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ARTICLE

Effects of Dietary Fish Protein on Serum and Liver Lipid Concentrations in Rats and the Expression of Hepatic Genes Involved in Lipid Metabolism

RYOTA HOSOMI,[†] KENJI FUKUNAGA,*[,†] HIROFUMI ARAI,[§] TOSHIMASA NISHIYAMA,[#] AND MUNEHIRO YOSHIDA[†]

[†]Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Osaka 564-8680, Japan, [§]Department of Biotechnology and Environmental Chemistry, Kitami Institute of Technology, Kitami, Hokkaido 090-8507, Japan, and [#]Department of Public Health, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

Dietary proteins influence the lipid metabolism of human subjects and animals. This study evaluated the effects of fish protein on lipid metabolism in rats. Alaska pollock fillets, widely supplied as raw materials of surimi, were used as fish protein. As parameters of lipid metabolism, cholesterol and triacylglycerol concentrations in the serum and liver, the fecal excretion of bile acids, and the hepatic expression of genes encoding proteins involved in lipid homeostasis were examined. Rats fed fish protein showed decreased cholesterol concentrations in the serum and liver, and fecal bile acid and cholesterol concentrations were increased. This was caused by the increased expression of cholesterol 7α-hydroxylase (CYP7A1) as the digested fish protein inhibited the absorption of bile acid and cholesterol in the small intestine. In addition, it was found that dietary fish protein affects the farnesoid X receptor/small heterodimer partner-dependent pathway, which is negatively regulated by the decreased reabsorption of bile acid. Furthermore, it increased the binding to the promoter of CYP7A1 through activated liver receptor homologue-1.

KEYWORDS: Fish protein; cholesterol; bile acid; triacylglycerol; lipid metabolism

INTRODUCTION

Traditionally, fish, vegetables, and rice were the main foods in Japanese cuisine, but recently the intake of other animal products has increased. Along with this change in dietary habits, the incidence of coronary heart disease (CHD) has increased (*1*). Epidemiological and experimental reports have shown a relationship between diet and the incidence of CHD. Metabolic phenotypes and Western-style diets containing low dietary fiber and high fat levels are both risk factors for the development of CHD (*2*, *3*).

Epidemiological evidence from Greenland Inuit and Japanese fishing villages suggests that eating fish and marine animals can prevent CHD (*4*, *5*). Dietary studies from many investigators have similarly shown that regular fish intake affects several humoral and cellular factors involved in CHD and may prevent atherosclerosis, thrombosis, and sudden cardiac death. Some reports have indicated that the beneficial effects of fish are attributable to n-3 polyunsaturated fatty acids such as icosapentaenoic acid (IPA) and docosahexaenoic acid (DHA) (*6*, *7*). However, Japanese dietary habits include not only fish oil but also the fish itself, which provides nutrients such as proteins as well as fat. Therefore, this study focused on fish protein, which is important as one of the main nutritional components of fish.

The effects of various nutrients on plasma cholesterol have been reported: initially with dietary cholesterol and later with amounts of dietary fat. Recently, dietary proteins such as plant and animal proteins have also been found to influence lipid metabolism in human subjects and animals (*8*, *9*). For example, rats fed soy protein had lower levels of plasma cholesterol accompanied by an increased fecal excretion of steroids when compared with rats fed casein (*10*). Similarly, the effect of dietary fish protein influenced blood lipid concentrations in rats or rabbits as experimental animals (*11*−*14*). However, few studies have focused on the influence of dietary fish protein on blood and liver lipid concentrations in experimental animals. In addition, few studies have evaluated the mRNA expression levels, enzyme activities of hepatic lipid metabolism-related enzymes, and fecal lipid concentrations with reference to dietary animal protein. Therefore, to obtain detailed information, this study examined the effects of fish proteins on gene expression levels and the activity of enzymes involved in cholesterol and fatty acid metabolism and the role of nuclear receptors in the metabolism of cholesterol and bile acids. The present study examined for the first time the effect of fish protein on the mRNA expression levels of nuclear receptors in the metabolism of cholesterol and bile acids.

Cholesterol homeostasis is maintained by a balance of uptake, biosynthesis, storage, catabolism, and export. To evaluate the

*Author to whom correspondence should be addressed (telephone +81-6-6368-0875; fax +81-6-6388-8609; e-mail fukunagk@kansai-u.ac.jp).

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constituents that could be responsible for the effects of fish protein on circulating cholesterol, this study examined the expression levels of sterol regulatory element-binding protein (SREBP)-2 in the liver; its target genes 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and low-density lipoprotein receptor (LDLR); the key enzyme required for cholesterol synthesis and cholesterol uptake into cells, acyl-coenzyme:cholesterol acyltransferase (ACAT); and the major enzymes required for cholesterol storage (*15*). To gain insights into cholesterol catabolism, the mRNA level of cholesterol 7α-hydroxylase (CYP7A1), the key enzyme in the synthesis of bile acids from cholesterol and the only metabolic route for the excretion of excess cholesterol from the body, was analyzed. In addition, the total amounts of bile acids in the feces could provide information about the possible effects of fish protein on cholesterol excretion via bile acids (*16*). Bile acids inhibit the transcription of CYP7A1 via the farnesoid X receptor (FXR)/small heterodimer partner (SHP)-dependent pathway. In the FXR/SHP-dependent pathway, bile acids activate the FXR-mediated transcription of SHP, which subsequently inhibits the transcription of CYP7A1 by interfering with liver receptor homologue-1 (LRH-1), a transcription factor for CYP7A1.

The antiarteriosclerotic effect, which is represented by a decrease in plasma triacylglycerol of fish lipids, that is, IPA and DHA, has been demonstrated in animal models and an epidemiological survey in humans. However, it is not possible to explain the health function of fish-based foods only in terms of IPA and DHA. In fact, it is not necessary to eat only fish oil in the daily diet. Currently, no information is available concerning the antiarteriosclerotic effects of fish protein. Therefore, this study investigated the antiarteriosclerotic disease inhibition mechanism derived from eating fish protein. In addition, there is a report that fish oil does not present an anticholesterol effect. In the present study, attention was paid to fish protein, which is also an important nutrient derived from fish. This study demonstrated that protein isolated from fish protein affects the serum and liver lipid concentrations and the activities of hepatic lipid metabolism. In the future, the creation of a new field of functional food materials in domestic and foreign can be expected using the finding obtained by this study on fish protein. This study also contributes to the development of food chemistry by clarifying a health functionality of fish protein.

MATERIALS AND METHODS

Sample Preparation. Alaska pollock (*Theragra chalcogramma*), widely supplied as raw material of surimi, were used as fish protein. Alaska pollock fillets were obtained from Suzuhiro Co., Ltd. (Odawara, Japan). The fillets were chopped into small pieces, mixed with an equal volume of distilled water, and homogenized in a Waring blender (Waring Products Division, New Hartford, CT) for 2 min after water deprivation. The resulting meat was treated with cold acetone, ethyl acetate, and *n*-hexane to remove protein-associated lipids. Then, the meat was dried under N_2 gas and stored at −30 °C.

The composition of fish protein was determined, and the crude protein content, which was assayed according to the Kjeldahl method, was 90.6%. The crude fat content, assayed by the Soxhlet method, was < 0.5%. The moisture content was determined as the loss in weight after drying at 105 °C for 24 h and was approximately 7%. Fish protein contained 1.5% ash as measured by the direct ignition method by heating at 550 °C for 24 h.

Animal Care. The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the "Guide for the Care and Use of Experimental Animals" of the Prime Minister's Office of Japan. Five-week-old male Wistar rats obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), were kept in an air-conditioned room (temperature, 20−22 °C; humidity, 55−65%; lights on, 8:00 a.m.−10:00 p.m.) and had free access to drinking water. The rats were fed a diet prepared according to the recommendations of the American Institute of Nutrition (AIN-93G) (*17*). After acclimation for 3 days with an AIN-93G diet, the rats were divided into the following eight dietary groups of seven rats each: control (Cont) diet; fish protein (FP) diet; high-fat (HF) diet; high-fat fish protein (HF-FP) diet; control plus cholesterol (Cont+C) diet; fish protein plus cholesterol (FP+C) diet; high-fat plus cholesterol (HF+C) diet; and high-fat fish protein plus cholesterol (HF-FP+C) diet. The experimental diets were prepared according to the AIN-93G scheme (**Table 1**). All of the diet ingredients were products of Oriental Yeast Co., Ltd. (Tokyo, Japan). After treatment with the experimental diets for 4 weeks, the rats were weighed and sacrificed under diethyl ether anesthesia. Blood was collected, and serum was harvested by centrifugation at 1500*g* for 15 min before being stored at −80 °C until analysis. Liver, abdominal white adipose tissue (WAT), and interscapular brown adipose tissue (BAT) were rapidly removed in their entirety and weighed, rinsed, frozen in liquid nitrogen, and kept at −80 °C. The liver was taken for mRNA expression analysis and stored in RNALater Storage Solution (Sigma Chemical Co., St. Louis, MO).

Table 1. Composition of Experimental Diets (Grams per Kilogram of Diet)[a]

	normal fat content diet				high fat content diet			
ingredient	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
α-corn starch	132.0	132.0	132.0	132.0	60.2	60.2	60.2	60.2
β-corn starch	397.5	397.5	391.5	391.5	181.3	181.3	175.3	175.3
casein	200.0	100.0	200.0	100.0	258.0	129.0	258.0	129.0
fish protein		100.0		100.0		129.0		129.0
sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
cellulose powder	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
mineral mixture[b]	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
vitamin mixture[b]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
L-cystine	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
soybean oil	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0
lard					230.0	230.0	230.0	230.0
cholesterol			5.0	5.0			5.0	5.0
cholic acid			1.0	1.0			1.0	1.0

[a] Diets were prepared on the basis of the AIN-93G composition. [b] AIN-93G formula.

Analysis of the Serum Parameters. Total lipid (TL), phospholipids (PL), triacylglycerol (TG), total cholesterol (T-Chol), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) analyses were performed by a commercial service (Japan Medical Laboratory, Osaka, Japan).

Analysis of the Liver Lipid Composition, Preparation of the Liver, and Enzyme Activity Measurements. Liver lipids were extracted with a mixture of chloroform/methanol/water (1:1:0.9, v/v). Phospholipids were separated by silica gel column chromatography using chloroform and methanol as elution solvents. The cholesterol concentration was analyzed by gas−liquid chromatography (GC-14B, Shimadzu, Kyoto, Japan) using 5α-cholestane as an internal standard. The triacylglycerol level was calculated by subtracting the phospholipid and cholesterol concentrations from the total lipid concentration.

The liver was homogenized in 6 volumes of a 125 mM NaCl containing 1 mM EDTA-2Na and 10 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 500*g* for 10 min at 4 °C. The supernatant was recentrifuged at 9000*g* for 10 min at 4 °C to sediment the mitochondria, and the remaining supernatant was collected. The activity of carnitine palmitoyl transferase (CPT) in the mitochondrial fraction was measured as described by Markwell et al. (*18*). The activities of fatty acid synthase (FAS) (*19*), acetyl-coenzyme A carboxylase (ACC) (*20*), glucose-6-phosphate dehydrogenase (G6PDH) (*21*), malic enzyme (ME) (*22*), ATP-citrate lyase (ACL) (*23*), and microsomal phosphatidate phosphatase (PAP) (*24*) in the supernatant fraction were measured spectrophotometrically. Protein concentrations were determined according to the method of Bradford (*25*) using bovine serum albumin as a standard.

Analysis of Fecal Bile Acid, Cholesterol, and Lipid Concentration. Feces were collected for 7 days after acclimation of the rats to the experimental conditions and diets. Total bile acids in the feces were determined as micromoles of 3α-hydroxysteroid based on the molar

Table 2. Body Weight, Energy Intake, Liver Weight, and WAT Weight of Rats Fed the Experimental Diets for 4 Weeks[a]

	normal fat content diet				high fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
initial BW (g)	101.4 ± 9.9	101.4 ± 3.5	108.6 ± 8.3	102.9 ± 4.5	108.6 ± 3.5	108.6 ± 6.4	110.0 ± 5.3	97.1 ± 4.5
final BW (g)	307.1 ± 26.6	311.4 ± 16.4	314.3 ± 16.8	302.9 ± 14.8	291.4 ± 23.6	290.0 ± 23.3	305.7 ± 23.2	315.7 ± 23.2
BW gain (g/day)	205.7 ± 21.5	210.0 ± 16.3	205.7 ± 12.7	200.0 ± 12.9	182.9 ± 25.6	181.4 ± 19.5	195.7 ± 22.3	218.6 ± 26.1
energy intake (kcal/day)	68.3 ± 7.2 ab	73.1 ± 7.7 b	67.0 ± 3.8 ab	65.2 ± 4.5 a	64.5 ± 9.1 a	64.8 ± 11.5 a	73.6 ± 9.0 ab	81.9 ± 16.5 b
EER[b] (g/kcal)	0.106 ± 0.010	0.101 ± 0.008	0.105 ± 0.009	0.105 ± 0.007	0.098 ± 0.011	0.099 ± 0.011	0.092 ± 0.011	0.095 ± 0.011
liver wt (g/100 g of BW)	3.9 ± 0.2	3.6 ± 0.2	5.4 ± 0.5	5.0 ± 0.2	3.8 ± 0.2	3.6 ± 0.4	5.3 ± 0.6	5.0 ± 0.3
WAT wt (g/100 g of BW)	4.8 ± 1.0	5.0 ± 0.7	4.4 ± 0.6	4.1 ± 0.4	5.5 ± 0.7	4.9 ± 0.7	5.3 ± 1.1	5.3 ± 0.8

[a] Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. [b] EER (energy efficiency ratio) = body weight gain (g/day)/energy intake (kcal/day).

Table 3. Lipid Concentrations in Serum of Rats Fed the Experimental Diets for 4 Weeks (Milligrams per Deciliter)[a]

	normal fat content diet				high fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
TL	244.1 ± 15.8	231.6 ± 26.0	251.7 ± 32.8	238.6 ± 22.5	257.6 ± 19.3	242.0 ± 22.1	270.9 ± 28.1	260.4 ± 24.8
TG	33.0 ± 3.2	30.6 ± 3.9	32.9 ± 6.0	32.9 ± 6.0	36.3 ± 5.7	32.3 ± 4.2	38.3 ± 6.8	35.1 ± 3.9
PL	131.0 ± 19.3	125.6 ± 20.1	127.9 ± 23.0	141.4 ± 23.4	129.0 ± 18.0	143.9 ± 23.7	145.0 ± 18.7	136.4 ± 20.3
T-Chol	77.6 ± 8.7 ac	70.7 ± 5.5 bc	84.9 ± 10.6 a	75.9 ± 6.7 c	83.4 ± 6.3 ac	75.9 ± 5.8 a	91.0 ± 8.9 ab	80.7 ± 6.9 c
HDL-C	67.9 ± 7.8	74.3 ± 5.8	66.3 ± 7.8	70.0 ± 7.8	61.7 ± 7.4	62.6 ± 9.8	65.7 ± 8.1	63.9 ± 6.5
LDL-C	5.3 ± 0.8 ab	4.8 ± 0.7 ab	5.7 ± 0.4 a	4.6 ± 1.1 b	5.4 ± 1.0 ab	4.7 ± 0.5 ab	5.7 ± 0.9 a	5.1 ± 0.8 b

[a] Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. TL, total lipid; TG, triacylglycerol; PL, phospholipid; T-Chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

extinction coefficient of NADH at 340 nm (*26*). Fecal cholesterol was determined using 5α-cholestane as an internal standard, and the lipid concentration was extracted with a mixture of chloroform/methanol/water (1:1:0.9, v/v).

Western Blot Analysis. BAT tissue was homogenized in 5 volumes of 10 mM Tris-HCl and 1 mM EDTA-2Na (pH 7.4) for 30 s using a Polytron (Pellet Pestles, Kontes, Vineland, NJ). After centrifugation at 1500*g* for 5 min, the fat cake was discarded, and the infranant (fat-free extract) was used for the Western blotting analysis of UCP-1. The total protein content in BAT was measured according to the method of Bradford (*25*). The supernatants (BAT 5 mg of protein/lane) were separated by 10% SDS−polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membrane. The membrane was incubated with an antibody against UCP-1 (Sigma) for 1 h and then incubated with a secondary rabbit IgG-antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The membranes were treated with ECL Western Blotting Detection reagent (GE Healthcare UK Ltd., U.K.) according to the manufacturer's instructions. β-Actin was detected as a control with a β-actin antibody (Santa Cruz Biotechnology).

mRNA Analysis. Total RNA was extracted from liver using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Then, cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Real-time quantitative RT-PCR analysis was performed with an automated sequence detection system (ABI Prism 7000; Applied Biosystems Japan Ltd.). PCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. HMGR, CYP7A1, ACAT1, SREBP-2, peroxisome proliferation activated receptor alpha (PPARα), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were measured using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). PCR Primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; ACAT1: Rn00567139_m1; SREBP-2: Rn01502638_m1; PPARα: Rn00566193_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems Japan Ltd. The expression signal of the housekeeping gene GAPDH served as an internal control for normalization.

Moreover, LDLR, FXR, SHP, LRH-1, and GAPDH mRNA expression levels were measured using SYBR Green PCR Master Mix (Applied Biosystems). The PCR solution (25 μL) was composed of 12.5 μL of SYBR Green PCR Master Mix solution, 5 μL of template cDNA, 1 μL of forward primer, 1 μL of reverse primer, and 5.5 μL of RNase free water. The primer sequences used for the detection of LDLR, FXR, SHP-1, LRH-1, and GAPDH were as follows: forward, 5′ CACCCCCTCGTTGAAAACCT3′, and reverse, 5′ CCTTAGCCAGCTCTTCCAGATC3′, for LDLR; forward, 5′ GGGCCTTGGACGTCTCTGA3′, and reverse, 5′ CTGGGATGGTGGTCTTCAAATAA3′, for FXR; forward, 5′ CGCCTGGCCCGAATC3′, and reverse, 5′ GAAGGGTACAGGAGATGTTCTTGAG3′, for SHP-1, forward, 5′TCCGGGCAATCAGCAAA3′, and reverse, 5′ CCCCATTCACGTGCTTGTAGT3′, for LRH-1; and forward, 5′ GAAGACACCAGTAGACTCCACGACATA3′, and reverse, 5′ GAAGGTCGGTGTGAACGGATT3′, for GAPDH. The PCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Statistical Analysis. Data are expressed as means ± SD of seven rats. Statistical analyses between multiple groups were determined by ANOVA. Statistical comparisons were made using the Tukey−Kramer test. Differences with $p < 0.05$ were considered to be significant. The analyses were performed using Stat View-J version 5.0 software (Abacus Concept, Berkeley, CA).

RESULTS

Growth Parameters. Body weight (BW) gain, food intake, energy efficiency ratio (EER), liver weight, and WAT weight are presented in **Table 2**. The diet intake tended to be higher in the HF-FP+C group than in the other groups, but the EER was not significantly different among the groups. No significant differences in these parameters were observed between the normal fat content and high fat content diets. There were no significant differences in BW, BW gain, liver weight, or WAT weight among the groups.

Serum Lipid Concentration. Serum lipid concentrations are presented in **Table 3**. TL and TG concentrations tended to be lower in rats fed the fish protein-containing diets compared with rats fed casein. When the rats were fed the normal fat content diet, HDL-C concentration tended to be higher in rats fed the fish protein-containing diet compared with the casein diet, but no differences were noted compared with rats fed the high fat content

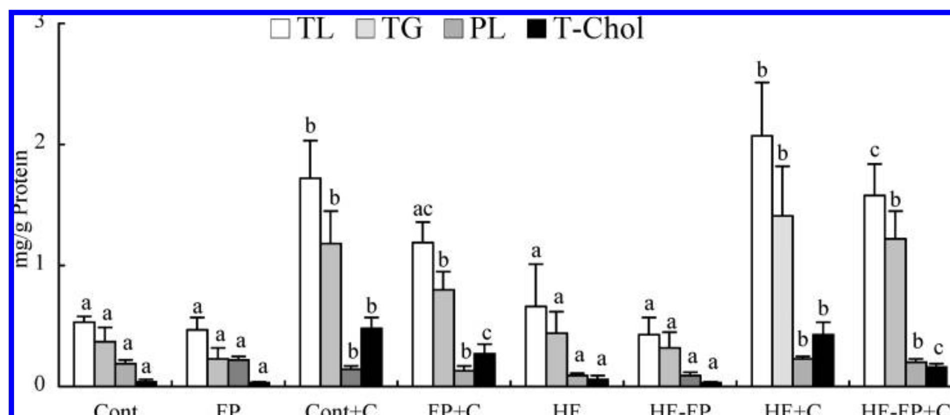

Figure 1. Lipid concentrations in the liver of rats fed the experimental diets for 4 weeks. Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. TL, total lipid; TG, triacylglycerol; PL, phospholipid; T-Chol, total cholesterol.

Table 4. Activities of Enzymes Related to the Fatty Acid Metabolic Pathways in the Livers of Wistar Rats (Nanomoles per Minute per Milligram of Protein)[a]

	normal fat content diet				high fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
FAS	1.91 ± 0.44 a	2.36 ± 0.51 ab	3.09 ± 0.39 b	2.72 ± 0.45 b	1.60 ± 0.35 a	2.04 ± 0.47 ab	1.76 ± 0.37 ab	2.51 ± 0.42 b
ACC	64.96 ± 7.59 ab	92.28 ± 8.79 a	72.38 ± 9.22 ab	75.93 ± 8.85 b	90.42 ± 9.30 a	89.84 ± 8.52 a	88.74 ± 12.9 a	118.19 ± 14.64 b
G6PDH	8.65 ± 2.34 ab	15.60 ± 5.94 a	5.59 ± 1.50 ab	5.18 ± 1.97 b	4.11 ± 1.27	4.74 ± 8.52 a	4.00 ± 0.89	4.19 ± 0.73
ME	2.84 ± 1.37 ab	6.50 ± 1.46 a	1.90 ± 0.49 ab	1.68 ± 0.38 b	1.42 ± 0.39	1.50 ± 0.44	1.15 ± 0.89	1.06 ± 0.37
ACL	26.67 ± 4.33 a	27.37 ± 4.44 a	13.35 ± 4.42 b	13.54 ± 4.62 b	8.95 ± 0.54	7.47 ± 1.80	5.69 ± 1.37	5.71 ± 0.70
PAP	6.58 ± 0.35	6.47 ± 1.16	7.09 ± 0.59	6.43 ± 0.42	5.62 ± 0.54	5.38 ± 0.49	5.41 ± 0.61	5.49 ± 0.58
CPT-II	2.40 ± 0.76	2.26 ± 0.64	1.95 ± 0.47	2.21 ± 0.62	1.57 ± 0.33	2.35 ± 0.43	2.02 ± 0.44	1.93 ± 0.65
ACD								
8:0-CoA	4.14 ± 1.54 a	5.90 ± 1.47 ab	5.29 ± 0.77 ab	6.25 ± 0.95 b	6.52 ± 1.00	8.09 ± 0.75	8.23 ± 0.58	8.23 ± 1.60
16:0-CoA	7.37 ± 1.19 a	5.50 ± 1.07 abc	4.42 ± 0.96 b	6.65 ± 0.91 ac	3.99 ± 1.10	5.50 ± 1.41	5.73 ± 1.52	5.61 ± 0.58

[a] Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. FAS, fatty acid synthase; ACC, acetyl-coenzyme carboxylase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme; ACL, ATP-citrate lyase; PAP, phosphatidate phosphatase; CPT-II, carnitine palmitoyl transferase II; ACD, acyl-coenzyme dehydrogenase.

diet. When the rats were fed cholesterol-enriched diets, serum T-Chol was lower in FP+C and HF-FP+C rats and was significantly lower than in the casein group, whereas rats fed the cholesterol-free diets of FP or HF-FP tended to show lower T-Chol levels. Dietary fish protein resulted in a reduction in LDL-C compared to casein diets. PL concentration was not significantly different among the groups.

Liver Lipid Concentration and Enzyme Activities. The liver lipid concentrations are presented in **Figure 1**. In the normal fat content diet, hepatic PL was significantly higher in rats fed the cholesterol-free diet than in rats fed the cholesterol-enriched diet, although in the high fat content diet hepatic PL was significantly lower in rats fed the cholesterol-free diet than in rats fed the cholesterol-enriched diet. When the rats were fed the cholesterol-enriched diet, TL concentration was significantly lower in rats fed the diet containing fish protein than in rats fed casein. There tended to be a lower concentration of TG in rats fed the fish protein diets than in rats fed the casein diets. When the rats were fed a cholesterol-enriched diet, the T-Chol concentration was markedly lower in rats fed the fish protein diet thanin rats fed the casein diet.

The hepatic enzyme activities of the fatty acid metabolic pathway are presented in **Table 4**. When the rats were fed the normal fat content diet, the hepatic activities of FAS, ACC, G6PDH, ME, and ACD were higher in rats fed the diet containing fish protein than in the rats that were fed casein. When the rats were fed the high fat content diet, the hepatic activities of FAS and ACC were higher in rats fed the fish protein diet than in rats fed the casein diet. There were no differences in the hepatic activities of G6PDH, ME, CPT-II, and ACD among the four groups. The activity of PAP, which is the rate-limiting enzyme for TG synthesis in the liver, showed no difference among all groups.

Fecal Lipid Concentration. The fecal lipid concentrations are presented in **Figure 2**. There was a tendency to higher concentrations of TL in rats fed the fish protein diet than in rats fed the casein diet. In the cholesterol-free diets, the fecal excretion of bile acid and cholesterol tended to be higher in rats fed the fish protein diet than in rats fed the casein diet, and for the cholesterol-enriched diets the rats fed the fish protein diet showed significantly greater fecal excretion of bile acid and cholesterol than those fed the casein diet.

UCP-1 Protein Expression in BAT. There was no difference in the UCP-1 protein expression level in the BAT or BAT weight among all groups.

mRNA Expression of Hepatic Lipid Metabolism Related Enzymes. To examine the effect of dietary fish protein on the level of mRNA lipid metabolism related enzymes, the expression levels of mRNAs related to cholesterol metabolism in the liver tissue were determined using quantitative real-time PCR (**Table 5**). The level of CYP7A1, which is the rate-limiting enzyme in the major biosynthetic pathway producing bile acid, was significantly increased by feeding fish protein. SHP-1, the nuclear receptor regulating CYP7A1 expression, tended to be less abundant in rats fed the fish protein diet than in rats fed the casein diet (**Figure 3**). The gene expression levels of HMGR, ACAT1, LDLR, SREBP-2, FXR, LRH-1, and PPARα were not significantly different among the groups.

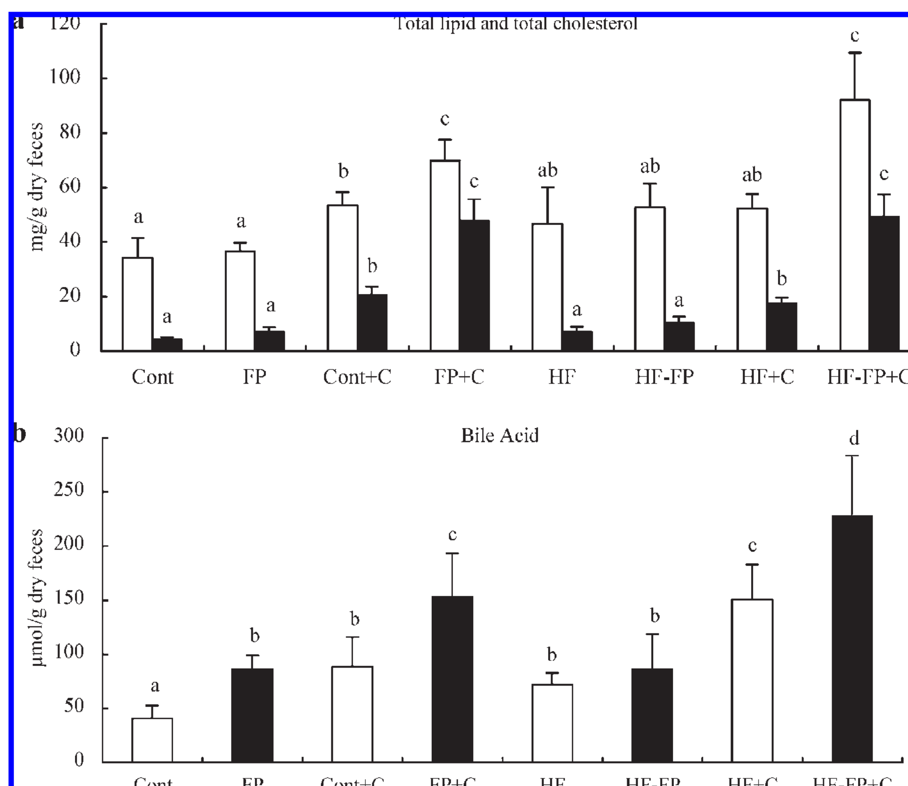

Figure 2. Total lipid and total cholesterol (**a**) and bile acid (**b**) in the feces of Wistar rats fed the diets containing casein (white bars) or fish protein (black bars). Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. TL, total lipid; T-Chol, total cholesterol.

Table 5. Expression Levels of mRNA in Rat Liver Estimated by Quantitative Real-Time RT-PCR (mRNA per GAPDH)[a]

	normal fat content diet				high fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
HMGR	1.00 ± 0.35	1.05 ± 0.18	0.84 ± 0.27	0.76 ± 0.09	1.49 ± 0.44	1.13 ± 0.45	1.12 ± 0.45	1.06 ± 0.24
ACAT	1.00 ± 0.14 a	1.24 ± 0.25 a	1.85 ± 0.32 b	1.48 ± 0.12 ab	1.70 ± 0.30	1.80 ± 0.34	2.10 ± 0.66	2.20 ± 0.42
LDL-R	1.00 ± 0.33	1.12 ± 0.24	1.17 ± 0.13	0.96 ± 0.18	1.45 ± 0.37	1.37 ± 0.26	1.96 ± 0.54	2.01 ± 0.61
SREBP-2	1.00 ± 0.25	1.17 ± 0.20	1.13 ± 0.13	0.93 ± 0.26	1.52 ± 0.39	1.66 ± 0.42	1.20 ± 0.35	1.25 ± 0.14
FXR	1.00 ± 0.37	1.36 ± 0.46	1.11 ± 0.31	0.79 ± 0.08	1.56 ± 0.65	1.51 ± 0.17	1.96 ± 0.32	1.96 ± 0.43
LRH-1	1.01 ± 0.60	0.96 ± 0.35	0.95 ± 0.34	0.73 ± 0.28	1.66 ± 0.43	1.35 ± 0.41	2.83 ± 0.91	2.18 ± 1.13
PPARα	1.00 ± 0.32	1.15 ± 0.28	1.35 ± 0.42	1.16 ± 0.22	1.79 ± 0.45	1.79 ± 0.32	2.05 ± 0.88	1.87 ± 0.30

[a] Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$. Relative values are presented as the ratio of mRNA to GAPDH mRNA. HMGR, 3-hydroxy-3methylglutaryl-coenzyme A reductase; ACAT-1, acyl-coenzyme:cholesterol acyltransferase-1; LDL-R, low-density lipoprotein receptor; SREBP-2, sterol regulatory element-binding protein-2; FXR, farnesoid X receptor; LRH-1, liver receptor homologue-1; PPARα, peroxisome proliferation activated receptor alpha.

DISCUSSION

The results of this study demonstrate that fish protein compared to casein led to a decrease in the liver T-Chol and serum T-Chol and LDL-C concentrations. Brandsch et al. suggested that protein extracts from beef, pork, and turkey meat did not alter the cholesterol concentration in the plasma or liver compared with casein (*8*). These results indicate a possibility that cholesterol metabolism changes in the liver and serum were involved in the antiatherosclerotic disease potential of fish protein compared with animal proteins such as casein, beef, pork, and turkey.

The liver TG concentration tended to be lower in fish protein-fed rats than in the casein-fed rats (**Table 4**). The reason for the decrease in TG concentration in the liver was thought to be the absorption of lipid from the small intestine and/or the metabolism of lipids and fatty acids in the liver. The enzyme activities of fatty acid β-oxidation in the mitochondria for fatty acid synthesis in the liver were examined when the rats were fed the normal fat content diet, and the hepatic activities of fatty acid synthesis in rats fed fish protein were higher than in rats fed casein, whereas the enzymes of β-oxidation in liver mitochondria showed no differences. When the rats were fed high fat content diets, there were no differences in the hepatic activities of fatty acid synthesis and the hepatic activities of fatty acid oxidation of the rats fed fish protein were greater than in those fed casein. However, the activity of PAP, which is the rate-limiting enzyme for TG synthesis in the liver, showed no differences among all of the diets. Moreover, the expression level of PPARα, which is a transcription factor that has important effects on lipid homeostasis via regulation of the expression of genes involved in hepatic fatty acid uptake and oxidation in peroxisomes, showed no differences among all of the groups (*27*). The UCP expression level in BAT, which is known as a significant component of whole body energy expenditure and its dysfunction contributes to the development of obesity, showed no differences among all of the diets (*28*). Therefore, it was considered that the decrease in liver TG was not caused by the enzyme activities of fatty acid β-oxidation and synthesis in the liver but rather by a decrease in the absorption of lipids from the small intestine in rats fed on fish protein. When the rats were fed

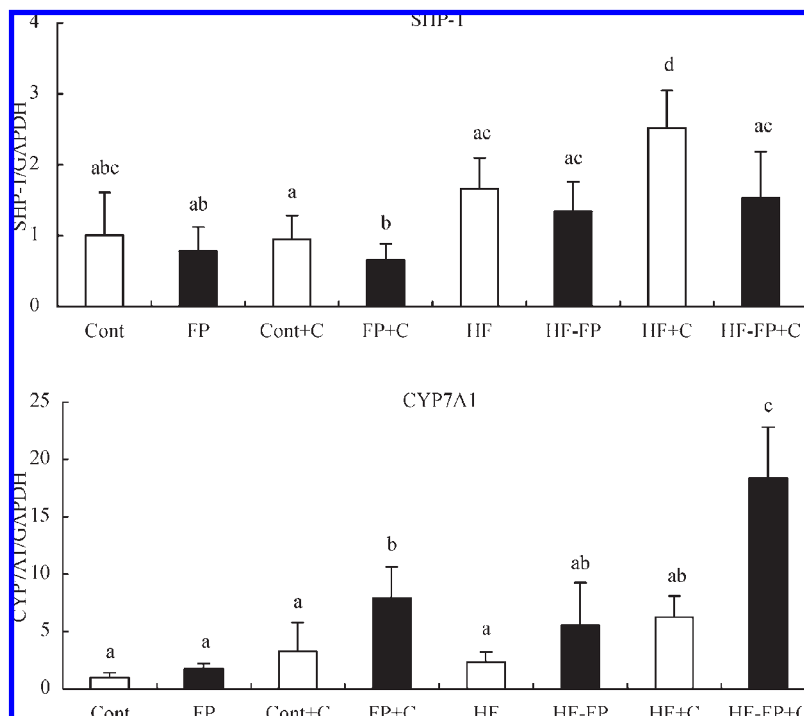

Figure 3. CYP7A1 and SHP-1 mRNA expression in the liver of Wistar rat fed diets containing casein (white bars) or fish protein (black bars). The expression levels of mRNA were determined by quantitative RT-PCR and expression relative to the control diet. Data are means $\pm$ SD. Values not sharing a common letter are significantly different at $p < 0.05$. CYP7A1, cholesterol 7α-hydroxylase; SHP-1, small heterodimer partner-1.

cholesterol-enriched diets, the hepatic activities of fatty acid synthesis and oxidation were not sufficient, and the livers of cholesterol-fed rats exhibited hypertrophy due to accumulated lipids. The fecal TL concentration of rats fed fish protein was greater than that of rats fed the casein diet (**Figure 2**). Consequently, it was hypothesized that the decrease in the liver TG concentration was due to the inhibition of lipid absorption from the small intestine by digested fish protein. The digested fish protein may inhibit lipase activity and micelle formation in the small intestine. Therefore, it is probable that the fish protein decreased the TG concentration in the liver.

Remarkable differences were observed in the cholesterol concentration in the serum and liver in rats fed fish protein (**Table 3**; **Figure 1**). In the cholesterol-enriched diet, dietary fish protein, as compared with dietary casein, significantly decreased the serum T-Chol and LDL-C and the liver T-Chol concentration. In the cholesterol-free diets, a similar decrease was observed. The reason for the decrease in cholesterol concentration in the liver was thought to be the down-regulation of genes involved in cholesterol synthesis and cholesterol uptake via decreased levels of mRNA coding for SREBP-2 and the up-regulation CYP7A1, which is the initial and rate-limiting enzyme in the conversion of cholesterol to 7α-hydroxylated bile acid, via a FXR-mediated pathway. In the present study, fish protein did not affect the hepatic gene expression levels of HMGR, LDLR, or SREBP-2, and the CYP7A1 expression level was higher in rats fed the fish protein diet than in rats fed the casein diet. Hence, in the livers of rats of fed fish protein, enhanced rates of the hepatic catabolism of cholesterol via bile acid were observed, but the synthesis and uptake of cholesterol were not changed.

In the present study, the fecal excretion of cholesterol and bile acid was greater in rats fed fish protein than in the rats fed casein. Hence, the dietary fish protein effectively inhibited the absorption cholesterol and bile acid in the small intestine. These results suggest the suppression of cholesterol absorption by the micellar solubility of cholesterol and the binding capacity of cholate of digested fish protein in the jejunal epithelia. Furthermore, the digestion of fish protein may also inhibit the reabsorption of bile acids in the ileum.

Recently, attention has been focused on the roles of nuclear receptors in the metabolism of cholesterol and bile acids (*29*). Bile acids negatively regulate the transcription of CYP7A1 via the activation FXR, that is, a SHP-dependent pathway. In this study, the mRNA expression level of hepatic SHP-1 was significantly lower in rats fed fish protein compared with rats fed a cholesterol-enriched diet. It is known that the reduction of SHP activates LRH-1, which binds to the promoter of CYP7A1 and increases its mRNA expression level. This study suggests that the CYP7A1 expression level was higher in rats fed the fish protein diet than in rats fed the casein diet, which resulted from the digested fish protein inhibiting the reabsorption of bile acids in the ileum. The FXR/SHP-dependent pathway was negatively regulated by the decreased reabsorption of bile acid, and increased binding to the promoter of CYP7A1 by LRH-1 activated the expression of CYP7A1.

The serum LDL-C concentration was lower in the fish protein fed than in the casein-fed rats. In the case of dietary soy protein, the LDL-C concentration was decreased as a result of an increase in LDLR activity (*30*). In this study, the mRNA expression level of hepatic LDLR was not significantly different among the diets. The decreased LDL-C concentration observed in the rats fed fish protein could possibly be related to a decrease in very low density lipoprotein (VLDL) secretion, which is the precursor of LDL, or an increase in the removal of VLDL by VLDL receptors in hepatocytes.(*31*). In a previous study, Shukla et al. showed that a decrease in the serum T-Chol concentration was due to reduced serum HDL-C concentration on feeding fish protein from Alaska pollock to rats (*11*). However, in the present study, dietary fish protein did not affect the serum HDL-C concentration. The reason for the difference in HDL-C metabolism due to the intake of fish protein is unclear at present.

In conclusion, the present study suggests that fish protein, compared to casein, promoted decreases in liver T-Chol and TG

and serum T-Chol and LDL-C concentrations. This study showed that fish protein exerted hypotriglyceridemic and hypocholesterolemic effects, which may aid in the prevention of arteriosclerosis.

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