RESEARCH ARTICLES

Effect of concomitantly used fish oil and cholesterol on lipid metabolism

Satoshi Hirako, Hyoun ju Kim*, Takeshi Arai, Hiroshige Chiba, Akiyo Matsumoto

Department of Clinical Dietetics & Human Nutrition, Faculty of Pharmaceutical Sciences, Josai University, Keyakidai, Sakado, Saitama 350-0295, Japan

Abstract

Although cholesterol plays various important roles in the body, when overconsumed, it causes atherosclerosis and results in ischemic heart disease. On the other hand, dietary fish oils contain n-3 fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid, which prevent ischemic heart disease. This effect of n-3 fatty acids mainly results from the combined effects of inhibiting lipogenesis via a decrease of the mature form of sterol regulatory element-binding proteins (SREBPs) and stimulating fatty acid oxidation via peroxisome proliferator–activator receptor (PPAR) α activation in the liver. In this study, we examined the interactive effects on lipid metabolism of dietary 2% cholesterol (w/w) and 20% or 50% energy fish oil. In a safflower oil diet with 2% cholesterol, hepatic lipids accumulated. On the other hand, hepatic lipids did not accumulate in the fish oil diets with cholesterol. Furthermore, in the groups with fish oil energy ratios of 20%, the negative feedback control of cholesterol affected SREBP-2, and the actions of fish oil and cholesterol were equivalent, but this was not observed in the cases with fish oil energy ratios of 50%. The results of this study suggest that differences in lipid accumulation in the body are due to differences in lipid source and energy ratios which differentially impact the control of transcription factors by cholesterol.
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1. Introduction

Atherosclerotic diseases such as coronary artery disease and cerebral infarction account for the major causes of death in many countries. It has been determined that hyper low-density lipoprotein (LDL) cholesterolemia is closely associated with these diseases [1]. On the other hand, it is known that high-density lipoprotein (HDL) cholesterol level shows negative correlation with development of coronary artery disease [2]. This means that decreases in LDL cholesterol levels and increases in HDL cholesterol levels in the blood are important for prevention of coronary artery disease.

Cholesterol is one of the components of animal cell membranes and a precursor for the synthesis of bile acid, steroid hormone and vitamin D. Much of the cholesterol produced in the body is synthesized from acetyl-CoA in the liver and is eventually excreted as cholesterol in bile or bile salt. Cholesterol and phytosterol in the inner cavity of the small intestine are imported into epithelial cells by Niemann-Pick C1 like-1 protein on the brush border of epithelial cells of the small intestine [3], and cholesterol is released into the lymph duct as chylomicron. On the other hand, phytosterol is considered to be drawn from epithelial cells to the inner cavity of the small intestine together with a cholesterol by a heterodimer of ATP-binding cassette transporter (ABC) G5 and G8 [4]. It

is known that cholesterols, including 22- and 24-hydroxy cholesterol, act as ligands for liver X receptor (LXR) α[5]. LXRα, a nuclear receptor, forms a heterodimer together with the retinoid X receptor and combines with the LXR element at the promoter of target genes to control transcription. The target genes of LXR include cholesterol 7α-hydroxylase (CYP7A1), ABCA1, ABCG1 and sterol regulatory element-binding proteins (SREBPs), genes which are highly involved in cholesterol metabolism [6,7].

It has been reported that fish oil, which includes high percentages of n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, decreases the risk of cardiovascular disease by decreasing blood triglyceride concentrations [8]. It is known that such effects of fish oil are caused by a combination of inhibition of lipogenesis and increased in fatty acid oxidation, mainly in the liver [9–11]. In the regulation of genes related to lipogenesis, SREBPs, which control cholesterol levels in cells as a function of their transcriptional levels, are involved [12]. SREBPs form a complex with SREBP cleavage-activating protein (SCAP) and are localized on the endoplasmic reticulum membrane, forming a hairpin structure. When intracellular cholesterol levels decrease, the complex of SCAP and membrane-bound precursor SREBP is transferred to the Golgi body and becomes active after a two-step cleavage event by site-1 protease and site-2 protease. The activated SREBP is transferred into the nucleus and binds to sterol regulatory elements in the gene promoter to facilitate the transcription [13,14]. SREBPs have three isoforms, -1a, -1c, and -2. It was reported that SREBP-1a and -1c mainly activate the transcription of fatty acid synthesis-related genes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD),

* Corresponding author. Department of Clinical Dietetics and Human Nutrition, Faculty of Pharmaceutical Sciences, Josai University 1-1, Saitama 350-0295, Japan. Tel.: +81 49 271 7234; fax: +81 49 271 7247.
   E-mail address: hyounju@josai.ac.jp (H. Kim).

while SREBP-2 mainly activates the transcription of cholesterol synthesis-related genes, such as the HMG-CoA-reducing enzyme, the HMG-CoA synthetic enzyme and the LDL receptor [10,15,16].

Based on the results of many studies, it is known that fish oil inhibits lipid synthesis by decreasing the expression of genes such as SREBP-1, FAS, ACC and SCD in the liver. In addition, it is known that fish oil may induce fatty acid oxidation by increasing the expression of genes, such as acyl-CoA oxidase (AOX), acetyl-CoA synthetase and uncoupling protein (UCP)-2 via peroxisome proliferator-activated receptor (PPAR) α activation [17–20].

To investigate the effective concentrations of fish oil that will improve cholesterol metabolism in the liver, groups of fish oil with lipid energy ratios of 20% and 50% which are found in high-cholesterol diets in which 2% cholesterol was added were prepared in this study. The lipid energy ratio of 20% was considered to be a ratio that can be taken in an ordinary diet, while the 50% ratio has often been used in past studies on high lipid diets and was considered to be a condition in which the effects of fish oil would be clearly observable in this study.

2. Materials and methods

2.1. Diet compositions

The composition of diets used in this study is shown in Table 1. We prepared diets that we classified as the SO group, in which lipid energy ratio was set to be 20% with safflower oil used as the fat source; the 20FO group, in which the ratio was set to be 20% with fish oil used as the fat source; and the 50FO group, in which the ratio was set to be 50% with fish oil used as the fat source. In addition, 2% cholesterol was added to individual groups to prepare the SO/CH, 20FO/CH and 50FO/CH groups. Casein, sucrose, β-starch, AIN-93G mineral mixture, AIN-93G vitamin mixture and cellulose powder were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan), and L-cystine, t-butylhydroquinone and cholesterol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Animals

Five 8-week-old C57BL/6 female mice (Tokyo Laboratory Animals Science Co., Ltd., Japan) were included in each group. Breeding was performed at the Josai University Life Science Center with conditions of light–dark cycle at 12-h intervals (light: 0700–1900), room temperature of $23\pm2°C$ and humidity of $55\pm10\%$. Feed and water were freely provided, and the feed was changed at 1100 every day and residual quantity was measured. Preliminary breeding was performed for 1 week (including a quarantine period) before commencement of the 8-week feeding. All animal studies were performed in accordance with the "Standards Relating to the Care and Management of Experimental Animals" (Notice No. 6 of the Office of the Prime Minister dated March 27, 1980) and the guidelines of Institutional Animal Care and Use Committee at the Josai University Life Science Center.

2.3. Collection of blood and tissue samples

At the end of the experiments, mice were anesthetized with injected pentobarbital sodium (Dainippon Pharmaceutical Co., Ltd., Japan), and animal X-ray CT (La Theta LCT 100, ALOKA Co., Ltd., Japan) was performed to measure body composition, which determined the fat level. After weight measurement, blood sampling from tail vein and assay of blood glucose level using a blood glucose reader (Ascensia Breeze, Bayel Medical), autopsy was performed. After blood sampling with EDTA-2Na from the inferior vena cava and removal of the liver, photographs of the liver were taken using a digital camera. Part of the removed liver was fixed with 10% neutral buffered formalin (Wako), and Kotobiken Medical Laboratories, Inc. (Tokyo, Japan) performed hematoxylin–eosin (H&E) staining for histopathological examination. Fat tissues (white adipose tissues around the uterus and brown adipose tissues) were removed for weight measurement. Sample blood was centrifuged (3000 rpm, 4°C, 10 min) to separate plasma and frozen at −80°C for storage until analysis. Removed organs were frozen with liquid nitrogen and stored at −80°C.

2.4. Measurement of lipid in plasma and liver

Hepatic lipid was extracted from approximately 100 mg of liver tissue from individual mice in accordance with the method of Folch et al. [21]. For the measurements of total cholesterol and triglyceride in the liver, Cholesterol E-Test Wako and Triglyceride E-Test Wako (Wako) were used, respectively. Measurement of plasma total cholesterol and triglyceride was performed using the same test kits. For the measurement of plasma HDL-cholesterol, HDL-Cholesterol E-Test Wako (Wako) was used.

2.5. Measurement of mRNA in the liver

Total RNA was extracted from the liver tissue of each mouse using Trizol (Invitrogen Co.) in accordance with the manufacturer's protocol. The measurement of mRNA levels by real-time PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems Co.). mRNAs were amplified with QuantiTect SYBP Green (QIAGEN, Hilden, Germany), Quantitect RT Mix and with the use of specific primers optimized at 100% by the manufacturer (Sigma-Aldrich Japan, Tokyo, Japan). The thermal cycling conditions were as follows: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min plus 40 cycles at 94°C for 15 s, 55°C for 30 s and at 72°C for 1 min. The PCR primers used were as follows:

SREBP-1c: forward primer, GGCCGAGATGTGCGAACT; reverse primer, TTGTTGATGAGCTGGAGCATGT;
Insig-1: forward primer, TCACAGTGACTGAGCTTCAGCA; reverse primer, TCATCTTCATCACACCCAGGAC;
Insig-2a: forward primer, CCCTCAATGAATGTACTGAAGGATT; reverse primer, TGTGAAGTGAAGCAGACCAATGT;
SREBP-2: forward primer, GCGTTCTGGAGACCATGGA; reverse primer, ACAAAGTTGCTCTGAAAACAAATCA;
FAS: forward primer, TCACCACTGTGGGCTCTGCAGAGAAGCGAG; reverse primer TGTCATTGGCCTCCTCAAAAAGGGCGTCCA;
SCD-1: forward primer, CCGGAGACCCCTTAGATCGA; reverse primer, TAGCCTGTAAAAGATTTCTGCAAACC;
HMG-CoA reductase: forward primer, CTTGTGGAATGCCTTGTGATTG; reverse primer, AGCCGAAGCAGCACATGAT;
LDL-receptor: forward primer, AGGCTGTGGGCTCCATAGG; reverse primer, TGCGGTCCAGGGTCATCT;
PPARα: forward primer, GTGGCTGCTATAATTTGCTGTG; reverse primer, GAAGGTGTCATCTGGATGGTT;
AOX: forward primer, TCAACAGCCCAACTGTGACTTCCATTA; reverse primer, TCAGGTAGCCATTATCCATCTCTTCA;
UCP-2: forward primer, GTTCCTCTGTCTCGTCTTGC; reverse primer, GGCCTTGAAACCAACCA;
CYP7A1: forward primer, CTGTGTTCACTTTCTGAAGCCATG; reverse primer, CCCAGGCATTGCTCTTTGAT;
CYP8B1: forward primer, TTCGACTTCAAGCTGGTCGA; reverse primer, CAAAGCCCCAGCGCCT.

2.6. Statistical analysis

A one-way analysis of variance was performed on the data, and comparison among groups used Fisher's protected least significant difference (PLSD) method. Differences were considered significant when $P<.05$.

3. Results

3.1. Body weight and tissue weight

Although final weights increased in all groups, increases in body weight (final weight−initial weight) were significantly less in the 20FO/CH and 50FO groups, compared with the SO group. The liver weight significantly decreased in the 20FO group, compared with the

Table 1
Composition of the experimental diets [a]

Ingredition	SO	SO/CH	20FO	20FO/CH	50FO	50FO/CH
Safflower oil	8	8	–	–	–	–
Fish oil	–	–	8	8	25	25
Casein	20	20	20	20	25	25
Sucrose	10.37	10.37	10.37	10.37	12.96	12.96
β-Starch	51.83	51.83	51.83	51.83	24.77	24.77
Vitamin mix [b]	1	1	1	1	1.25	1.25
Mineral mix	3.5	3.5	3.5	3.5	4.38	4.38
Cellulose powder	5	5	5	5	6.25	6.25
L-Cystine	0.3	0.3	0.3	0.3	0.38	0.38
tert-Butyl hydroquinone	0.0016	0.0016	0.0016	0.0016	0.005	0.005
Cholesterol	–	2	–	2	–	2
Energy (kJ/100 g)	1564.9	1534.2	1564.9	1534.2	1941.5	1903.4
Fat energy ratio (%)	19.70	20.01	19.70	20.01	49.62	50.61

[a] All ingredients are in grams per 100 g of diet.
[b] Vitamin mix and mineral mix were based on the AIN-93G formation. Vitamin mix substituted 0.25% sucrose for choline bitartrate.

Table 2
Body weight, liver weight and adipose weight of 16-week-old female mice

Parameter	SO	SO/CH	20FO	20FO/CH	50FO	50FO/CH
Initial body weight (g)	17.04±1.01	17.10±1.02	17.06±0.98	17.00±1.10	17.06±0.86	17.02±0.84
Final body weight (g)	22.12±1.88[a]	21.49±1.18[ab]	21.54±0.98[ab]	20.97±2.56[b]	20.49±0.79[b]	20.80±1.98[ab]
Liver weight (g)	0.98±0.19[a]	0.85±0.51[a]	0.84±0.10[b]	0.83±0.04[b]	1.06±0.06[a]	1.13±0.09[a]
Liver weight/body weight (%)	4.62±0.83[bc]	4.56±0.62[bc]	3.93±0.53[c]	4.24±0.19[c]	5.17.±0.31[ab]	5.56±0.98[a]
White adipose tissue weight (g)	0.31±0.16	0.36±0.08	0.29±0.10	0.24±0.12	0.23±0.16	0.27±0.08
Brown adipose tissue weight (mg)	59.5±11.2[ab]	58.6±9.0[ab]	58.9±7.8[ab]	49.7±6.2[b]	60.6±6.2[ab]	65.4±4.7[a]

Values represent means±S.D. (n=4–5). Means with different letters are different at the $P<.05$ level by Fisher's PLSD test.

SO group, regardless of the addition of cholesterol. No large difference in the weight of white adipose tissues around the uterus and brown adipose tissues between the groups was observed (Table 2).

3.2. Liver appearance and tissue histology

To investigate the effects of fish oil and cholesterol on lipid accumulation in the liver, the appearance and tissue specimens of the liver were compared (Fig. 1). Although the liver weight in the SO/CH group was similar to that in the SO group, the liver size was enlarged, and the entire surface and inside of the liver changed color. Tissue specimens of the SO/CH group showed deposition of many lipid droplets over the entirety of the hepatic cells. However, such deposition of lipid droplets was not observed in the fish oil groups, regardless of the addition of cholesterol.

3.3. Assessment of abdominal fat tissues using X-ray CT

Although visceral fat (pink parts) in the SO/CH group was clearly larger than in the SO group, visceral fat in the 20FO and 50FO groups decreased, and even in the 20FO/CH and 50FO/CH groups, in which fish oil and cholesterol were concomitantly used, no increase of visceral fat was observed. A similar tendency was observed regarding subcutaneous fat (yellow parts) (Fig. 2). A graph of the digitalized X-ray CT images is shown in Fig. 2.

3.4. Plasma and hepatic lipid levels

To examine the effects of dietary fish oil and cholesterol on lipid metabolism in the body, plasma and hepatic lipid levels were measured, and the results are shown in Figs. 1 and 3. Although total cholesterol level in plasma significantly decreased in the 20FO group, compared with the SO group, the level significantly increased in the 20FO/CH and 50FO/CH groups, compared with the 20FO and 50FO groups, respectively. Although plasma HDL-cholesterol level tended to decrease in the SO/CH group, levels in the 20FO/CH and 50FO/CH groups significantly increased. These data suggested that increases in total plasma cholesterol in the 20FO/CH and 50FO/CH groups were caused by increased HDL cholesterol. As for hepatic lipid levels, it was found that triglyceride levels and total cholesterol levels in the SO/CH group increased 2.1-fold and 9-fold compared to the SO group, respectively. On the other hand, no large increase was observed in total cholesterol and triglyceride levels in the 20FO/CH and 50FO/CH groups, in which cholesterol was used in addition to fish oil (Fig. 1).
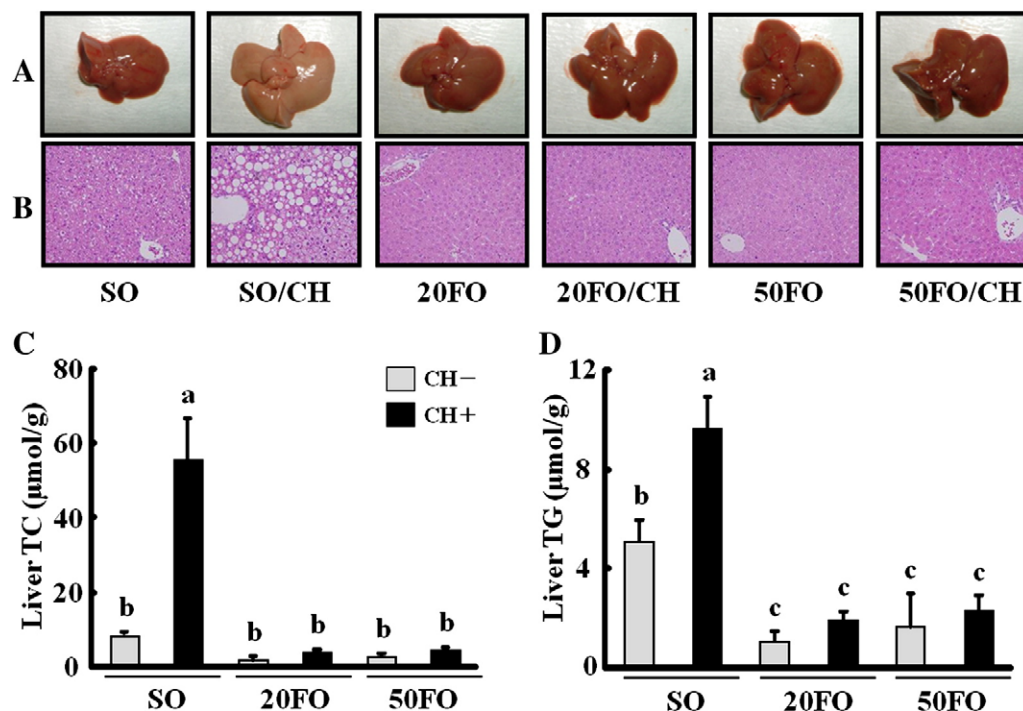

Fig. 1. Liver appearance, tissue histology and lipid levels of 16-week-old female mice. Liver appearance (A) , H&E-stained liver sections (B), total cholesterol (TC) (C) and triglycerides (TG) (D) in mice fed SO, SO/CH, 20FO, 20FO/CH, 50FO and 50FO/CH for 8 weeks. Values represent means±S.D. (n=4–5). Means with different letters are different at the $P<.05$ level by Fisher's PLSD test.
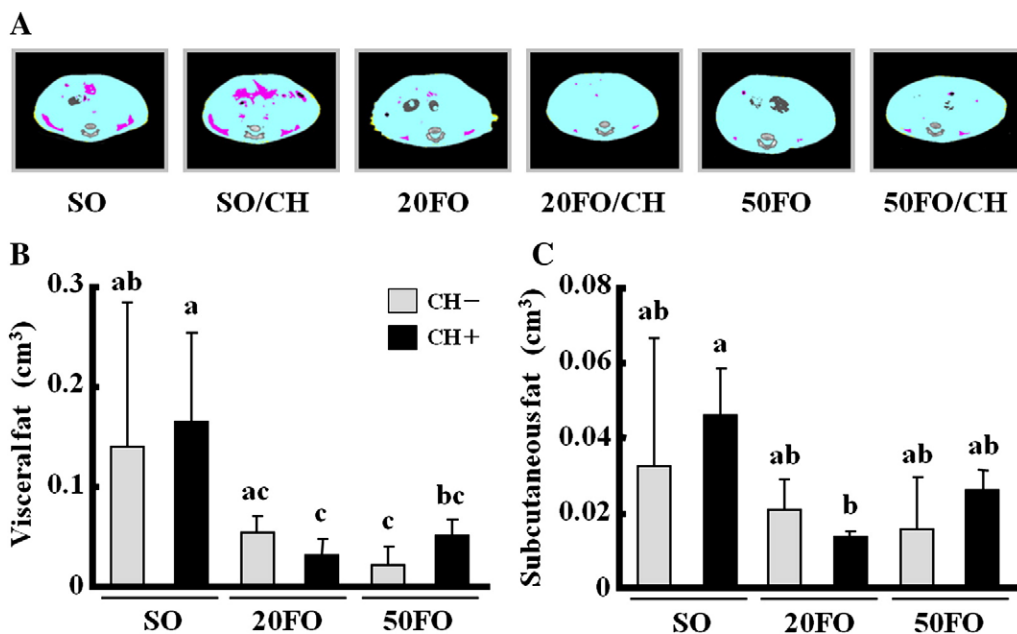
Fig. 2. CT-based fat tissues composition analysis of 16-week-old female mice. Representative X-ray CT images of mice fed SO, SO/CH, 20FO, 20FO/CH, 50FO and 50FO/CH for 8 weeks, at the L3 level (A). The areas indicated with pink, yellow and light-blue are visceral fat, subcutaneous fat and muscle, respectively. CT-estimated amounts of visceral fat (B) and subcutaneous fat (C) in the abdominal area of L2–L4. Values represent means±S.D. (n=4–5). Means with different letters are different at the $P<.05$ level by Fisher's PLSD test.

3.5. Hepatic expression levels of genes involved in lipid metabolism

Expression levels of SREBPs, transcription factors that regulate lipid metabolism-related genes and SREBP target genes are shown in Table 3. Although SREBP-1c mRNA level tended to decrease in the 50FO group, compared with the SO group, the decrease in the expression caused by fish oil was abrogated when cholesterol was added. Insig-1 and Insig-2a mRNA, which strongly respond to nutrients and insulin, significantly decreased in the 20FO and 50FO groups, compared with the SO group. The tendency for further decrease was observed in the 20FO/CH and 50FO/CH groups, in which cholesterol was added. On the other hand, SREBP-2 mRNA expression levels tended to decrease in the 50FO group, compared with the SO group, and the level significantly decreased when
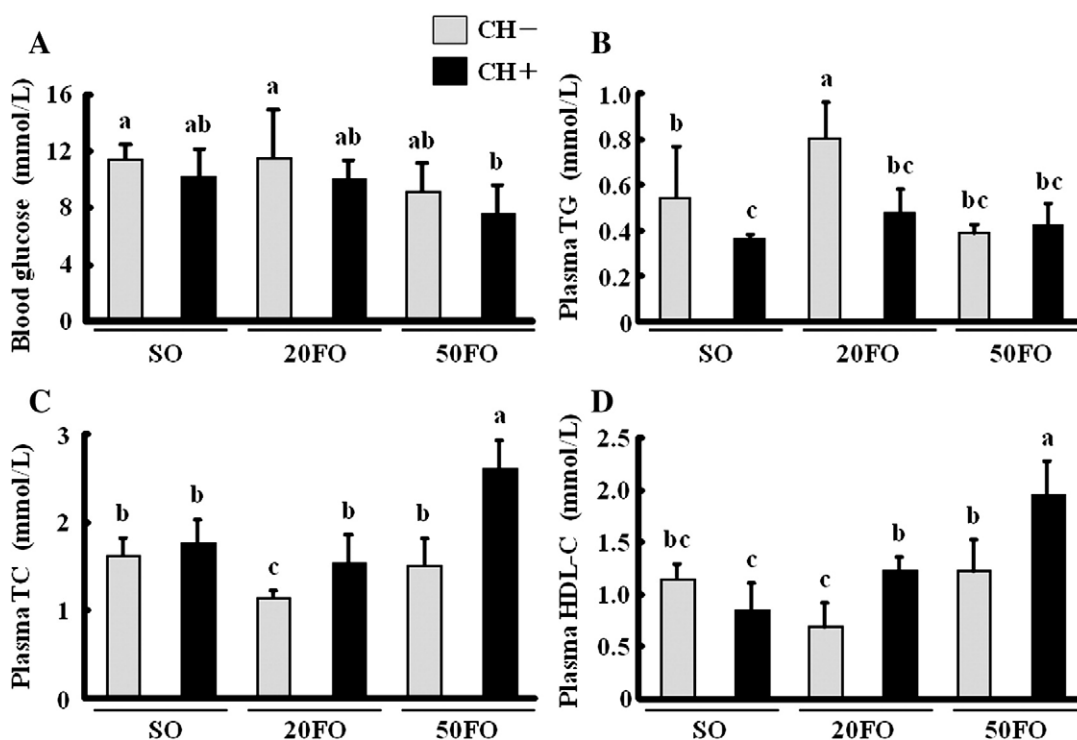

Fig. 3. Blood glucose and plasma lipid levels of 16-week-old female mice. Blood glucose (A), TG (B), TC (C) and HDL-C (D) in mice fed SO, SO/CH, 20FO, 20FO/CH, 50FO and 50FO/CH for 8 weeks. Values represent means±S.D. (n=4–5). Means with different letters are different at the $P<.05$ level by Fisher's PLSD test.

Table 3
The mRNA expression of SREBP-1c and other lipogenic genes

	SO	SO/CH	20FO	20FO/CH	50FO	50FO/CH
SREBP pathway						
SREBP-1c	1.00±0.14[cd]	2.57±0.61[a]	0.93±0.13[cd]	1.29±0.31[bc]	0.83±0.54[d]	1.49±0.05[b]
SREBP-2	1.00±0.08[ab]	0.54±0.11[c]	1.11±0.17[a]	0.64±0.14[c]	0.86±0.26[b]	0.56±0.43[c]
Insig-1	1.00±0.23[a]	0.42±0.34[b]	0.50±0.21[b]	0.16±0.20[c]	0.19±0.23[c]	0.12±0.16[c]
Insig-2a	1.00±0.17[a]	0.81±0.29[b]	0.62±0.27[b]	0.51±0.37[c]	0.44±0.35[c]	0.28±0.29[c]
Fatty acid biosynthesis						
FAS	1.00±0.51[a]	0.38±0.29[b]	0.33±0.35[b]	0.12±0.18[b]	0.08±0.48[b]	0.06±0.28[b]
SCD-1	1.00±0.22[a]	0.97±1.75[a]	0.20±0.88[bc]	0.27±0.50[b]	0.00±0.71[c]	0.06±0.58[bc]
Cholesterol homeostasis						
HMG-CoA Reductase	1.00±0.65[a]	0.29±0.36[b]	0.39±0.24[b]	0.24±0.14[b]	0.24±0.22[b]	0.22±0.14[b]
LDL-Receptor	1.00±0.23[a]	0.94±0.19[a]	1.04±0.25[a]	0.70±0.37[b]	0.70±0.09[b]	0.59±0.14[b]
Bile acid biosynthesis						
CYP7A1	1.00±0.55[cd]	2.13±0.37[bc]	0.71±0.60[d]	2.78±0.42[b]	2.19±0.24[bc]	4.20±0.21[a]
CYP8B1	1.00±0.10[d]	0.88±0.19[d]	2.04±0.11[c]	2.32±0.34[bc]	3.57±0.26[a]	2.93±0.14[a]
Fatty acid β-oxidation						
PPAR α	1.00±0.12[c]	1.42±0.21[b]	1.75±0.06[a]	1.87±0.07[a]	1.75±0.08[a]	1.77±0.12[a]
AOX	1.00±0.12[d]	1.26±0.10[d]	2.61±0.22[c]	2.95±0.12[bc]	3.91±0.12[ab]	3.72±0.12[a]
UCP-2	1.00±0.23[e]	0.79±0.07[e]	2.75±0.08[c]	1.68±0.10[d]	5.02±0.11[a]	3.88±0.06[b]

The mRNA expression levels in livers of mice fed SO, SO/CH, 20FO, 20FO/CH, 50FO and 50FO/CH for 8 weeks. Values represent means±S.D. (n=4–5). Means with different letters are different at the $P<.05$ level by Fisher's PLSD test.

cholesterol was added. Such decreases were observed not only in the fish oil groups, such as the 20FO/CH and 50FO/CH groups, but also in the SO/CH group.

The level of FAS mRNA, a target gene of SREBP-1c, decreased in the 20FO and 50FO groups by 22% and 8%, respectively. Regarding the effects of cholesterol on FAS mRNA expression, it was found that the expression level in the SO/CH and 20FO/CH groups decreased by more than 50%, compared with the SO and 20FO groups, respectively, but no difference was observed between the 50FO/CH and 50FO groups. Although the mRNA level of SCD-1 decreased dose dependently in the 20FO and 50FO groups, as seen in FAS, no further decrease was observed for SCD-1 when cholesterol was added. Although both FAS and SCD-1 are target genes of SREBP-1c, these findings suggest that these genes might be controlled by separate mechanisms when cholesterol is added.

The expression of HMG-CoA reductase mRNA, a gene involved in cholesterol synthesis, significantly decreased in the 20FO and 50FO groups, compared with the SO group, and no further decrease was observed when cholesterol was added.

The expression of PPARα mRNA, a transcription factor for fatty acid oxidation-related genes, increased in the fish oil groups, regardless of the addition of cholesterol. The mRNA expression of AOX, a target gene of PPARα involved in fatty acid oxidation, and UCP-2, involved in heat production, significantly increased in the 20FO and 50FO groups, compared to the SO group. However, although no changes were observed in AOX mRNA expression when cholesterol was added, UCP-2 mRNA expression decreased.

In the mRNA expression of CYP7A1 and CYP8B1, genes involved in bile acid synthesis, CYP7A1 tended to increase in the 50FO group and CYP8B1 significantly increased in the 20FO and 50FO groups, compared with the SO group. The effects of cholesterol differed between the two genes: the expression of CYP7A1 mRNA clearly increased when cholesterol was added, compared with the groups in which only fish oil was used, while no such increase was observed in the expression of CYP8B1 mRNA when cholesterol was added. Furthermore, the expression of CYP8B1 mRNA tended to decrease in the 50FO group, when cholesterol was added.

4. Discussion

In this study, the body weight gain was small in groups where fish oil was used as the lipid source, compared with the SO group. The liver

weight and the ratio of the liver to body weight were significantly decreased in the 20FO group, but these values in the 50FO group were similar to those in the SO group, with a lipid energy ratio of 20%. In some studies on physiological action of fish oil, it has been reported that liver weight would increase when liver peroxisomes increased [22], but no significant increase was observed in this study. On the other hand, the effect of cholesterol was largest in the liver of the safflower oil groups. The liver in the SO/CH group showed an increase in size and a dramatic change in color. Hepatic tissue specimens also indicated deposition of many lipid droplets distributed throughout the hepatic cells. Such deposition of lipid droplets caused by the addition of cholesterol was not seen in groups where fish oil was used as the lipid source, and this confirmed that differences in oil type have a large effect on cholesterol metabolism in the liver. While hepatic triglyceride and total cholesterol levels were extremely high in the SO/CH group, no large increase due to cholesterol was observed in the 20FO/CH and 50FO/CH groups, and this finding was reflected in the histopathological results. In addition, the accumulation of cholesterol in the liver was similar in the 20FO/CH and 50FO/CH groups which suggest that the effects of fish oil on inhibition of cholesterol accumulation would be sufficient, even in cases with energy ratios of 20%.

The main catabolic pathway of cholesterol is the synthesis of bile acid in the liver. The synthetic pathway of bile acid is controlled by CYP7A1, which is induced by LXR [4]. In this study, CYP7A1 mRNA level in the liver increased in the 50FO group, compared with the SO group, and increases were seen in all groups when cholesterol was added. It is speculated that CYP7A1 mRNA level is increased by the addition of cholesterol due to negative feedback mechanisms which maintain the homeostasis of cholesterol in the body. On the other hand, it is known that cholic acid is the major primary bile acid, and CYP8B1 is involved in the enzymes of cholic acid synthesis [23,24]. Several studies have reported that cholesterol feeding regulates the expression of CYP7A1 and CYP8B1 genes in different directions. We also observed that the expression of CYP8B1 mRNA suppressed when cholesterol was added in the 50% fish oil energy ratios.

In the SO/CH group, high cholesterol levels in the blood were prevented by an increase in the catabolism of cholesterol, but, as a result, high levels of lipid accumulated in the liver, causing fatty liver and increased visceral fat and subcutaneous fat in the abdomen. On the other hand, in the 20FO/CH and 50FO/CH groups in which fish oil and cholesterol were used concomitantly, almost no accumulation of

lipid was observed in hepatic cells, and abdominal fat was also decreased. Increases in catabolism of cholesterol and other factors are speculated to account for these findings. Further investigation is required on this matter.

Comparison of visceral fat and subcutaneous fat levels based on analysis of X-ray CT images showed that visceral fat and subcutaneous fat in all fish oil groups tended to decrease, regardless of the addition of cholesterol. The mRNA levels of SREBP-1c and -2 decreased in fish oil groups, compared with the safflower oil group. The mRNA level of FAS and SCD-1 also decreased in the fish oil groups, regardless of the addition of cholesterol. Fish oil inhibited lipid synthesis even when cholesterol was added, which suggests that such an effect is similar for lipid energy ratios of 20% and 50%. Regarding fish oil to inhibition of lipid accumulation, it is known that target genes of SREBP-1c decrease when the expression of SREBP-1c is decreased [8,9], and we obtained similar results in this study. PPARα mRNA is increased by highly unsaturated fatty acid and fibrate drugs that decrease neutral fat [18], and we saw increases in PPARα mRNA in fish oil groups, regardless of addition of cholesterol. Also, AOX and UCP-2 mRNA increased in the fish oil groups. Our data indicate that fish oil may effectively inhibit lipid accumulation in the body both by inhibiting fatty acid synthesis and by facilitating oxidation of fatty acid. On the other hand, when cholesterol was concomitantly used with fish oil, both might have affected fatty acid metabolism and cholesterol metabolism. In this study, it was shown that AOX and UCP-2 mRNA expression in the liver increased in both cases with lipid energy ratios of 20% and 50% in groups where only fish oil was used. However, when cholesterol was added, differences in expression of AOX and UCP-2 mRNA were seen. While the expression level of AOX mRNA was not changed by cholesterol, UCP-2 mRNA decreased when cholesterol was added. Due to the SRE in the promoter region of the UCP-2 gene [25,26], it has been hypothesized that the expression level of UCP-2 mRNA might decrease when SREBP-2 mRNA decreased in a state with excessive cholesterol. HMG-CoA reductase mRNA decreased in the 20FO and 50FO groups, compared with the SO group, and a tendency of further decrease was observed in the 20FO/CH group. On the other hand, in the 50FO group, no further decrease was observed when cholesterol was added. There is a report that shows fish oil may decrease mRNA levels of cholesterol synthesis-related genes through a decrease in SREBP-2 [9]. In the present study, expressions of genes controlled by SREBP-2 were inhibited by the combined actions of fish oil and cholesterol in the 20FO/CH group, while expressions of these genes did not show additional decrease in the 50FO/CH group compared to the 50FO group. This suggests that the effect of fish oil was larger in the 50FO/CH group than in the 20FO/CH group. Based on these data, it might be difficult to observe the changes caused by cholesterol when the concentration of fish oil was high.

When the intracellular concentration of cholesterol was increased by adding cholesterol to the diet, lipid synthesis decreased along with the expression of SREBP's target genes. However, when cholesterol was added to a group in which safflower oil was used as the lipid source, almost no decrease in lipid synthesis was observed, and significant lipid accumulation in hepatic cells and increases of white adipose tissue were observed. In contrast, when cholesterol was added to a group in which fish oil was used as the lipid source, SREBPs were affected by negative feedback, resulting in inhibition on lipid accumulation in hepatic cells, and no increase was observed in abdominal visceral fat and subcutaneous fat tissues. Such changes were larger in the cases with fish oil energy ratios of 20% than in the cases with ratios of 50%.

In this study, it was shown that lipid accumulation in tissues was inhibited by fatty acid degradation, which was facilitated by inhibited lipid synthesis through a decrease in SREBP expression and increased expression of fatty acid oxidation-related genes, such as AOX and UCP-2, both in the groups with fish oil energy ratios of 20% and 50%, regardless of addition of cholesterol. The results of this study suggest that differences in lipid accumulation in the body are due to differences in lipid source and energy ratios which differentially impact the control of transcription factors by cholesterol.

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