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Ezetimibe Markedly Reduces Hepatic Triglycerides and Cholesterol in Rats Fed on Fish Oil by Increasing the Expression of Cholesterol Efflux Transporters[S]

Yuji Tanaka, Takanori Ikeda, Hiroshi Ogawa, and Toshinori Kamisako

Department of Clinical Laboratory Medicine, Kindai University Faculty of Medicine, Osakasayama, Osaka, Japan (Y.T., T.K.), and Faculty of Human Sciences, Tezukayama Gakuin University, Sakai, Osaka, Japan (T.I., H.O.)

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ABSTRACT

Besides diet therapy, hypolipidemic pharmacological therapy may be a crucial component of nonalcoholic fatty liver disease (NAFLD) treatment. Ezetimibe may be a promising drug for treatment of NAFLD. n-3 polyunsaturated fatty acids, which are abundant in fish oil, reduce serum and hepatic cholesterol and triglycerides in rodents. The aim of this study was to examine the combined effects of dietary fish oil and ezetimibe on lipid metabolism in rats. Seven-week-old male Sprague-Dawley rats were allocated to four different diets containing 1) 10% soybean oil (C), 2) 10% fish oil (F), 3) 10% soybean oil + 0.005% ezetimibe, and 4) 10% fish oil + 0.005% ezetimibe (F+E) for 4 weeks, when the liver, jejunum, blood, and fecal samples were collected. Compared with the C group, the F+E diet decreased hepatic triglycerides and cholesterol 84% and 86%, but it did not increase fecal cholesterol. In liver, the expression of lipogenic enzymes was decreased in the F+E diet, whereas β-oxidation–related genes were not increased. Abcg5/g8 mRNA expression was increased 1380%/442% when ezetimibe was added to the F diet. These gene expression changes are related to the decrease in hepatic lipids. In jejunum, Abcg5/g8 mRNA was increased 244%/841% when ezetimibe was added to the F diet. Hepatic induction of Abcg5/8 rather than intestinal induction correlates with the marked decrease in liver cholesterol when ezetimibe was added to the F diet. These data suggest that fish oil diet and ezetimibe in combination may be a beneficial therapy for NAFLD by increasing hepatic Abcg5/g8 and decreasing lipogenic genes.

SIGNIFICANCE STATEMENT

There is currently no single treatment for NAFLD. Thus, lifestyle modifications including dietary regulation and physical activity are also important options. In this study, ezetimibe, a cholesterol absorption inhibitor, was evaluated for the treatment of liver steatosis in rats fed on the different diets. We found that ezetimibe and fish oil in combination markedly improved fatty liver by increasing cholesterol efflux transporters. The combination therapy of fish oil agents and ezetimibe may be effective for NAFLD.

Introduction

Nonalcoholic fatty liver (NAFL) disease (NAFLD) is the hepatic manifestation of metabolic syndrome, and it includes a wide spectrum of liver diseases, ranging from NAFL to nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma (Preiss and Sattar, 2008). The prevalence of NAFLD is rapidly increasing worldwide owing to the rising incidence of obesity. Apart from lifestyle modifications, including dietary regulation and exercise, several pharmacological agents have been evaluated for the treatment of NAFLD (Armstrong et al., 2016; Anushiravani et al., 2019; Aso et al., 2019). The American Association for the Study of Liver Diseases guidelines state that pioglitazone or vitamin E improves liver histology in patients with biopsy-proven NASH and therefore may be used to treat these patients (Chalasani et al., 2018). However, there are no established treatment options for NAFLD at present.

As patients with NAFLD frequently have dyslipidemia, lipid-lowering agents, including statins and ezetimibe, are expected to have potential therapeutic benefits in these patients. Ezetimibe is a cholesterol-lowering drug that inhibits NPC1L1, which is responsible for intestinal cholesterol absorption. Previous studies have demonstrated that ezetimibe prevents liver steatosis in mice fed on a high-fat diet and also in obese Zucker rats (Deushi et al., 2007; Ushio et al.,

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ABBREVIATIONS: Abc, ATP-binding cassette transporter; ACC, acetyl-CoA carboxylase; Acox1, acyl-CoA oxidase 1; Asbt, apical sodium-dependent bile acid transporter; C, 10% soybean oil; CI, confidence interval; E, 10% soybean oil + 0.005% ezetimibe; EPA, eicosapentaenoic acid; F, 10% fish oil; FAS, fatty acid synthase; F+E, 10% fish oil + 0.005% ezetimibe; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Mdr2, multidrug resistance protein 2; NAFL, nonalcoholic fatty liver; NAFLD, NAFL disease; NASH, nonalcoholic steatohepatitis; NPC1L1, Niemann-Pick C1-Like 1; Ost, organic solute transporter; PCR, polymerase chain reaction; PPARα, peroxisome proliferator–activated receptor α; SCD, stearoyl-CoA desaturase; SR-B1, scavenger-receptor class B, type 1; SREBP, sterol regulatory element-binding protein.

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2013). The long-term administration of ezetimibe in human subjects improves the levels of serum lipids and alanine aminotransferase and also improves the histologic features of hepatic steatosis and inflammation, but not fibrosis (Park et al., 2011). In contrast, the Magnetic Resonance Imaging and Elastography in Ezetimibe Versus Placebo for the Assessment of Response to Treatment in NASH trial demonstrated that ezetimibe does not reduce liver fat content as quantified by the magnetic resonance imaging–derived proton density-fat fraction and does not improve liver histology in NASH (Loomba et al., 2015).

The n-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid, are PPARα ligands that are abundant in fish oil and have been shown to improve dyslipidemia and increase insulin sensitivity; therefore, they have been suggested as potential treatment options for NAFLD (Lombardo et al., 2007; Fakhrzadeh et al., 2010). A previous study demonstrated that an EPA-rich diet reduces the serum and hepatic triglyceride levels in leptin-deficient ob/ob mice, which is a commonly studied model of obesity and liver steatosis (Sekiya et al., 2003). A study on rats demonstrated that the administration of a high-fat diet supplemented with fish oil lowers the hepatic triglyceride and cholesterol levels and improves hepatic steatosis, as observed in a group fed on a high-fat diet without fish oil supplementation (Yuan et al., 2016). A previous study on human subjects demonstrated that the administration of 2.7 g of EPA on a daily basis for 12 months reduces the serum levels of alanine aminotransferase and improves hepatic steatosis, as revealed by ultrasound imaging and analysis of the histologic features of hepatic steatosis, inflammation, and fibrosis (Tanaka et al., 2008). In contrast, a recent multicenter trial performed in North America demonstrated that ethyl-EPA, a synthetic polyunsaturated fatty acid, does not improve the levels of liver enzymes or the hepatic histology (Sanyal et al., 2014).

The present study aimed to examine the combined effects of fish oil and ezetimibe on the serum and hepatic levels of lipids by administering a menhaden fish oil diet or a control soybean oil diet, either unsupplemented or supplemented with ezetimibe, to male Sprague-Dawley rats for 4 weeks, after which the changes in the triglyceride and cholesterol status were analyzed by studying the lipid profiles and the expression of lipid and bile acid metabolism–related genes in the liver and jejunum.

Materials and Methods

Chemicals and Reagents. TRIzol reagent was purchased from Life Technologies (Tokyo, Japan), the PrimeScript RT reagent kit and TB Green Premix Ex Taq were purchased from Takara Bio (Shiga, Japan), and ezetimibe was purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Diet. A control diet, containing 10% soybean oil as the fat source by weight, and three different experimental diets, containing 10% fish oil, 10% soybean oil with 0.005% ezetimibe, or 10% fish oil with 0.005% ezetimibe, were all purchased from Research Diets (New Brunswick, NJ). The composition of the control and experimental diets is shown in Supplemental Table 1.

Animals. Six-week-old male Sprague-Dawley rats were obtained from CLEA Japan (Shizuoka, Japan) and allowed to acclimate for 1 week in housing before beginning experiments. All rats were housed in the same animal care facility controlling for light, temperature, and humidity. Rats (n = 7/group) were allocated to four different diets containing 1) 10% soybean oil (C; control group), 2) 10% menhaden oil (F; fish oil group), 3) 10% soybean oil plus 0.005% ezetimibe (E; ezetimibe group), and 4) 10% fish oil plus 0.005% ezetimibe (F+E; fish oil plus ezetimibe group) for 4 weeks. The dose of ezetimibe was chosen with reference to previous studies (Zheng et al., 2008). After 4 weeks, fecal samples were collected, blood was taken from the abdominal aorta under anesthesia with isoflurane, and livers were harvested as well as jejunum. Studies were approved by Kindai University Faculty of Medicine Animal Care and Use Committee.

Quantification of Serum, Hepatic, and Fecal Triglycerides and Cholesterol. Serum cholesterol and triglycerides were quantified by an AutoAnalyzer. Hepatic and fecal lipid content was extracted according to the method reported previously (Folch et al., 1957). The amount of triglycerides and cholesterol in the liver and feces was determined enzymatically using commercially available kits (Cholesterol E-test and Triglyceride E-test, respectively; Wako Pure Chemical Industries).

Histopathology. Liver tissue was fixed in 10% formalin and embedded in paraffin. H&E was performed on each sample. Liver sections were stained with H&E and evaluated for hepatocellular steatosis.

RNA Isolation. Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Life Technologies). The concentration of total RNA in each sample was determined spectrophotometrically at 260 nm.

Real-Time Polymerase Chain Reaction. The mRNA expression level of 18s ribosomal RNA and cholesterol, bile acid, and fatty acid metabolism–related genes, including sterol regulatory element–binding protein (SREBP)-1c, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD), fatty acid translocase (CD36), peroxisome proliferator–activated receptor α (PPARα), acyl-CoA oxidase 1 (Acox1), carnitine palmytoyltransferase 1, low-density lipoprotein receptor, scavenger-receptor class B, type 1 (SR-B1), Niemann-Pick C1-like 1 (NPC1L1), ATP-binding cassette transporter g5 (Abcg5), Abcg8, Abca1, multidrug resistance protein 2 (Mdr2), cholesterol 7α-hydroxylase (Cyp7a1), sterol-27α-hydroxylase (Cyp27a1), sterol-12α-hydroxylase (Cyp8b1), 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase), HMG-CoA reductase, apical sodium-dependent bile acid transporter (Asbt), organic solute transporter α (Ostα), and Ostβ, were quantified by SYBR real-time polymerase chain reaction (PCR). One of the primers was designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA). Other primers were used as indicated in previous reports, and the sequences of these primers are shown in Supplemental Table 2 (Ballatori *et al.*, 2005; Baumgardner *et al.*, 2008; Chen *et al.*, 2009; Boone *et al.*, 2011; Gao *et al.*, 2013; Wang *et al.*, 2013; Sun *et al.*, 2014; Tu *et al.*, 2014; Madsen *et al.*, 2015; Michihara *et al.*, 2015; Kawashima *et al.*, 2018; van Golen *et al.*, 2018; Wei *et al.*, 2018). The amplification reactions were performed in an ABI Prism 7900 sequence detection system (Applied Biosystems). The amount of mRNA was calculated using the comparative cycle threshold method, which determines the amount of target normalized to an endogenous reference. Each gene was normalized to 18s ribosomal RNA.

Statistical Analysis. The number of animals was determined based on power analysis and previous reports (van Heek et al., 2001; Wang et al., 2014). The software package SYSTAT, version 11 (Systat Inc., Evanston, IL), was used for statistical analysis. All data were analyzed using one-way ANOVA, followed by Tukey's post hoc test. Differences were considered statistically significant at $P < 0.05$.

Results

Weight Gain and Liver Weight. After feeding each diet for 4 weeks, weight gain did not differ among the groups. The liver weight was decreased 14% in the F-fed rats (95% CI:

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TABLE 1

Body weight increase; liver weight; and serum, hepatic, and fecal concentrations of lipids.

Data are presented as means ± S.D. (each group, n = 7 animals). Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Significance was set at $P < 0.05$.

	C	F	E	F+E
Body weight increase (g)	337.4 ± 34.2	316.6 ± 19.0	307.0 ± 31.9	306.1 ± 40.5
Liver weight (g)	22.6 ± 2.5	19.5 ± 1.5[a]	19.8 ± 2.0	19.5 ± 2.1[b]
Serum triglyceride (mg/dl)	392.4 ± 157.1	106.3 ± 31.8[c]	241.0 ± 102.0[d]	68.0 ± 27.4[e]
Serum total cholesterol (mg/dl)	74.3 ± 11.5	50.1 ± 8.7[f]	66.7 ± 6.9	47.4 ± 9.5[g]
Serum HDL cholesterol (mg/dl)	25.9 ± 3.1	15.3 ± 2.4[h,i]	27.8 ± 1.2	20.0 ± 3.3[j]
Liver triglyceride conc. (mg/g tissue)	23.0 ± 8.7	13.9 ± 5.4	32.2 ± 10.6	3.6 ± 1.2[k]
Whole-liver triglyceride content (mg/whole liver)	524.0 ± 228.6	270.5 ± 115.4[l]	624.3 ± 172.6	70.8 ± 28.4[m]
Liver cholesterol conc. (mg/g tissue)	1.6 ± 0.5	1.2 ± 0.7	1.9 ± 0.9	0.2 ± 0.1[n,o]
Whole-liver cholesterol content (mg/whole liver)	36.0 ± 10.2	23.4 ± 14.2	36.3 ± 12.5	4.4 ± 1.3[p,q]
Fecal triglyceride (mg/g feces)	0.8 ± 0.2	0.4 ± 0.2[r]	0.6 ± 0.2	0.4 ± 0.1[s]
Fecal cholesterol (mg/g feces)	2.5 ± 0.5	3.6 ± 0.5[t]	3.2 ± 0.7	2.6 ± 0.4[u]

[a] $P = 0.041$, significant difference from C.
[b] $P = 0.045$, significant difference from C.
[c] $P < 0.001$, significant difference from C.
[d] $P = 0.033$, significant difference from C.
[e] $P < 0.001$, significant difference from C.
[f] $P < 0.001$, significant difference from C.
[g] $P < 0.001$, significant difference from C.
[h] $P < 0.001$, significant difference from C.
[i] $P < 0.001$, significant difference from E.
[j] $P = 0.002$, significant difference from C.
[k] $P < 0.001$, significant difference from C.
[l] $P = 0.026$, significant difference from F.
[m] $P < 0.001$, significant difference from C.
[n] $P = 0.001$, significant difference from C.
[o] $P = 0.026$, significant difference from F.
[p] $P < 0.001$, significant difference from C.
[q] $P = 0.015$, significant difference from F.
[r] $P = 0.002$, significant difference from C.
[s] $P < 0.001$, significant difference from C.
[t] $P = 0.005$, significant difference from C.
[u] $P = 0.009$, significant difference from F.

9%–19%; $P = 0.041$) and 14% in the F+E group (95% CI: 7%–21%; $P = 0.045$), whereas the E-fed rats (absolute mean difference 13%; 95% CI: 6%–19%; $P = 0.071$) tended to reduce liver weight compared with the C group (Table 1).

Serum, Hepatic, and Fecal Concentrations of Triglyceride and Cholesterol. Compared with the C group, feeding the F diet reduced serum triglyceride levels 73% (95% CI: 67%–79%; $P < 0.001$), 39% when ezetimibe

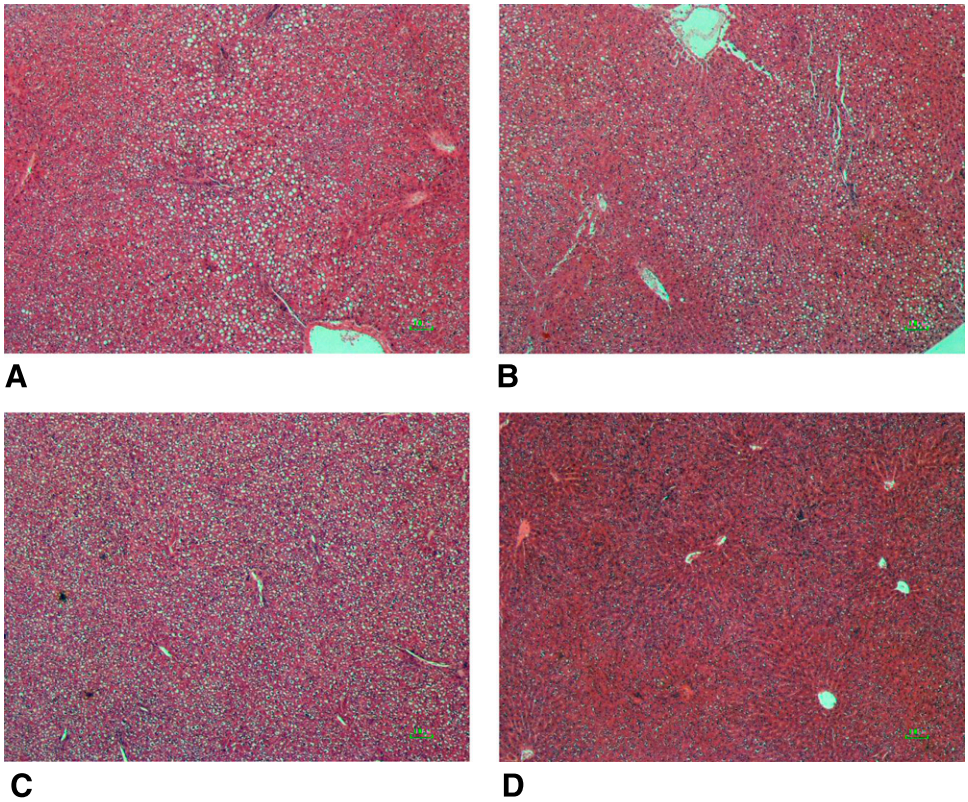

Fig. 1. Histologic examination in rats after 4 weeks of each diet. Liver H&E staining is presented as follows: C-fed (A), F-fed (B), E-fed (C), and F+E-fed (D) rats. Representative images are shown. Scale bar, 100 μm.

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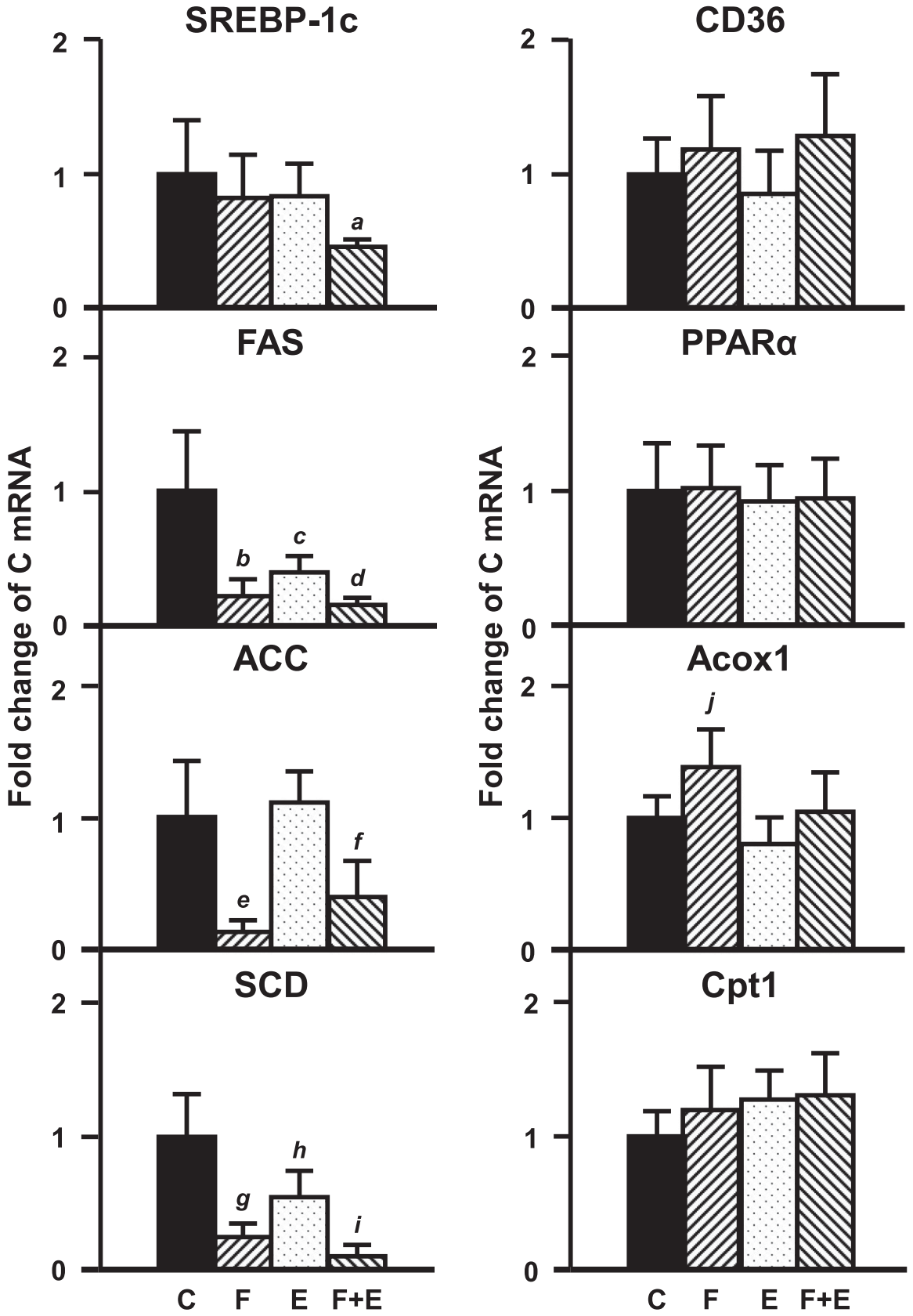

Fig. 2. mRNA expression of fatty acid–related genes in rat liver after 4 weeks of each diet. Total hepatic RNA was isolated from rats, and mRNA levels were quantified by real-time PCR as described in *Materials and Methods*. Data are presented as means ± S.D. (each group, $n = 7$ animals). Rats were allocated to four different diets containing 1) 10% soybean oil (C; control group), 2) 10% menhaden oil (F; fish oil group), 3) 10% soybean oil plus 0.005% ezetimibe (E; ezetimibe group), and 4) 10% fish oil plus 0.005% ezetimibe (F+E; fish oil plus ezetimibe group) for 4 weeks. Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Significance was set at $P < 0.05$. *a*, $P = 0.005$, significant difference from C; *b*, $P < 0.001$, significant

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was added to the C diet (95% CI: 19%–58%; $P = 0.033$), and 83% when ezetimibe was added to the F diet (95% CI: 78%–88%; $P < 0.001$). Serum total cholesterol and the HDL cholesterol were approximately the same in the rats fed the C diet and those that had ezetimibe added to the C diet, but the F diet reduced total cholesterol 33% (95% CI: 24%–41%; $P < 0.001$) and HDL cholesterol 41% (95% CI: 34%–48%; $P < 0.001$), and when ezetimibe was added to the F diet, total cholesterol was reduced 36% (95% CI: 27%–46%; $P < 0.001$) and HDL cholesterol was reduced 23% (95% CI: 13%–32%; $P = 0.002$). Serum HDL cholesterol was 45% (95% CI: 38%–51%; $P < 0.001$) lower in rats fed the F diet than the E diet. The content of triglyceride in the whole liver was decreased 48% (95% CI: 31%–66%; $P = 0.026$) when the C diet was replaced with the F diet, but liver triglyceride concentrations were not decreased. Ezetimibe did not decrease the concentrations and total content of triglyceride in the liver of the rats fed the C diet, but ezetimibe markedly decreased liver triglyceride concentrations 84% (95% CI: 80%–88%; $P < 0.001$) and whole-liver triglyceride content 86% (95% CI: 82%–91%; $P < 0.001$) when added to the F diet. Similarly, liver cholesterol concentrations and whole-liver cholesterol content were not decreased when the C diet was replaced with the F diet or when ezetimibe was added to the C diet, but ezetimibe markedly decreased liver cholesterol concentrations 86% (95% CI: 83%–89%; $P = 0.001$) and whole-liver cholesterol content 88% (95% CI: 85%–91%; $P < 0.001$) when added to the diet of the F-fed rats. Fecal triglycerides were reduced 47% (95% CI: 33%–61%; $P = 0.002$) when the C diet was replaced with the F diet, and ezetimibe did not reduce the triglycerides in the feces of the rats fed the C diet, but ezetimibe reduced fecal triglyceride levels 55% (95% CI: 44%–66%; $P < 0.001$) when added to the F diet. Fecal excretion of cholesterol was 41% (95% CI: 25%–57%; $P = 0.005$) higher in rats fed the F-diet and tended to be higher (absolute mean difference 28%; 95% CI: 9%–48%; $P = 0.078$) in rats fed the E diet than the C diet; however, this increase in fecal cholesterol levels in the F group was attenuated in the F+E group (Table 1).

Histopathology. To determine differences in hepatic steatosis histologically, H&E was conducted. Photomicrographs of H&E-stained liver sections from the C-fed and E-fed rats (Figs. 1A and 1C) demonstrated predominant macrovesicular steatosis in the hepatic lobules, and the degree of steatosis was mild or moderate. H&E-stained liver section from the F-diet rat (Fig. 1B) showed less fat deposition compared with the C-fed and E-fed rats. Furthermore, the F+E-fed rat (Fig. 1D) hardly showed fat deposition in the liver. These results were similar to whole-liver triglyceride content.

Hepatic Expression of Fatty Acid–Related Genes. The mRNA expression of SREBP-1c and its target lipogenic enzymes was quantified. The mRNA expression of SREBP-1c was decreased 55% (95% CI: 51%–59%; $P = 0.005$) in the F+E diet. The mRNA expression of FAS, ACC, and SCD was decreased 78% (95% CI: 70%–86%; $P < 0.001$), 87% (95% CI: 81%–92%; $P < 0.001$), and 76% (95% CI: 69%–83%; $P < 0.001$) in the F-fed rats and 85% (95% CI: 79%–90%; $P < 0.001$), 60% (95% CI: 40%–80%; $P = 0.003$), and 90% (95% CI: 83%–97%; $P < 0.001$) in the F+E diet. However, there were no differences in the mRNA expression of these genes between the F group and the F+E group. The mRNA expression of the fatty acid oxidation genes was determined. The mRNA expression of PPARα target gene Acox1 was increased 38% (95% CI: 18%–59%; $P = 0.038$) in the F-fed rats compared with the C-fed rats (Fig. 2).

Hepatic Expression of Cholesterol Homeostatic Genes and Bile Acid Synthetic Enzymes. Cholesterol levels are maintained by the balance of uptake, synthesis, catabolism, and export. Compared with the C-fed rats, mRNA expression of cholesterol efflux transporters Abcg5 and Abcg8 was induced 1380% (95% CI: 965%–1795%; $P < 0.001$) and 442% (95% CI: 270%–613%; $P < 0.001$) in the F+E diet. The mRNA expression of phospholipid transporter Mdr2 was increased 92% (95% CI: 45%–140%; $P = 0.006$) in the F+E-fed rats compared with the C-fed rats. The mRNA expression of cholesterol synthesis gene HMG-CoA synthase was increased 481% (95% CI: 391%–571%; $P < 0.001$) in the F+E-fed rats compared with the C-fed rats. The mRNA expression of Cyp7a1, the rate-limiting enzyme of bile acid synthesis, was decreased 66% (95% CI: 51%–82%; $P = 0.022$) in the F+E-fed rats compared with the F-fed rats. The mRNA expression of Cyp27a1, a key enzyme in the alternative acidic pathway of bile acid synthesis, tended to be decreased (absolute mean difference 19%; 95% CI: 10%–27%; $P = 0.065$) in the F+E-fed rats compared with the F-fed rats. In contrast, compared with the C-fed and the F-fed rats, the mRNA expression of the cholic acid–synthesizing enzyme Cyp8b1 was increased 89% (95% CI: 61%–117%; $P < 0.001$) and 45% (95% CI: 23%–67%; $P = 0.006$) in the F+E-fed rats, respectively (Fig. 3).

Jejunal Expression of Cholesterol and Bile Acid Metabolism–Related Genes. The mRNA expression of Abcg5 and Abcg8 in jejunum was increased 244% (95% CI: 77%–411%; $P = 0.006$) and 841% (95% CI: 302%–1381%; $P = 0.008$) in the F+E-fed rats compared with the C-fed rats. The mRNA expression of bile acid uptake transporter Asbt was increased 970% (95% CI: 345%–1602%; $P = 0.004$) in the F+E-fed rats compared with the C-fed rats (Table 2). The mRNA expression of the HDL receptor SR-B1 was decreased 56% (95% CI: 48%–65%; $P = 0.044$) in the F-fed rats compared with the E-fed rats.

Discussion

To date, studies have demonstrated that weight loss through dietary regulation and exercise is effective for the treatment of NAFLD. However, the therapeutic efficacy of drugs against NAFLD has not been verified (Huang et al., 2005; Vilar-Gomez et al., 2015). Therefore, to investigate the combined effects of dietary fish oil and ezetimibe on lipid metabolism in rats, four different diets were fed to rats for 4 weeks. On the basis of the data in previous studies, a diet containing 10% soybean oil was used to induce fatty liver in the control group (Ataide et al., 2009; Farias Santos et al.,

difference from C; *c*, $P = 0.001$, significant difference from C; *d*, $P < 0.001$, significant difference from C; *e*, $P < 0.001$, significant difference from C; *f*, $P = 0.003$, significant difference from C; *g*, $P < 0.001$, significant difference from C; *h*, $P = 0.001$, significant difference from C; *i*, $P < 0.001$, significant difference from C; *j*, $P = 0.038$, significant difference from C.

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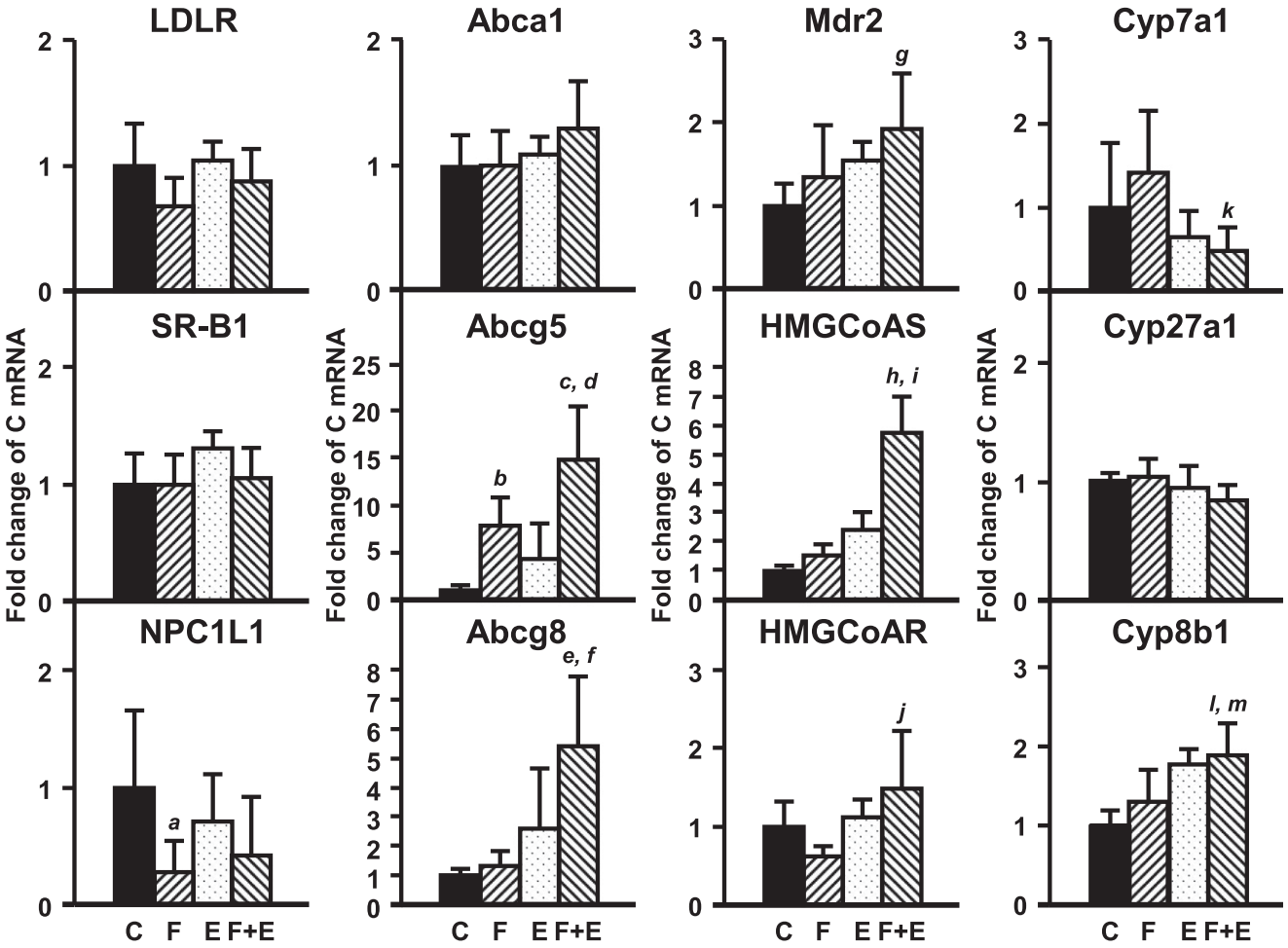

Fig. 3. mRNA expression of cholesterol homeostatic genes, bile acid synthetic enzymes, and phospholipid transporter in rat liver after 4 weeks of each diet. Total hepatic RNA was isolated from rats, and mRNA levels were quantified by real-time PCR as described in *Materials and Methods*. Data are presented as means ± S.D. (each group, $n = 7$ animals). Rats were allocated to four different diets containing 1) 10% soybean oil (C; control group), 2) 10% menhaden oil (F; fish oil group), 3) 10% soybean oil plus 0.005% ezetimibe (E; ezetimibe group), and 4) 10% fish oil plus 0.005% ezetimibe (F+E; fish oil plus ezetimibe group) for 4 weeks. Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Significance was set at $P < 0.05$. *a*, $P = 0.049$, significant difference from C; *b*, $P = 0.01$, significant difference from C; *c*, $P < 0.001$, significant difference from C; *d*, $P = 0.008$, significant difference from F; *e*, $P < 0.001$, significant difference from C; *f*, $P < 0.001$, significant difference from F; *g*, $P = 0.006$, significant difference from C; *h*, $P < 0.001$, significant difference from C; *i*, $P < 0.001$, significant difference from F; *j*, $P = 0.004$, significant difference from F; *k*, $P = 0.022$, significant difference from F; *l*, $P < 0.001$, significant difference from C; *m*, $P = 0.006$, significant difference from F. LDLR, low-density lipoprotein receptor.

2015). The present study revealed that the combined administration of ezetimibe, a medication used for treating dyslipidemia, and fish oil, which is rich in n-3 polyunsaturated fatty acids, significantly reduced the hepatic levels of lipids and suppressed the formation of lipid droplets in rats, as revealed by histologic analysis. This suppression was stronger in the

TABLE 2

Gene expression of cholesterol and bile acid metabolism–related genes in jejunum.

Genes were categorized according to biologic mechanism. Data are presented as means ± S.D. (each group, $n = 7$ animals). Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Significance was set at $P < 0.05$. The 95% CI and the *P*-value are listed.

		Fold Change of C					
Category	Gene Name	C	F	E	F+E	95% CI	*P*-Value
Cholesterol homeostasis							
	NPC1L1	1.0 ± 0.4	1.1 ± 0.4	1.8 ± 1.1	1.2 ± 0.7	−39% to 70%	0.978, C vs. F+E
	Abca1	1.0 ± 0.9	0.9 ± 0.6	0.6 ± 0.2	0.8 ± 0.2	8%–36%	0.891, C vs. F+E
	Abcg5	1.0 ± 0.4	2.1 ± 0.8	1.9 ± 0.5	3.4 ± 2.3	77%–411%	0.006, C vs. F+E
	Abcg8	1.0 ± 0.4	6.4 ± 3.3	3.8 ± 3.7	9.4 ± 7.3	302%–1381%	0.008, C vs. F+E
	LDLR	1.0 ± 0.4	1.1 ± 0.4	1.4 ± 0.6	1.0 ± 0.3	−16% to 27%	0.996, C vs. F+E
	SR-B1	1.0 ± 0.4	0.6 ± 0.2	1.5 ± 0.9	0.7 ± 0.5	48%–65%	0.044, F vs. E
Bile acid transporter							
	Asbt	1.0 ± 0.5	7.1 ± 3.5	4.1 ± 2.3	10.7 ± 8.5	345%–1602%	0.004, C vs. F+E
	Ostα	1.0 ± 0.5	1.2 ± 0.6	1.9 ± 1.0	1.6 ± 1.3	−36% to 154%	0.612, C vs. F+E
	Ostβ	1.0 ± 0.3	1.4 ± 0.8	2.3 ± 1.1	1.7 ± 1.1	−9% to 156%	0.419, C vs. F+E

LDLR, low-density lipoprotein receptor.

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F+E group than the F group and the group that received only ezetimibe (Fig. 1; Table 1).

Dyslipidemia is observed in some patients with NAFLD. Ezetimibe, a lipid-lowering agent, is considered to be a therapeutic option for NAFLD because it inhibits NPC1L1-mediated cholesterol uptake in the small intestine (Yamagishi et al., 2006; Ostovaneh et al., 2015). NAFLD improves when the production of chylomicrons is reduced, and because cholesterol is necessary for chylomicron synthesis in the small intestine, the inhibition of cholesterol uptake by ezetimibe is thought to be the underlying mechanism for the improvement in NAFLD after ezetimibe treatment (de Bari et al., 2012). A study demonstrated that the improvement in the serum and hepatic levels of triglycerides and cholesterol and fatty liver histology was more significant in Zucker rats fed on a high-fat diet supplemented with ezetimibe in comparison with that of the rats that were fed on a high-fat diet only (Deushi et al., 2007). However, the levels of serum and hepatic cholesterol did not show any improvement in the group that received only ezetimibe in this study, and this was also true for the fatty liver. However, the serum levels of triglycerides did show improvements (Fig. 1; Table 1). The difference in the composition of fatty acids in the feed and the dosage of ezetimibe may have resulted in the discrepancy between the results of the study by Deushi and coworkers and those obtained in the present study.

It is considered that n-3 polyunsaturated fatty acid (PUFA), which is abundant in fish oil, can effectively reduce the serum levels of triglycerides and prevent cardiovascular disorders (Harris et al., 1988; Yagi et al., 2017). A study reported that the diets of patients with NAFLD contain large amounts of saturated fatty acids and cholesterol and small amounts of n-3 PUFA (Musso et al., 2003). Another study reported a decrease in the levels of n-3 PUFA and an increase in the ratio of n-6 fatty acids to n-3 fatty acids in the hepatic triglycerides of patients with NASH (Puri et al., 2007). Although fish oil ameliorates obesity, insulin resistance, and fatty liver in rodents, no consensus has been reached regarding its effect on the levels of hepatic enzymes and fatty liver in human subjects (Sekiya et al., 2003; Lombardo et al., 2007; Tanaka et al., 2008; Sanyal et al., 2014). In this study, the serum levels of triglycerides, cholesterol, and HDL cholesterol were reduced in the F group. However, the difference in the hepatic triglyceride levels between the F group and the control group was not statistically significant, and the levels of hepatic triglycerides were lower in the F group than in the group that received only ezetimibe (Table 1). Considering the decrease in serum HDL cholesterol in the F group, hepatic and jejunal mRNA expression of Abca1, which is involved in HDL particle formation, was not changed among all groups. Compared with the E group, the mRNA expression of the HDL receptor SR-B1 was not induced in the liver but was reduced in the jejunum in the F group, which likely contributed to the decreased serum HDL cholesterol levels by inhibiting its absorption (Bietrix et al., 2006; Duong et al., 2006) (Fig. 3; Table 2).

The hepatic levels of triglycerides and cholesterol were not reduced in the F group and the group that received only ezetimibe. However, for the F+E group, the hepatic levels of lipids were markedly reduced, and the fatty liver histology also showed improvements. The above findings suggested that fish oil and ezetimibe worked synergistically when administered together (Fig. 1; Table 1). The expression of the genes related to lipid metabolism were quantified in this study for elucidating the mechanism underlying the marked suppression in the hepatic lipid levels by fish oil and ezetimibe. The results obtained in this study corresponded to those of existing studies. It was observed that the expression of the fatty acid synthesis genes FAS, ACC, and SCD in the liver decreased in the F group; however, no further reduction was observed for the F+E group (Katsurada et al., 1990; Ntambi, 1992; Xu et al., 1999). Analysis of the expression of the genes associated with β-oxidation revealed that only the expression of Acox1 was increased in the F group (Fig. 2).

The notable changes in gene expression observed in this study were the increased expression of the cholesterol excretion transporters Abcg5/g8 in the liver and the small intestine and the increased hepatic expression of Mdr2 in the F+E group (Fig. 3; Table 2). We have previously reported that the mRNA expression of Abcg5/g8 is increased in the liver and small intestine of mice fed on fish oil (Kamisako et al., 2012). Another study reported that the expression of Abcg5/g8 is increased in the liver of mice that were administered ezetimibe (Altemus et al., 2014). These findings suggest that the combined administration of fish oil and ezetimibe has an additive effect in increasing the expression of Abcg5/g8. It has been observed that a high-fat, high-cholesterol diet causes fatty liver and induces progression to steatohepatitis in rodents (Côté et al., 2013; Savard et al., 2013). In a recent study, a high-fat, high-cholesterol diet was fed to wild-type mice and transgenic mice with liver-specific overexpression of NPC1L1 over the course of 2 weeks (Toyoda et al., 2019). The study reported a marked formation of fatty liver in the transgenic mice but not in their wild-type counterparts. It was additionally observed that Mdr2 is necessary for the Abcg5/g8-mediated secretion of hepatic cholesterol into the bile (Langheim et al., 2005). These findings together with the fact that there was no significant difference in the hepatic expression of fatty acid synthesis genes between the F group and the F+E group suggest that the increased hepatic expression of Abcg5/g8 and Mdr2 in the F+E group contributed to the marked improvement in the fatty liver (Figs. 1 and 3).

A recent study reported the phenomenon of transintestinal cholesterol excretion, in which cholesterol is directly excreted from the epithelial cells of the small intestine into the intestine (van der Velde et al., 2008). The transintestinal cholesterol excretion pathway is different from that of biliary cholesterol excretion. In this study, the increase in the levels of fecal cholesterol was higher in the F group than in the control group. On the other hand, there was no increase in the fecal cholesterol levels of the F+E group. It was therefore considered that the increased expression of Abcg5/g8 in the liver instead of in the small intestine contributed to the reduction in the levels of serum and hepatic lipids and the improvement in fatty liver. Cyp8b1 is required for synthesis of cholic acid, which plays a role in intestinal cholesterol absorption. Asbt is responsible for the absorption of bile acids from the small intestine. The expression of these two genes was increased in the F+E group. This, together with cholesterol reabsorption, may explain the observation that the fecal cholesterol levels were not increased in the F+E group (Fig. 3; Tables 1 and 2) (Murphy et al., 2005; van de Peppel et al., 2019).

There may be some possible limitations in this study. For gene expression analysis, we only quantified mRNA levels of

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fatty acid, cholesterol, and bile acid metabolism–related genes. mRNA levels do not always directly correlate with protein levels because of translational and post-translational regulation. Therefore, Western blot analysis should be performed to determine whether changes in mRNA expression corresponded with protein levels in future research.

In conclusion, the combined administration of fish oil and ezetimibe significantly decreased the serum and hepatic levels of triglycerides and cholesterol and markedly improved fatty liver histology. It was considered that the marked improvement in fatty liver histology in the F+E group was associated with the increase in the expression of Abcg5/g8 and Mdr2 in the liver. The results of this study suggest that the combination therapy of ezetimibe and fish oil agents may be effective in treating NAFLD.

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Authorship Contributions

Participated in research design: Tanaka, Kamisako.

Conducted experiments: Tanaka, Ikeda.

Contributed new reagents or analytic tools: Tanaka, Ikeda, Ogawa, Kamisako.

Performed data analysis: Tanaka.

Wrote or contributed to the writing of the manuscript: Tanaka, Kamisako.

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Address correspondence to: Dr. Yuji Tanaka, Department of Clinical Laboratory Medicine, Kindai University Faculty of Medicine, 377-2, Ohnohigashi, Osakasayama 589-8511, Japan. E-mail: ytanaka@med.kindai.ac.jp

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