
FATP5	F: TTGCGAACGTACGGCAAGTAG	NM_024143
	R: AAGGCGGTCTCGGAAGTAGAAG	
FABPpm	F: TCTGCCAATCCTATGCCAA	NM_013177
	R: CACCCTTTTGGCTTCTTC	
FABP1	F: CGGCAAGTACCAAGTGCAGAG	BC086947
	R: CTGACACCCCCTTGATGTCCT	
ACSL1	F: TCAGAGCAGTTCATCGGCATC	NM_012820
	R: GTCGGTTCCAAGCGTGTCATA	
ACSL3	F: GGTGGCCAAAATGTGACAATG	NM_057107
	R: AAACTCTCCAATATCGCCAGT	
ACSL5	F: CAAACATGGCTGCTTTCCTCA	NM_053607
	R: ACCCTGGACAAGCCTCTCAAA	
FAS	F: CGCCGACCAGTATAAACCCA	M76767
	R: GTTGTAATCGGCACCCAAGTC	
SCD1	F:TCACCTTGAGAGAAGAATTAGCA	J02585
	R: TTCCCATTCCCTTCACTCTGA	
Elovl6	F: AGAACACGTAGCGACTCCGAA	AB071986
	R: CAAACGCGTAAGCCCAGAAT	
DGAT1	F: CCGTGGTATCCTGAATTGGT	NM_053437
	R: GGCGCTTCTCAATCTGAAAT	
DGAT2	F: ATCTTCTCTGTCACCTGGCT	NM_001012345
	R: ACCTTTCTTGGGCGTGTTCC	
GPAT1	F: AGACACAGGCAGGGAATCCAC	AF021348
	R: AATTCCCGGAGAAGCCCAG	
GPAT4	F: TTGGAGTCCTGGAATTTGCTGA	NM_001047849
	R: GGCTAATCCCTGTGAATGCCA	

Acot1	F: ACTACGACGACCTCCCCAAGA	NM_031315.1
	R: TGGCCACGCAGGTAGTTCA	
β-Actin	F: TGCAGAAGGAGATTACTGCC	V01217
	R: CGCAGCTCAGTAACAGTCC	

Table 2. Effects of fibrates on gene expression in the liver

Genes	Control	Fenofibrate	Bezafibrate	Clofibric acid
Lipid degradation				
ATGL	1.0 ± 0.05 [a]	4.45 ± 0.69 [b]	4.25 ± 0.82 [b]	3.32 ± 0.12 [b]
CGI-58	1.0 ± 0.12 [a]	3.95 ± 0.24 [b]	3.13 ± 0.87 [bc]	2.10 ± 0.96 [ab]
CPT1a	1.0 ± 0.28[a]	2.95 ± 0.44[b]	3.19 ± 0.37[b]	2.76 ± 1.24[b]
MCAD	1.0 ± 0.18[a]	4.53 ± 0.58[b]	3.58 ± 0.33[bc]	2.63 ± 0.64[c]
LCAD	1.0 ± 0.40 [a]	3.68 ± 0.30 [b]	3.39 ± 0.40 [b]	2.88 ± 0.46 [b]
Acox1	1.0 ± 0.06 [a]	27.56 ± 3.95 [b]	33.08 ± 10.58 [b]	9.42 ± 2.52 [a]
Fatty acid trafficking				
FAT/CD36	1.0 ± 0.32[a]	17.26 ± 1.17[b]	9.32 ± 1.77[c]	23.05 ± 3.67[d]
FATP2	1.0 ± 0.02[a]	5.15 ± 0.65[b]	4.24 ± 0.36[b]	4.26 ± 1.11[b]
FATP4	1.0 ± 0.09	1.34 ± 0.35	1.35 ± 0.25	1.14 ± 0.30
FATP5	1.0 ± 0.09[a]	0.38 ± 0.08[b]	0.41 ± 0.08[b]	0.33 ± 0.11[b]
FABPpm	1.0 ± 0.14	1.68 ± 0.30	0.61 ± 0.56	1.46± 0.30
FABP1	1.0 ± 0.25[a]	6.72 ± 0.78[b]	5.12 ± 0.91[c]	2.53 ± 0.53[d]
ACSL1	1.0 ± 0.15 [a]	3.64 ± 0.34 [b]	3.11 ± 0.19 [c]	2.23 ± 0.22 [d]
ACSL3	1.0 ± 0.05[a]	10.76 ± 3.39[b]	7.53 ± 0.76[bc]	5.05 ± 1.43[c]
ACSL5	1.0 ± 0.15[a]	1.54 ± 0.37[b]	0.72 ± 0.05[a]	0.69± 0.13[a]
Lipogenesis				
FAS	1.0 ± 0.49 [a]	2.52 ± 1.07 [b]	1.19 ± 0.27 [ab]	1.84 ± 0.33 [ab]
SCD1	1.0 ± 0.24 [a]	17.56 ± 4.58 [b]	19.84 ± 6.61 [b]	20.73 ± 1.44 [b]
Elovl6	1.0 ± 0.55 [a]	18.35 ± 7.43 [b]	17.60 ± 5.33 [b]	27.81 ± 9.50 [b]
DGAT1	1.0 ± 0.49 [a]	2.62 ± 1.05 [b]	1.73 ± 0.83 [ab]	1.80 ± 0.41 [ab]
DGAT2	1.0 ± 0.12 [a]	0.69 ± 0.09 [b]	0.75 ± 0.07 [b]	0.59 ± 0.07 [b]
GPAT1	1.0 ± 0.03	1.68 ± 0.33	1.19 ± 0.16	1.12 ± 0.37
GPAT4	1.0 ± 0.21 [a]	1.46 ± 0.25 [b]	1.42 ± 0.12 [b]	0.49 ± 0.16 [c]

Rats were fed on the standard diet or diet containing 0.1% (w/w) fenofibrate (67.7 ± 10.4 mg/kg body weight per day), 0.1% (w/w) bezafibrate (66.6 ± 11.7 mg/kg body weight per day) or 0.3% (w/w) clofibric acid (202.1 ± 40.2 mg/kg body weight per day) for 14 days. Values represent means ± S.D. (n =4–6). Differences in horizontal means without a common superscript (a, b, c, d) are significant ($p < 0.05$). In the absence of a superscript, the differences in the means are not significant ($P > 0.05$).

Fig. 1

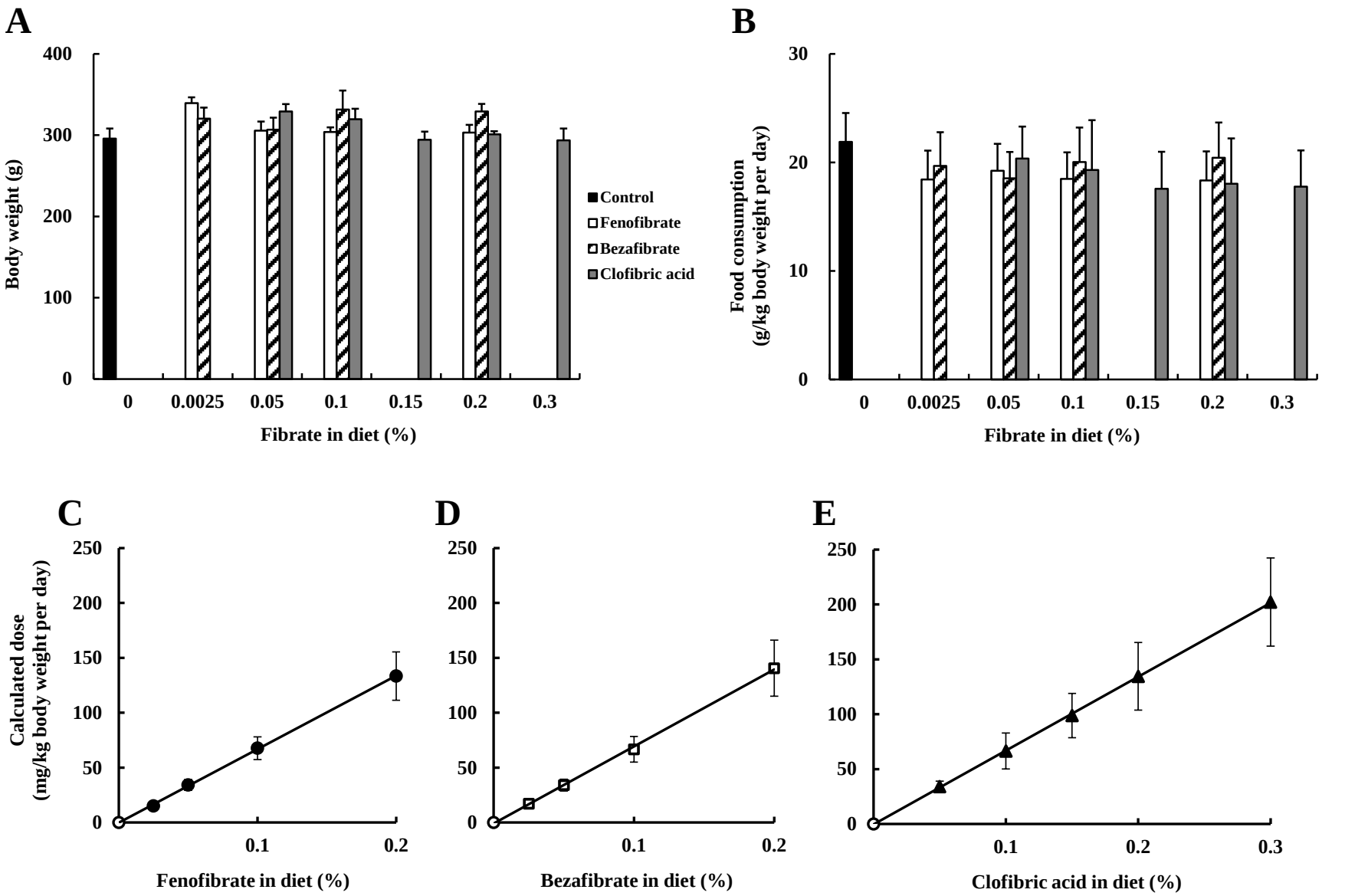

A
Body weight (g)
400
300
200
100
0
0
0.0025
0.05
0.1
0.15
0.2
0.3
Fibrate in diet (%)
Control
Fenofibrate
Bezafibrate
Clofibric acid
B
Food consumption (g/kg body weight per day)
30
20
10
0
0
0.0025
0.05
0.1
0.15
0.2
0.3
Fibrate in diet (%)
C
Calculated dose (mg/kg body weight per day)
250
200
150
100
50
0
0.1
0.2
Fenofibrate in diet (%)
D
250
200
150
100
50
0
0.1
0.2
Bezafibrate in diet (%)
E
250
200
150
100
50
0
0.1
0.2
0.3
Clofibric acid in diet (%)

Fig. 2

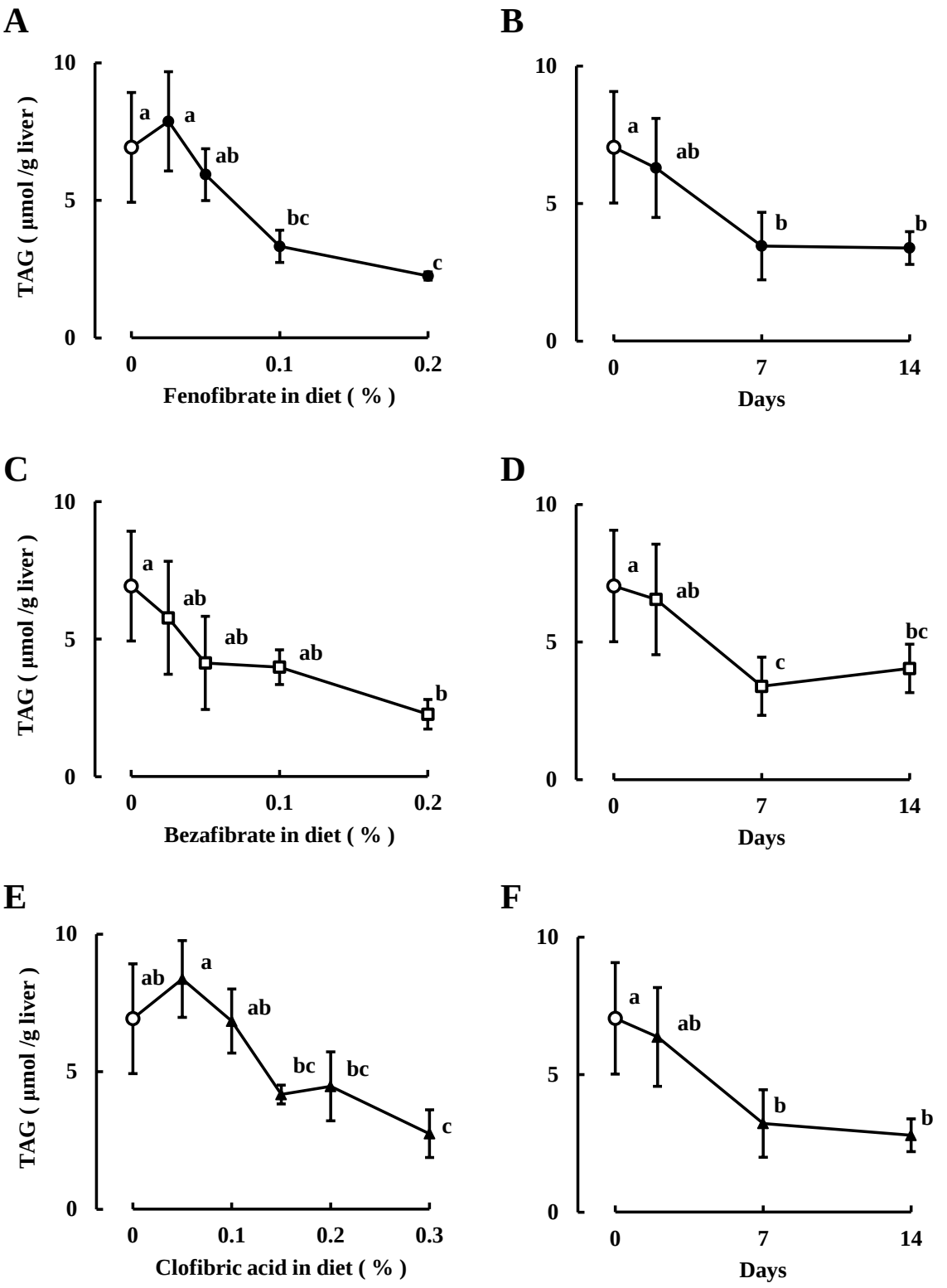

A
B
C
D
E
F
TAG (µmol /g liver)
10
5
0
a
a
ab
bc
c
0
0.1
0.2
Fenofibrate in diet (%)
ab
b
b
7
14
Days
ab
ab
ab
b
Bezafibrate in diet (%)
ab
c
bc
ab
a
ab
bc
bc
c
0.3
Clofibric acid in diet (%)

Fig. 3

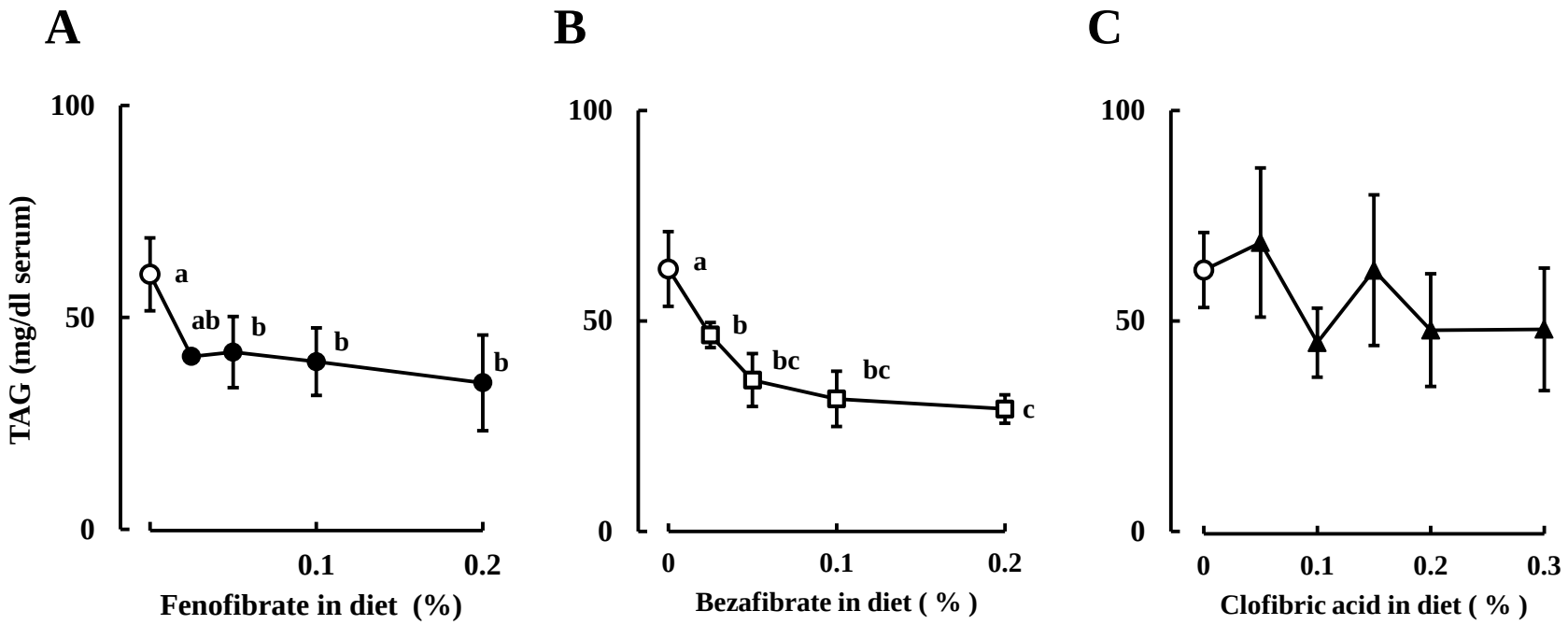

A
B
C
TAG (mg/dl serum)
100
50
0
a
ab
b
b
b
0.1
0.2
Fenofibrate in diet (%)
a
b
bc
bc
c
0
0.1
0.2
Bezafibrate in diet (%)
0
0.1
0.2
0.3
Clofibric acid in diet (%)

Fig. 4

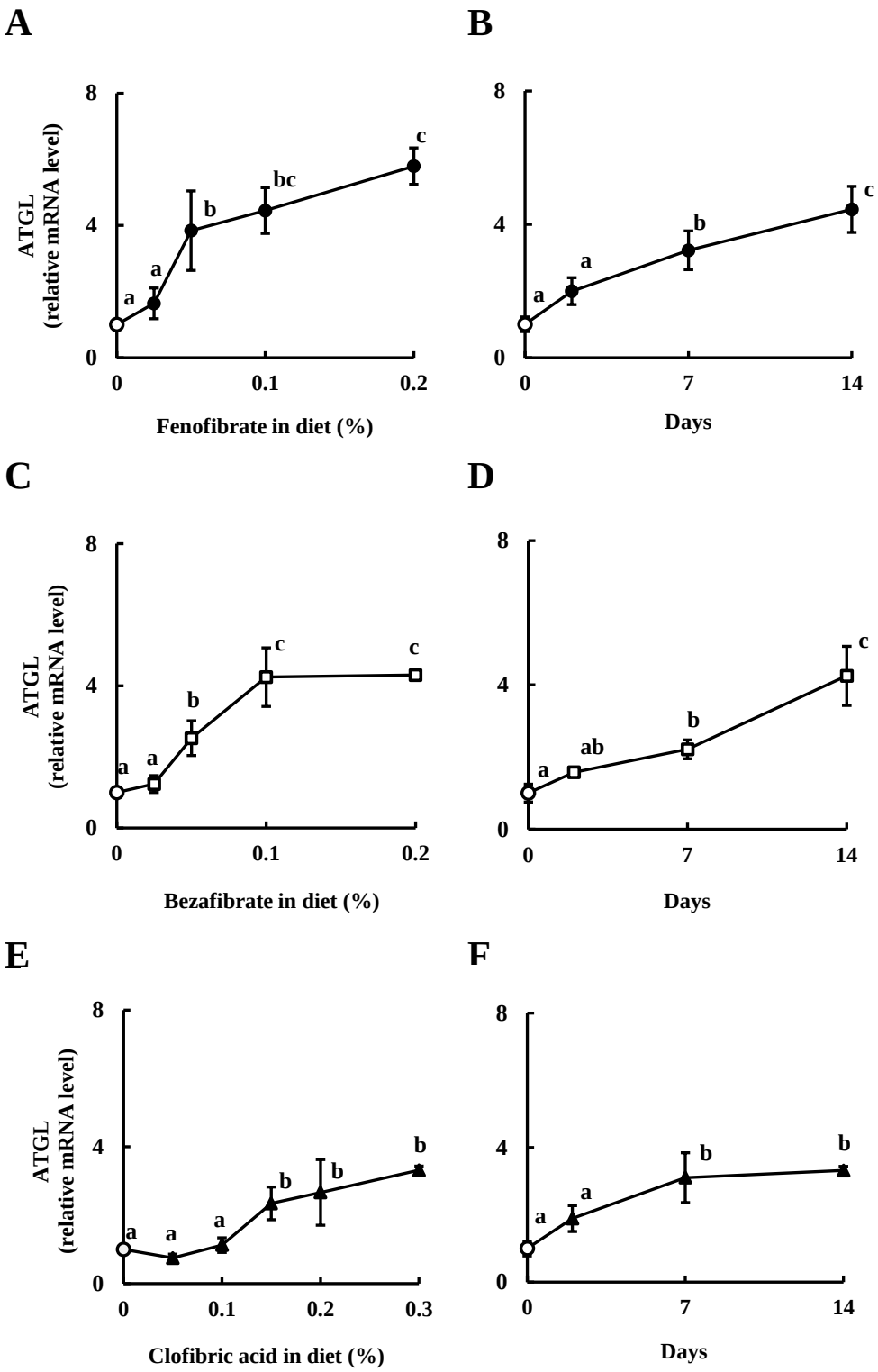

A
B
C
D
E
F
ATGL
(relative mRNA level)
Fenofibrate in diet (%)
Days
Bezafibrate in diet (%)
Clofibric acid in diet (%)
0
4
8
0.1
0.2
0.3
7
14
a
ab
b
bc
c

Fig. 5

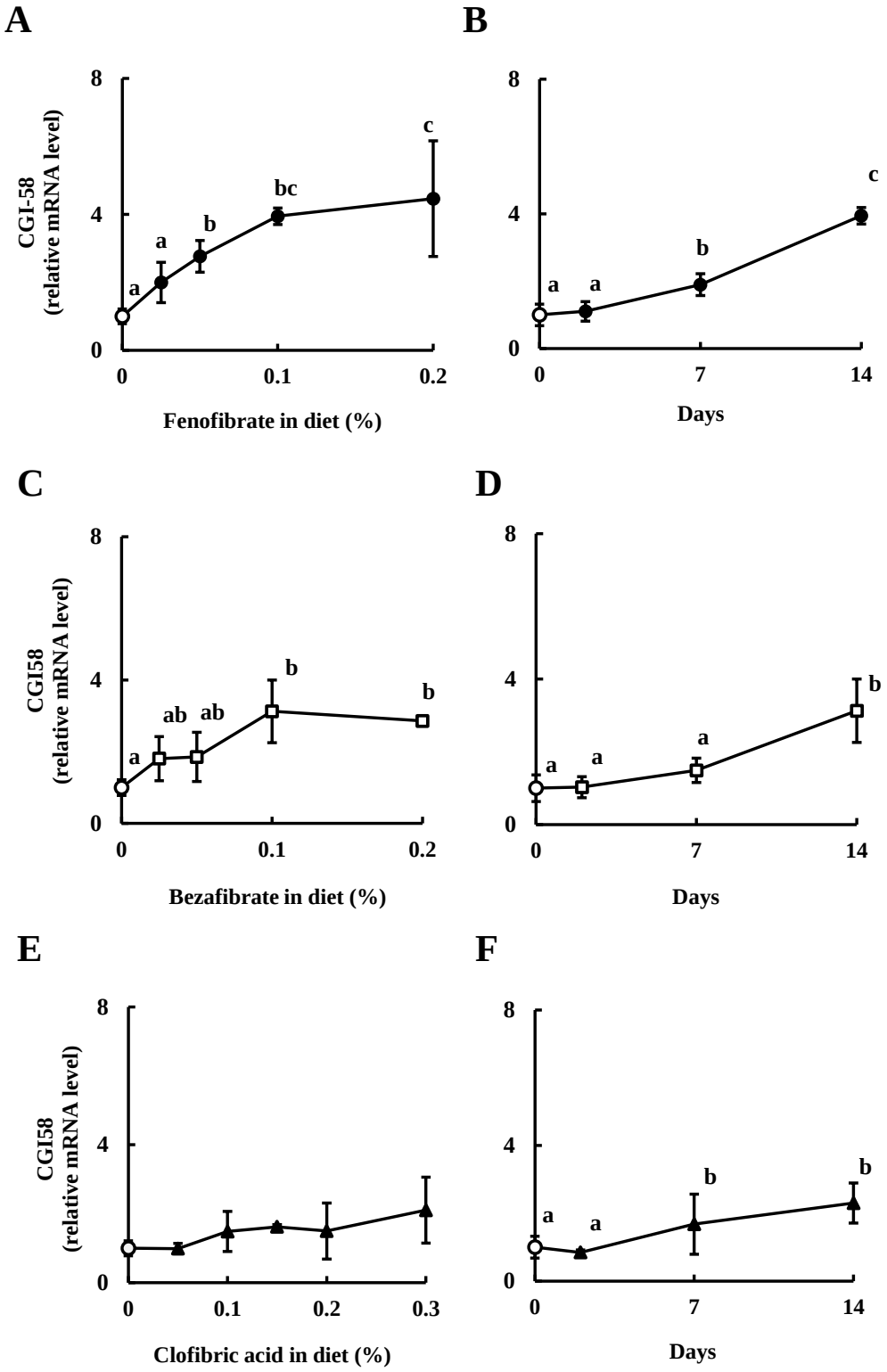

A
B
C
D
E
F
CGI-58 (relative mRNA level)
CGI58 (relative mRNA level)
Fenofibrate in diet (%)
Bezafibrate in diet (%)
Clofibric acid in diet (%)
Days
0
4
8
0.1
0.2
0.3
7
14
a
ab
b
bc
c

Fig. 6

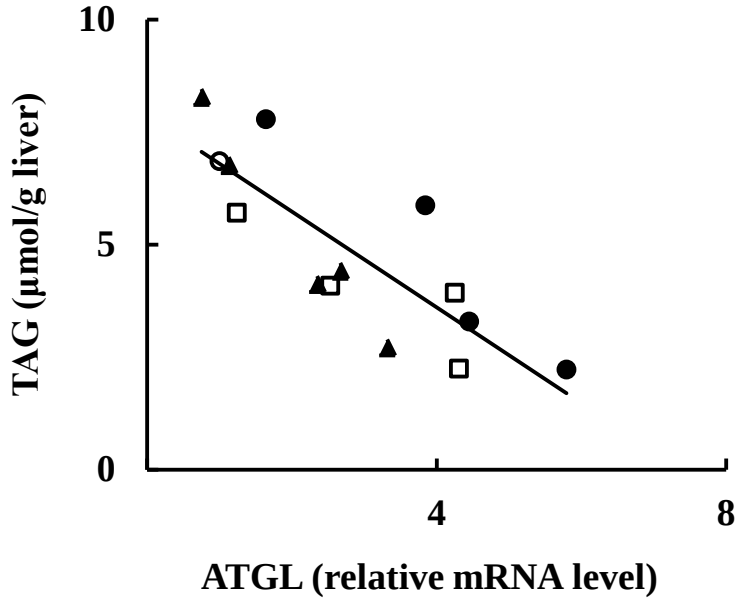


Fig. 7

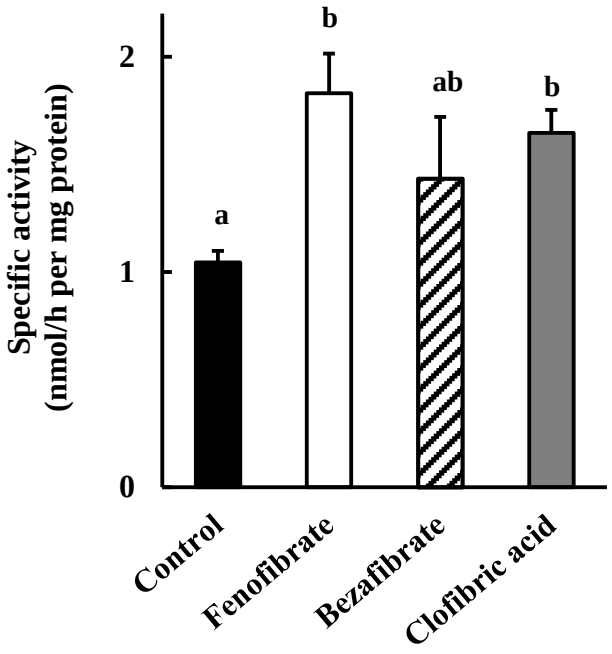
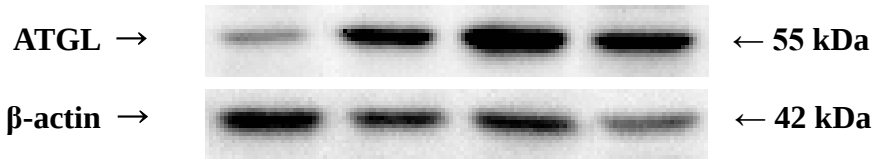

Specific activity
(nmol/h per mg protein)
2
1
0
a
b
ab
b
Control
Fenofibrate
Bezafibrate
Clofibric acid

Fig. 8


ATGL →
← 55 kDa
β-actin →
← 42 kDa

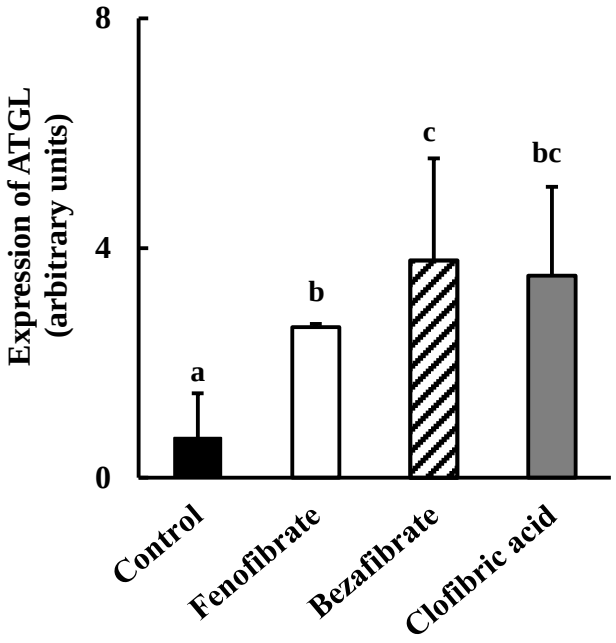
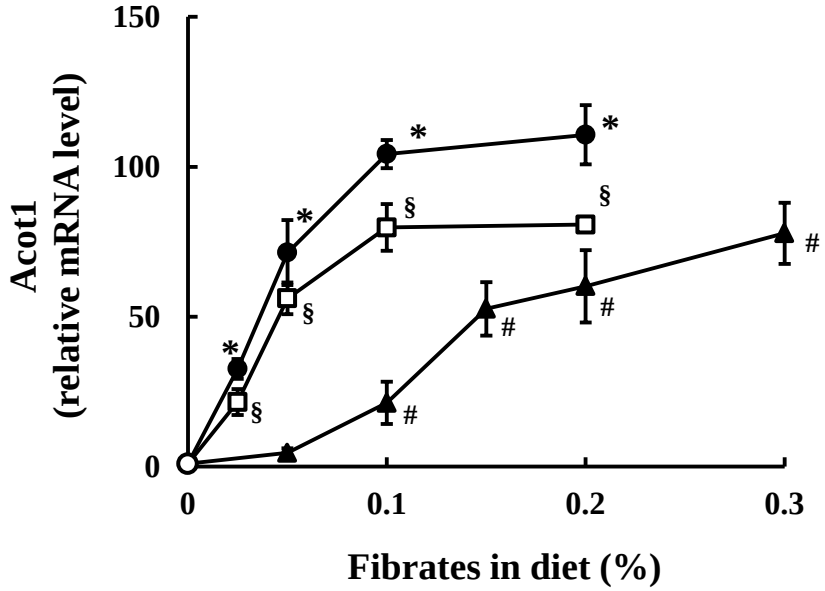

Expression of ATGL (arbitrary units)
8
4
0
a
b
c
bc
Control
Fenofibrate
Bezafibrate
Clofibric acid

Fig. 9

A


150
100
50
0
Acot1
(relative mRNA level)
0
0.1
0.2
0.3
Fibrates in diet (%)
*
§
#

B

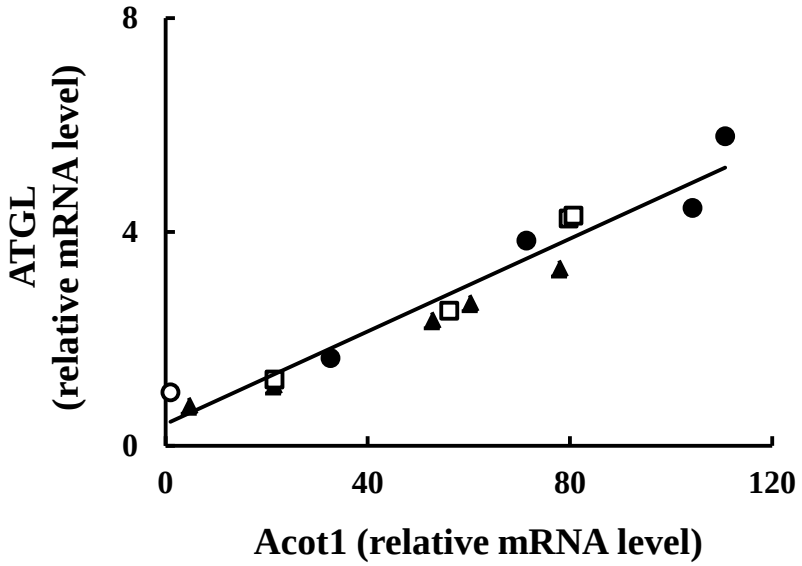
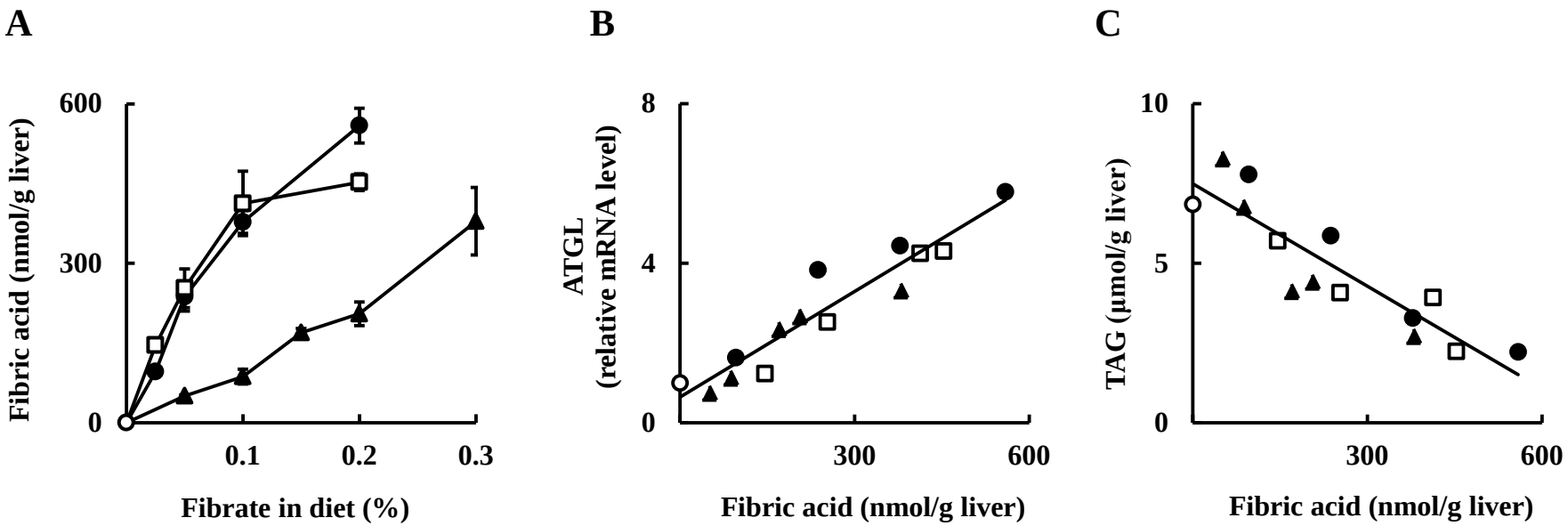

8
4
0
ATGL
(relative mRNA level)
0
40
80
120
Acot1 (relative mRNA level)

Fig. 10


A
Fibric acid (nmol/g liver)
600
300
0
0.1
0.2
0.3
Fibrate in diet (%)
B
ATGL
(relative mRNA level)
8
4
0
300
600
Fibric acid (nmol/g liver)
C
TAG (μmol/g liver)
10
5
0
300
600
Fibric acid (nmol/g liver)