Effect of Simultaneous Intake of Fish Protein and Fish Oil on Cholesterol Metabolism in Rats Fed High-Cholesterol Diets

Ryota Hosomi[1], Kenji Fukunaga[1,*], Hirofumi Arai[2], Seiji Kanda[3], Toshimasa Nishiyama[3] and Munehiro Yoshida[1]

[1]*Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Osaka, 564-8680, Japan*

[2]*Department of Biotechnology and Environmental Chemistry, Kitami Institute of Technology, Kitami, Hokkaido 090-8507, Japan*

[3]*Department of Public Health, Kansai Medical University, Moriguchi, Osaka, 570-8506, Japan*

Abstract: This study demonstrated the effect of the simultaneous dietary administration of fish protein and fish oil, two macronutrients found in fish meat, on cholesterol metabolism in rats. Male Wistar rats were divided into four groups and fed an AIN-93G modified hypercholesterolmic diet with casein (20%) + soybean oil (7%), casein (10%) + fish protein (10%) + soybean oil (7%), casein (20%) + soybean oil (5%) + fish oil (2%), and casein (10%) + fish protein (10%) + soybean oil (5%) + fish oil (2%) for four weeks. Cholesterol metabolism was measured through serum and liver cholesterol contents, fecal cholesterol and bile acid excretion levels, and liver mRNA expression levels of enzymes and nuclear receptors involved in cholesterol homeostasis. Dietary fish protein decreased serum and liver cholesterol contents, perhaps through increasing fecal cholesterol and bile acid excretion and liver cholesterol 7α-hydroxylase expression level. Dietary fish oil, on the other hand, decreased liver cholesterol content, perhaps due to the suppression of cholesterol synthesis through a decrease in the 3-hydroxy-3methylglutaryl-coenzyme A reductase expression level; the serum cholesterol content was unchanged. This study found that the simultaneous dietary administration of fish protein and fish oil, which is achieved by the intake of intact fish muscle, has hypocholesterolemic effects that help prevent hyper-lipidemia and atherosclerosis.

Keywords: Fish protein, fish oil, cholesterol metabolism, bile acid, nuclear receptor.

INTRODUCTION

In several cohort studies [1, 2] and interventional studies [3, 4], the consumption of fish or fish oil containing n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was associated with reduced risk of coronary heart disease (CHD). Fish oil is also known to beneficially suppress inflammation [5] and platelet aggregation [6]. Accordingly, most of the research on dietary fish consumption has focused on the effects of EPA and DHA. Several mechanisms have been proposed to explain how n-3 PUFA might beneficially influence cardio-vascular disease [7]; these include suggestions that n-3 PUFA can prevent arrhythmias [8] or decrease serum triacyl-glycerol (TG) by stimulating β-oxidation and inhibiting lipogenesis in the liver [9]. Yet it is difficult to explain the health benefits associated with fish consumption only in terms of EPA and DHA because their effect on plasma cholesterol (CHOL) levels is still controversial [10].

*Address correspondence to this author at the Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Osaka, 564-8680, Japan Fax: +81-6-6388-8609
E-mail: fukunagk@kansai-u.ac.jp

In recent years, dietary fish protein, another macro-nutrient occurring in fish meat, has attracted much attention. It has been shown that dietary fish protein decreases blood CHOL levels in laboratory animals [11-13] and exerts antihypertensive [14] and anti-obesity effects [15]. Most people who eat fish eat fish muscle, and thereby consume fish oil and fish protein together, but few studies have focused on the combined effects of dietary fish oil and fish protein on lipid metabolism or CHOL levels.

The present study investigated the effects of the simultaneous intake of fish protein and fish oil on serum and liver CHOL levels in rats fed high-CHOL diets. Other recent studies have suggested that various nuclear receptors, such as liver X receptor-α (LXRα), farnesoid X receptor-α (FXRα), and hepatocyte nuclear factor-4α (HNF-4α), play important roles in the regulation of CHOL and bile acid metabolism enzymes and transporters [16-18]. To further elucidate the mechanisms underlying the beneficial effects of the intake of fish protein and oil together, we also examined the liver mRNA expression levels of the above-mentioned nuclear receptors associated with CHOL metabolism-related enzymes, as well as 3-hydroxy-3methylglutaryl-coenzyme A reductase (HMGR), cholesterol 7α-hydroxylase (CYP7A1),

1876-3960/11 2011 Bentham Open

CHOL metabolism-related transporters, and ATP-binding cassette A1 (ABCA1).

MATERIALS AND METHODS

Materials

Fish protein was prepared from Alaska pollock (*Theragra chalcogramma*) fillet, as described previously [11]. Fish oil extracted from Bigeye tuna (*Thunnus obesus*) was provided by Yashima Shiyoji Co., Ltd (Shizuoka, Japan). AIN-93 vitamin mix, AIN-93G mineral mix, dextrinized cornstarch, cornstarch, cellulose, sucrose and casein were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). L-Cystine, choline bitartrate and soybean oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were obtained from common commercial sources and were reagent grade.

Animal Subjects

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 housed in plastic cages in an air-conditioned room (temperature, 21-22˚C; humidity, 55-65%; lights on, 08:00-20:00 h). After 5 days of acclimation, during which rats were fed a diet prepared according to the recommendations of the American Institute of Nutrition (AIN-93G) [19], rats were divided into 4 groups of 7 rats each with similar mean body weights, and each group was fed one of the four experimental diets (casein diet, CAS; fish protein diet, FP; fish oil diet, FO; fish protein and fish oil diet, FP+FO). Rats had free access to drinking tap water. The compositions of the experimental diets, which were prepared according to AIN-93G with hypercholesterolemic modification, are shown in Table **1**. The amino acid compositions of the casein and fish protein, as determined through analysis by a commercial service (Japan Food Research Laboratories, Tokyo, Japan), are shown in Table **2**. Table **3** shows the fatty acid compositions of the soybean oil and fish oil, as determined through gas chromatography (GC-14B, Shimadzu Co., Kyoto, Japan) using an Omegawax 250 fused silica capillary column (Supelco Co., Ltd., Bellefonte, PA, USA) after methylation with sodium methoxide.

Food intake and body weights were recorded every two days. Feces were collected from each group every 24 hours for 7 days prior to sacrifice. After consuming the experimental diets for 4 weeks, rats were weighed and sacrificed under Pentobarbital (Nembutal®, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) anesthesia. Rats were not fasted before being sacrificed because food deprivation prior to sacrifice leads to a significant down-regulation of the genes involved in fatty acid synthesis and CHOL metabolism [20]. Blood was collected, and serum was obtained by centrifugation at 1,500 g for 15 min and stored at –80˚C until analysis. Liver and abdominal white adipose tissues (WAT) from the epididymis, mesentery, and perinephria were excised, weighed, rinsed, frozen in liquid nitrogen, and then

Table 1. Composition of the Experimental Diets (g/kg)

Components	CAS	FP	FO	FP+FO
Dextrinized corn starch	132	132	132	132
Corn starch	391.5	391.5	391.5	391.5
Casein	200	100	200	100
Fish protein	-	100	-	100
Sucrose	100	100	100	100
Cellulose	50	50	50	50
AIN-93G mineral mixture	35	35	35	35
AIN93 vitamin mixture	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butylhydroquinone	0.01	0.01	0.01	0.01
Soybean oil	70	70	50	50
Fish oil	-	-	20	20
Cholesterol	5	5	5	5
Sodium cholate	1	1	1	1

Diets were prepared based on AIN-93G recommendations.

Table 2. Amino Acid Composition of Dietary Proteins (g/100g Protein)

Amino Acid	Casein	Fish Protein
Alanine	2.9	5.8
Arginine	3.5	6.6
Aspartic acid*	6.7	10.6
Cystine	0.4	1.0
Glutamic acid†	20.2	17.2
Glycine	1.7	3.6
Histidine	2.9	2.3
Isoleucine	5.2	4.7
Leucine	8.9	8.6
Lysine	7.6	10.3
Methionine	2.8	3.2
Phenylalanine	4.8	3.7
Proline	10.6	3.3
Serine	4.9	4.2
Threonine	3.9	4.7
Tryptophan	1.2	1.1
Tyrosine	5.3	3.9
Valine	6.4	5.1

* Aspartic acid: Aspartic acid + Asparagine
† Glutamic acid: Glutamic acid + Glutamine

Table 3. Fatty Acid Composition of Experimental Oils (wt%)

Fatty Acid	Soybean Oil	Fish Oil
14:0	N.D.	3.3
15:0	N.D.	0.7
16:0	10.6	15.8
16:1 n-7	N.D.	0.8
16:1 n-9	Tr.	5.1
17:0	N.D.	1.1
17:1	N.D.	0.5
18:0	3.8	4.6
18:1 n-7	1.4	2.2
18:1 n-9	21.8	12.5
18:2 n-6	53.7	1.3
18:3 n-3	5.8	1.7
18:3 n-6	Tr.	0.6
20:1 n-9	Tr.	1.2
20:4 n-6	N.D.	2.0
20:5 n-3	N.D.	10.9
22:5 n-3	N.D.	1.9
22:5 n-6	N.D.	1.9
22:6 n-3	N.D.	28.8
Others	2.9	3.1

N.D., not detected; T., trace (less than 0.5%).

stored at –80˚C until analysis. An aliquot of liver was taken for mRNA expression analysis and stored in RNA-Later Storage Solution (Sigma-Aldrich, St. Louis, MO, USA).

Analysis of Lipid Indexes

Serum CHOL, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), TG and contents were measured in triplicate using an Olympus AU5431 automatic analyzer with AU regent (Beckman Coulter Inc., Brea, CA, USA). Liver lipid was extracted according to the method of Bligh and Dyer [21]. Liver and fecal CHOL contents were analyzed through gas chromatography using an SE-30 column (Shinwa Chemical Industries Ltd., Kyoto, Japan), with 5α-cholestane as an internal standard. Liver TG content was determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries, Ltd.) after the total liver lipid had been dissolved in an equal volume of dimethyl sulfoxide. Fecal bile acid content was measured according to the method of Bruusgaard *et al.* [22].

Analysis of mRNA Expression Levels

Total liver RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. cDNA was then synthesized from the total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Real-time quantitative PCR analysis was performed with an automated sequence detection system (ABI Prism 7000, Applied Biosystems Japan Ltd.). HMGR, CYP7A1, sterol regulatory element binding protein-2 (SREBP-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were measured with TaqMan® Gene Expression Assays (Applied Biosystems Japan Ltd.). PCR Primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; SREBP-2: Rn01502638_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems Japan Ltd. mRNA expression levels of ABCA1, ABCG5, ABCG8, LDL receptor (LDLR), scavenger receptor class B type 1 (SR-B1), LXRα, FXRα, small heterodimer partner-1 (SHP-1), liver receptor homolog-1 (LRH-1), HNF-4α and GAPDH were measured using SYBR®Green PCR Master Mix (Applied Biosystems Japan Ltd.). The primer sequences used for the detection of ABCA1, ABCG5, ABCG8, LDLR, SR-B1, LXRα, FXRα, SHP-1, LRH-1, and HNF-4α and GAPDH were as follows: forward: 5' CCCGGCGGAGTAGAAAGG 3' and reverse: 5' AGGGCGATGCAAACAAAGAC 3' for

ABCA1, forward: 5' CCTCAAGGGCTCCGAGAACT 3' and reverse: 5' ACCACACTGCCCCATAAGCT 3' for ABCG5, forward: 5' GCCATGGACCTGAACTCACA 3' and reverse: 5' GCTGATGCCAATGACGATGA 3'; for ABCG8, forward: 5' CACCCCCTCGTTGAAAACCT 3' and reverse: 5' CCTTAGCCAGCTCTTCCAGATC 3' for LDLR, forward: 5' GCATTCGGAACAGTGCAACA 3' and reverse: 5' TCATGAATGGTGCCCACATC 3' for SR-B1, forward: 5' TGCATGCCTATGTCTCCATCA 3' and reverse: 5' GCATCCGTGGGAACATCAGT 3' for LXRα, forward: 5' GGGCCTTGGACGTCTCTGA 3' and reverse: 5' CTGGGATGGTGGTCTTCAAATAA 3' for FXRα, forward: 5' CGCCTGGCCCGAATC 3' and reverse: 5' GAAGGGTACAGGAGATGTTCTTGAG3'; for SHP-1, forward: 5'TCCGGGCAATCAGCAAA 3' and reverse: 5' CCCCATTCACGTGCTTGTAGT 3' and for LRH-1, forward: 5' TGCAGGCAGAGGTCCTGTCT 3' and reverse: 5' TCGCCATTGATCCCAGAGA 3' for HNF-4α, forward: 5' GAAGACACCAGTAGACTCCACGACATA 3' and reverse: 5' GAAGGTCGGTGTGAACGGATT 3' for GAPDH using Primer Express 3.0 software (Applied Biosystems Japan Ltd.). The expression level of GAPDH, the housekeeping gene, served as an internal control for the normalization.

Statistical Analysis

Data are expressed as means ± standard error of mean (SEM) in the 7-rat groups. The types of statistical analysis used were two-way analysis of variance (ANOVA) and Tukey-Kramer test; these were performed using StatView-J version 5.0 software (Abacus Concept, Berkeley, CA, USA). Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Epidemiological studies among Greenland Inuits and in Japanese fishing villages have suggested that the consumption of fish and marine animals can prevent CHD [2, 23]. Other evidence suggests that elevated serum TG and LDL-C levels significantly increase CHD risk, while there is an inverse correlation between serum HDL-C level and CHD risk [24, 25]. Many researchers have shown that dietary fish oil containing EPA and DHA can suppress serum TG levels [9]. In recent years, we and other researchers have found that dietary fish protein, unlike dietary casein, can decrease plasma CHOL concentrations in laboratory animals [11-13]. Yet few studies have focused on the effect of the simultaneous intake of fish protein and fish oil on lipid metabolism. The present study investigated the effect of the simultaneous intake of fish protein and fish oil on CHOL and bile acid metabolism in rats fed high-CHOL diets. It also sought to analyze gene expression levels of enzymes and nuclear receptors related to the metabolism of CHOL.

Table **4** showed that initial body weight, final body weight, body weight gain, energy intake, food efficiency, and relative weights of liver and WAT (sum of epididymal, mesentery, and perirenal WAT) in rats fed experimental diets for 4 weeks. There were no significant differences among the groups in any of these measurements.

Table **5** showed that serum, liver, and fecal lipid indices. Serum CHOL content was significantly lower in the FP+FO group than in the FO group. Fish protein affected serum CHOL content, but fish oil alone did not (P=0.003). Serum LDL-C contents were significantly lower in the FP and FP+FO groups than in the CAS group. Fish protein affected serum LDL-C content, but fish oil alone did not (P=0.002). Dietary fish oil (FO and FP+FO) tended to decrease serum TG content, but this tendency was not significant (P=0.073). Liver CHOL content was markedly decreased by the intake of an FP, FO, or FP+FO diet as compared with a CAS diet. Liver TG content was significantly lower in the FO group than in the CAS, FP, and FP+FO groups.

As shown in Table **5**, our results indicate that the simultaneous intake of fish protein and fish oil decreases serum CHOL, LDL-C, and TG levels in rats; the decreases in

Table 4. Growth Parameters and Organs Weights of Rats Fed the Experimental Diets for 4 Weeks

	CAS	FP	FO	FP+FO	ANOVA (*P* values)		
					Protein(P)	Lipid(L)	P×L
Growth parameters							
Initial BW (g)	130.0±2.2	128.6±2.4	130.0±3.1	130.0±1.5	0.765	0.787	0.796
Final BW (g)	327.9±4.0	317.9±6.3	322.1±9.6	327.1±5.2	0.710	0.790	0.270
BW gain (g/day)	7.1±0.4	6.8±0.5	7.1±0.5	7.0±0.6	0.591	0.948	0.063
Energy intake (kcal/day)	71.8±2.4	69.6±2.0	68.8±2.2	72.9±2.5	0.577	0.546	0.269
Food efficiency (g/kcal)	0.098±0.002	0.097±0.003	0.099±0.003	0.097±0.003	0.256	0.495	0.128
Organ weight (g/kg BW)							
Liver weight	0.49±0.06	0.49±0.03	0.51±0.11	0.53±0.03	0.136	0.598	0.069
WAT weight	0.46±0.07	0.44±0.05	0.49±0.09	0.46±0.05	0.373	0.350	0.869

Data are means ± SEM (n=7). BW, body weight; WAT, white adipose tissue.
WAT represents the sum of WAT weights from the epididymis, mesentery, and perinephria.

Table 5. Lipid Indexes in Serum, Liver, and Feces of Rats Fed the Experimental Diets

	CAS	FP	FO	FP+FO	ANOVA (*P* values)		
					Protein (P)	Lipid (L)	P×L
Serum (mg/dl)							
CHOL	73.3±3.1 [ab]	64.8±2.8 [ab]	74.4±3.4 [a]	63.0±2.2 [b]	0.003	0.904	0.634
HDL-C	52.7±2.1	57.0±2.1	49.9±2.0	54.7±2.4	0.049	0.256	0.898
LDL-C	8.2±0.3 [a]	7.0±0.3 [b]	7.8±0.4 [ab]	6.9±0.2 [b]	0.002	0.480	0.723
TG	40.3±2.1	39.2±2.2	36.7±1.6	33.5±3.0	0.389	0.073	0.689
Liver (mg/g Liver)							
CHOL	13.1±0.9 [a]	8.1±0.6 [bc]	6.3±0.7 [c]	10.3±0.7 [b]	0.505	0.071	<0.001
TG	141.1±1.9 [a]	147.6±7.6 [a]	100.6±5.7 [b]	143.9±3.2 [a]	0.002	<0.001	0.001
Feces							
CHOL (mg/day)	89.7±5.8 [a]	120.6±5.9 [b]	100.3±7.4 [ab]	88.3±11.5 [a]	0.275	0.213	0.018
Bile acid (μmol/day)	192±11.2 [a]	252±12.9 [b]	188.7±9.3 [a]	270±19.3 [b]	<0.001	0.656	0.527

Data are means ± SEM (n=7). Values not sharing a common letter are significantly different at $P<0.05$.
TG, triacylglycerol; CHOL, cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

serum CHOL and LDL-C may be due to dietary fish protein intake, while the decrease in serum TG may be due to dietary fish oil intake. In addition, the intake of dietary fish protein elevates serum HDL-C (P=0.049, Table **5**), which is known to be the principal vehicle by which surplus CHOL is removed from the peripheral tissues to the liver. These results suggest that the simultaneous intake of fish protein and fish oil may contribute to a decreased risk of CHD. Wergedahl *et al.* and Shukla *et al.*, however, have reported that dietary fish protein from Salmon and Alaska pollock decreases serum HDL-C in hyperlipidemic obese Zucker rats and Wistar rats [12, 13]. In addition, Bergeron *et al.* have reported that dietary fish protein elevates HDL-C concentrations in rabbits [26]. Therefore, the effect of dietary fish protein on HDL-C metabolism is still controversial.

Two main mechanisms have been proposed to explain the decreases in CHOL levels in the serum and liver that are associated with dietary fish protein and fish oil. One is an increase in excretion of steroids, including CHOL and bile acid [27]; the other is a change in CHOL metabolism in the liver, which is maintained through a balance of excretion, biosynthesis, storage, catabolism, and uptake of steroids. To test the first mechanism, we measured the fecal CHOL and bile acid levels in rats fed fish protein and fish oil. The simultaneous intake of fish protein and fish oil (FP+FO) had no effect on fecal CHOL excretion, while fecal bile acid excretion was increased compared with CAS group (Table **5**). The intake of fish protein only (FP), on the other hand, increased both fecal CHOL and bile acid excretion, but the intake of fish oil only (FO) had no effect on either fecal CHOL or bile acid excretion. Consequently, it has been suggested that the decreases in serum and liver CHOL in rats fed fish protein may be attributable to the suppression of CHOL and bile acid absorption through the enhancement of fecal CHOL and bile acid excretion, while the decrease in liver CHOL associated with dietary fish oil may be due not to the excretion of CHOL and bile acid but to changes in CHOL metabolism.

To test the second proposed mechanism, we analyzed liver mRNA expression levels of genes encoding proteins involved in CHOL metabolism (Table **6**). ABCA1 is a transporter involved in the production of HDL, SR-B1 is responsible for the selective uptake of HDL, and LDLR is required for LDL uptake into cells. As shown in Table **6**, there were no differences in the expression levels of ABCA1, SR-B1, and LDLR among the groups. A previous study has shown that fish oil intake suppresses the activity of HMGR, a rate-limiting enzyme involved in CHOL synthesis [28]; our results likewise show that fish oil (FO and FP+FO) decreases HMGR expression level (P=0.002). Liver ABCG5 and ABCG8 in their heterodimer forms play major roles in the excretion of CHOL into bile [29], and it has been reported that an increase in biliary CHOL excretion is induced by an increase in ABCG5 and ABCG8 expression [16]. Our results show that fish oil intake tends to increase ABCG5 and ABCG8 expression levels (P=0.097 and 0.061, respectively), which might increase biliary CHOL excretion, though fish oil intake did not affect fecal CHOL excretion. These results suggest that the decreased liver CHOL levels in rats fed diets containing fish oil could be attributable to the suppression of CHOL synthesis through decreased liver HMGR expression and to an increase in the excretion of liver CHOL into bile through increased ABCG5 and ABCG8 expression. Excess CHOL in the liver is eliminated mainly through conversion to bile acid by CYP7A1, the initial and rate-limiting enzyme involved in the conversion of CHOL to 7α-hydroxylated bile acid [30]. The expression level of CYP7A1 in the liver was increased by fish protein intake but

Table 6. Liver mRNA Expression Levels of Genes Encoding Proteins Involved in Cholesterol Metabolism

	CAS	FP	FO	FP+FO	ANOVA (*P* values)		
					Protein(P)	Lipid(L)	P×L
Relative expression levels							
ABCA1	1.00±0.06	0.89±0.04	0.93±0.04	0.91±0.04	0.264	0.787	0.223
ABCG5	1.00±0.15	1.03±0.27	1.38±0.09	1.24±0.11	0.754	0.097	0.643
ABCG8	1.00±0.15	1.07±0.27	1.62±0.12	1.47±0.19	0.893	0.061	0.679
SR-B1	1.00±0.07	1.02±0.05	1.00±0.04	0.97±0.05	0.995	0.621	0.666
LDLR	1.00±0.08	0.98±0.07	0.97±0.05	0.97±0.05	0.873	0.820	0.836
HMGR	1.00±0.08 [ab]	1.14±0.06 [a]	0.81±0.05 [b]	0.79±0.08 [b]	0.432	0.002	0.317
CYP7A1	1.00±0.08	1.34±0.13	0.91±0.13	1.23±0.13	0.019	0.444	0.937

Data are means ± SEM (n=7). Values not sharing a common letter are significantly different at $P<0.05$.
Total RNA was extracted from liver and mRNA expression levels were determined by real-time PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA expression level for normalisation. mRNA expression levels of gene are shown relative to the livers of rats fed CAS diet (= 1.00).
ABCA1, ATP-binding cassette A1; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; SR-B1, scavenger receptor class B type 1; LDLR, low density lipoprotein receptor; HMGR, 3-hydroxy-3methylglutaryl-coenzyme A reductase; CYP7A1, cholesterol 7α-hydroxylase.

not by casein diet intake; this indicates that dietary fish protein can reduce the excess CHOL in the liver that accumulates due to a high-CHOL diet (P=0.019). In the FP+FO group, HMGR expression decreased while ABCG5, ABCG8 and CYP7A1 expression levels increased, though the effect of liver CHOL reduction was weaker in the FP+FO group than in the FP and FO groups. It has previously been suggested that decreases in lipoprotein formation along with the subsequent lowering of plasma lipids could lead to the accumulation of lipids in the liver [31]. The FP+FO diet strongly decreased serum CHOL compared to the FO diet and strongly decreased serum TG compared to the FP diet. Thus these serum lipid decreases may be due to a reduction in lipoprotein formation in the liver, which results in an accumulation of CHOL and TG in the liver. In addition, the lack of any change to the fecal CHOL excretion levels associated with the FP+FO diet may be due to an accumulation of CHOL in the liver. The reason why fecal CHOL excretion was unchanged in the FP+FO group in contrast to the CAS group is not yet clear.

The effects of diet on transcription factors and nuclear receptors have recently begun to be studied [16-18]. We analyzed the mRNA expression levels of SREBP-2, a transcription factor, and of LXRα, FXRα, SHP-1, LRH-1, and HNF-4α, all of which are nuclear receptors (Table **7**). Many transcriptional factors have been reported to play important roles in regulating CYP7A1 transcription [32]. CYP7A1 expression is negatively regulated by the FXRα/SHP-1-dependent pathway, which is regulated by changes in bile acid reabsorption rates, and positively regulated by LXRα and HNF-4α. In the present study, CYP7A1 expression levels increased in groups consuming fish protein (P=0.019). Dietary fish protein did not affect the expression levels of LXRα and HNF-4α, but the expression of SHP-1 was decreased in groups consuming fish protein compared with groups consuming casein (P=0.035). In addition, fecal bile acid excretion was increased in the FP and FP+FO groups compared with the CAS and FO groups (P<0.001). Therefore, dietary fish protein may affect the FXRα/SHP-1-dependent pathway through inhibition of the absorption of bile acid in the small intestine as a result of the increased expression level of CYP7A1.

Dietary fish oil tended to increase ABCG5 and ABCG8 expression levels compared with dietary soybean oil (P=0.097 and 0.061, respectively). ABCG5 expression and ABCG8 expression are regulated by LXRα, which also regulates the expression of many other key genes in lipid metabolism, including ABCA1 [33], but there was no difference in liver LXRα expression among the groups in the present study, meaning that the increase in ABCG5 and ABCG8 expression in the FO and FP+FO groups was not caused by an increase in LXRα expression. Freeman *et al*. have suggested that one positive transcription factor regulating ABCG5 and ABCG8 is LRH-1, which is also involved in sterol and bile acid secretion from the liver, but there was no difference in LRH-1 expression among the groups, either [34]. Clearly, further experimentation will be necessary to clarify the mechanisms involved in this phenomenon.

SREBPs regulate the transcription of genes encoding enzymes in the biosynthesis of CHOL and TG [35]. SREBP-2 mainly activates the transcription of CHOL synthesis-related genes, such as HMGR and LDLR. Dietary fish oil decreased HMGR expression due to a decrease in liver SREBP-2 expression, while LDLR expression was unchanged. This shows that the simultaneous intake of fish protein and fish oil may increase CYP7A1 expression in the liver through suppression of the FXRα/SHP-1 pathway due to fish protein intake while also decreasing liver HMGR expression through suppression of SREBP-2 expression due to fish oil intake. We suggest that the simultaneous intake of fish protein and fish oil results in an additive effect on CHOL metabolism, but not a synergistic effect.

Table 7. Liver mRNA Expression Levels of a Transcriptional Factor (SREBP-2) and Several Nuclear Receptors (LXRα, FXRα, SHP-1, LRH-1 and HNF-4α)

	CAS	FP	FO	FP+FO	ANOVA (*P* values)		
					Protein(P)	Lipid(L)	P×L
Relative expression levels							
SREBP-2	1.00±0.07	1.05±0.09	0.87±0.07	0.87±0.06	0.733	0.042	0.757
LXRα	1.00±0.05	1.02±0.06	0.98±0.06	0.94±0.05	0.888	0.088	0.762
FXRα	1.00±0.09	1.00±0.10	0.88±0.07	0.98±0.07	0.513	0.433	0.559
SHP-1	1.00±0.17	0.73±0.13	1.11±0.07	0.79±0.09	0.035	0.532	0.822
LRH-1	1.00±0.07	1.13±0.06	1.06±0.02	1.02±0.06	0.466	0.704	0.176
HNF-4α	1.00±0.09	0.99±0.05	0.92±0.05	0.91±0.04	0.855	0.223	0.996

Data are means ± SEM (n=7). Values are significantly different at $p<0.05$.
Total RNA was extracted from liver and mRNA expression levels were determined by real-time PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA expression level for normalisation. mRNA expression levels of gene are shown relative to the livers of rats fed CAS diet (= 1.00).
SREBP-2, sterol regulatory element binding protein-2; LXRα, liver X receptor-α; FXRα, farnesoid X receptor-α; SHP-1, small heterodimer partner-1; LRH-1, liver receptor homolog-1; HNF-4α, hepatocyte nuclear factor-4α.

CONCLUSION

Dietary fish protein decreased serum CHOL, LDL-C levels, and liver CHOL content through increased fecal CHOL and bile acid excretion and increased liver CYP7A1 expression level due to the suppression of the FXRα/SHP-1 pathway. Furthermore, dietary fish oil did not affect serum CHOL content, though liver CHOL levels were decreased through the suppression of CHOL synthesis due to a decrease in HMGR expression and export of CHOL into bile from the liver, which were due in turn to an increase in ABCG5 and ABCG8 expression. We confirmed that the diet containing both fish protein and fish oil conveyed an additive effect. This study found that the simultaneous intake of fish protein and fish oil, which is achieved through the intake of intact fish muscle, has hypocholesterolemic effects that can aid in the prevention of CHD, hyperlipidemia, and atherosclerosis.

ABBREVIATIONS

ABC = ATP-binding cassette
CHD = Coronary heart disease
CHOL = Cholesterol
CYP7A1 = Cholesterol 7α-hydroxylase
DHA = Docosahexaenoic acid
EPA = Eicosapentaenoic acid
FXRα = Farnesoid X receptor-α
GAPDH = Glyceraldehyde-3-phosphate dehydrogenase
HDL-C = HDL-cholesterol
HMGR = 3-hydroxy-3methylglutaryl-coenzyme A reductase
HNF-4α = Hepatocyte nuclear factor-4α
LDL-C = LDL-cholesterol
LDLR = LDL receptor
LHR-1 = Liver receptor homolog-1
LXRα = Liver X receptor-α
SHP-1 = Small heterodimer partner-1
SREBP-2 = Sterol regulatory element binding protein-2
SR-B1 = Scavenger receptor class B type 1
TG = Triacylglycerol
WAT = White adipose tissue

ACKNOWLEDGEMENT

We thank Hayato Maeda of Hirosaki University and Masashi Hosokawa and Kazuo Miyashita of Hokkaido University for their assistance with the real-time PCR analyses, and Kenta Hayashi and Takanori Shimizu of Kansai University for their assistance with the lipid analyses.

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Received: January 03, 2011 Revised: January 11, 2011 Accepted: January 12, 2011