Effect of combination of dietary fish protein and fish oil on lipid metabolism in rats

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Abstract This study examined the effects of fish protein in combination with fish oil on rat lipid metabolism. Male Wistar rats were divided into four groups and fed an AIN93G-based 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 4 weeks. The dietary combination of fish protein and fish oil decreased the contents of serum triacylglycerol, serum cholesterol, liver triacylglycerol and liver cholesterol in addition to altering liver lipid fatty acid composition. These effects are partly due to the increase in fecal cholesterol, bile acid excretion, and increased enzyme activities of fatty acid β -oxidation in the liver. These data suggest that combined intake of fish protein and fish oil lead to both hypocholesterolemic and hypotriglyceridemic in serum and the liver, while sole intake of fish protein or fish oil decrease only cholesterol and triglyceride levels, respectively. These results suggest that combined intake of fish protein and fish oil may play beneficial roles in the

prevention of lifestyle-related diseases as compared with sole fish protein intake.

Keywords Fish protein · Fish oil · n-3 polyunsaturated fatty acid · Lipid metabolism

Introduction

In developed countries, there is an increased incidence of lifestyle-related diseases, such as coronary heart disease (CHD), hyperlipidemia, atherosclerosis, diabetes, and hypertension. Epidemiological and experimental reports have shown the relationship between diet and the incidence of CHD (Osler et al. 2002; Pereira et al. 2004). Dietary therapy is the first-choice treatment for CHD and is considered to be as important as medical treatment.

Dietary proteins have also been found to influence lipid metabolism in human subjects and animals (Sugano et al. 1990; Zhang and Beynen 1993; Wergedahl et al. 2004; Brandsch et al. 2006; Shukla et al. 2006; Hosomi et al. 2009). Most studies have focused on the effects of plant protein compared with those of casein (Sugano et al. 1990). Hence, it is often assumed that the effects of dietary animal proteins are like those of dietary casein, although dietary animal protein also affects lipid metabolism (Zhang and Beynen 1993; Wergedahl et al. 2004; Brandsch et al. 2006; Shukla et al. 2006; Hosomi et al. 2009). Fish protein, which is a major macronutrient in fish, plays an important role in human nutrition worldwide. Fish protein has been used as a main ingredient in processed seafood, such as kamaboko and fish sausage, in Japan. Some researchers have suggested that fish protein diets reduce plasma cholesterol levels in laboratory animals as compared with those of a casein

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diet (Zhang and Beynen 1993; Wergedahl et al. 2004; Shukla et al. 2006; Hosomi et al. 2009). Our previous study showed that dietary fish protein prepared from Alaska pollock fillet decreased the serum cholesterol concentration in rats (Hosomi et al. 2009). There have been, however, few reports on the combined effect of fish protein and other compounds.

Fish oil is rich in n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish oil has attracted widespread attention as a functional oil, and it has been suggested that dietary fish oil decreases serum triacylglycerol by stimulating lipid oxidation (Berge et al. 1999) and inhibiting lipogenesis in the liver (Grønn et al. 1992). It has also been demonstrated that fish oil has beneficial anti-inflammatory (Simopoulos 2002) and anti-cancer properties (Minoura et al. 1988). Furthermore, fish oil has been reported to exhibit a synergistic effect with sesamin on hepatic fatty acid oxidation in rats (Ide et al. 2004) and attenuates fatty liver induced by conjugated linoleic acid in mice (Yanagita et al. 2005). Thus, the dietary combination of fish protein with fish oil may be more effective in the prevention and improvement of hyperlipidemia and CHD than fish protein alone.

In this study, we investigated the effects of fish protein diets in combination with fish oil on lipid metabolism in the blood and the liver of rats. In addition, we investigated whether the effects of fish protein and fish oil on lipid metabolism in rats could exhibit additive or synergistic effects by combining these two components in their diets.

Materials and methods

Materials

Fish protein (lipid content is less than 0.1%) was prepared from Alaska pollock (*Theragra chalcogramma*) as described previously (Hosomi et al. 2009). For fish oil we used purified *tuna oil* (99.5% triacylglycerol) 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 care and diets

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 acclimation for 5 d by feeding a diet prepared according to the recommendation of the American Institute of Nutrition (AIN-93G) (Reeves et al. 1993), the rats were divided into four groups of seven rats each and given free access to drinking water and fed the experimental diets. As shown in Table 1, to distinguish between the separate effects of fish protein and fish oil and any combined effect, we fed groups of rats on 10% fish protein (FP diet), 2% fish oil (FO diet), or a combination of 10% fish protein and 2% fish oil (FPO diet). Table 2 shows the amino acid composition of casein and fish protein analyzed by a commercial service (Japan Food Research Laboratories, Tokyo, Japan). After comparing the amino acid composition of casein and fish protein, it was found that in fish protein, the levels of alanine, arginine, aspartic acid, glycine, and lysine were high, while the level of proline was low. However, the levels of branched-chain amino acids, such as valine, leucine, and isoleucine, were nearly identical, and the polarity of amino acids did not reveal any significant differences.

Table 3 shows that the fatty acid compositions of soybean oil and fish oil were analyzed with a fused silica capillary column (Omegawax 250, Supelco Co., Ltd., Bellefonte, PA, USA) after methylation by sodium meth-

Table 1 Composition of experimental diets (g/kg)

Components	Dietary groups			
	Control	FP	FO	FPO
Dextrinized corn starch	132	132	132	132
Corn starch	397.5	397.5	397.5	397.5
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
Soybean oil	70	70	50	50
Fish oil	–	–	20	20

Diets were prepared based on the composition of AIN-93G

FP fish protein group, FO fish oil group, FPO fish protein and fish oil group

Table 2 Amino acid composition of casein and fish protein

Amino acid	Dietary protein (g/100 g protein)	
	Casein	Fish protein
Alanine	2.7	6.2
Arginine	3.3	7.0
Aspartic acid	6.3	11.3
Cystine	0.4	1.1
Glutamic acid	19.0	18.3
Glycine	1.6	3.8
Histidine	2.7	2.4
Isoleucine	4.9	5.0
Leucine	8.4	9.1
Lysine	7.1	10.9
Methionine	2.6	3.5
Phenylalanine	4.5	3.9
Proline	10.0	3.5
Serine	4.6	4.6
Threonine	3.7	5.0
Tryptophan	1.1	1.2
Tyrosine	5.0	4.1
Valine	6.0	5.4

Table 3 Fatty acid composition of soybean oil and fish oil

Fatty acid	Dietary lipids (wt%)	
	Soybean oil	Fish oil
14:0	N.D	3.4
15:0	N.D	0.7
16:0	10.6	16.1
16:1 n-7	N.D	0.8
16:1 n-9	N.D	5.3
17:0	N.D	1.1
17:1	N.D	0.5
18:0	3.8	4.6
18:1 n-7	1.3	2.3
18:1 n-9	21.8	12.7
18:2 n-6	53.7	1.4
18:3 n-3	5.8	0.6
18:3 n-6	N.D	1.8
20:1 n-9	N.D	1.2
20:4 n-6	N.D	2.1
20:5 n-3	N.D	11.1
22:5 n-3	N.D	1.9
22:5 n-6	N.D	1.9
22:6 n-3	N.D	29.1
Others	3.0	1.2

N.D. not detected

oxide using a gas-liquid chromatograph (GC-14B, Shimadzu, Kyoto, Japan) .

Food consumption and body wt were recorded every 2 d. Feces were collected from each group every 24 h for 7 d prior to sacrifice. After feeding for 4 weeks with the experimental diets, rats were weighed and sacrificed under Nembutal (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) anesthesia. Rats were not fasted before sacrifice because food deprivation leads to a significant down regulation of the genes involved in fatty acid synthesis and cholesterol metabolism (Horton et al. 1998). 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 tissue (WAT) from the epididymis, mesentery, and perinephria were removed rapidly, weighed, rinsed with saline, frozen in liquid nitrogen, and then stored at -80°C . An aliquot of liver was taken for mRNA expression analysis and stored in RNA-Later Storage Solution (Sigma Chemical Co., St. Louis, MO, USA).

Analysis of lipids

Serum triacylglycerol, cholesterol, high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesterol (LDL-C) were measured using an Olympus AU5431 automatic analyzer (Olympus Co., Tokyo, Japan). Liver lipids were extracted using the method described by Bligh and Dyer (1959). Total lipid samples were dissolved in an equal volume of dimethyl sulfoxide, and the content of triacylglycerol was determined by using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries, Ltd.). Cholesterol contents in liver and feces were analyzed with a SE-30 column (Shinwa Chemical Industries LTD., Kyoto, Japan) using a GC-14B gas-liquid chromatograph (Shimadzu Co.) and 5α -cholestane as an internal standard. Liver protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Fecal bile acid content was measured according to the method of Bruusgaard et al. (1977). The fatty acid composition of liver lipids were analyzed with a fused silica capillary column (Omegawax 250, Supelco Co., Ltd.) after methylation by sodium methoxide using a GC-14B gas-liquid chromatograph (Shimadzu Co.).

Analysis of liver enzyme activity

Each liver was homogenized in 10 volumes of 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA-2Na. The homogenate was centrifuged at 500 g for 10 min at 4°C , and the supernatant was obtained (fraction 1). The supernatant was recentrifuged at 9,000 g for 10 min

at 4 °C to sediment the mitochondria (fraction 2). The supernatant was ultracentrifuged at 105,000 g for 60 min at 4 °C, and the supernatant was obtained (fraction 3). Acyl-CoA oxidase (ACOX, EC 1.3.3.6) activity of fraction 1 was measured as described previously (Ide et al. 1987). Carnitine palmitoyltransferase-2 (CPT-2, EC 2.3.1.21) activity in the mitochondrial fraction (fraction 2) was measured as described by Markwell et al. (1973). Fatty acid synthase (FAS, EC 2.3.1.85) (Kelley et al. 1986) and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) (Kelley and Kletzien 1984) activities in fraction 3 were measured spectrophotometrically. The protein content of each fraction was determined according to the method of Lowry et al. (1951) as described previously.

Analysis of mRNA expression

Total RNA was extracted from the livers using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Real-time quantitative polymerase chain reaction (PCR) analysis was performed using an automated sequence detection system (ABI Prism 7000, Applied Biosystems). 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), cholesterol 7 α -hydroxylase (CYP7A1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were measured using TaqMan Gene Expression Assays (Applied Biosystems). PCR primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems. mRNA expression levels of ATP-binding cassette (ABC) A1 (ABCA1), ABCG5, ABCG8, low density lipoprotein receptor (LDLR), stearyl-Coenzyme A desaturase (SCD)-1 scavenger receptor class B type 1 (SR-B1), and GAPDH were measured using SYBR Green PCR Master Mix (Applied Biosystems). PCR primers were as follows: forward: 5'-CCCGCGGAGTAGAAAGG-3' and reverse: 5'-AGGGC GATGCAAACAAAGAC-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'-CCGTGGCTTTTCTTCTCTCA-3' and reverse: 5'-GCATTCGGAACAGTGCAACA-3'; for SCD-1; forward: 5'-CACCCCTCGTTGAAAACCT-3' and reverse: 5'-CCTTAGCCAGCTCTTCCAGATC-3' for LDLR; forward: 5'-GCATTCGGAACAGTGCAACA-3' and reverse: 5'-TCATGAATGGTGCCACATC-3' for SR-B1; and forward: 5'-GAAGACACCAGTAGACTCCACGACATA-3' and reverse: 5'-GAAGGTCCGTGTGAACGGATT-3' for

GAPDH. All primers were designed using Primer Express 3.0 software (Applied Biosystems). The expression level of the housekeeping gene GAPDH served as an internal control for normalization.

Statistical analysis

Data are expressed as means $\pm$ standard error (SE) for seven rats. To determine the main protein and lipid effects, the interactions between dietary proteins and lipids were subjected to two-way analysis of variance (ANOVA). Statistical comparisons were made using the Tukey-Kramer test. Differences were considered significant at $P < 0.05$.

Results and discussion

Table 4 shows growth parameters and relative organ wt. Initial body wt, final body wt, body wt gain, food consumption and food efficiency were not found to be significantly different among the groups. There were also no significant differences in relative liver wt and various WAT wt among the groups.

Table 5 shows lipids parameters in serum, liver, and feces. Serum triacylglycerol content was lower in rats fed fish oil diets than in rats fed soybean oil diets ($P = 0.044$). The protein source did not independently affect serum triacylglycerol content. Serum cholesterol content was lower in dietary fish protein fed groups than in dietary casein fed groups ($P = 0.001$). There were, however, no synergistic effects of dietary fat source and protein. The combination of fish protein and fish oil also revealed no synergistic effect, but it had an independent effect on serum triacylglycerol and cholesterol contents. Fish protein fed groups exhibited higher HDL-C ($P = 0.021$) and lower LDL-C contents ($P < 0.0001$) as compared with casein fed groups. Many epidemiological and clinical studies have demonstrated that increases in LDL-C levels and decreases in HDL-C levels in blood are important risk factors for CHD (Cromwell and Otvos 2004). It was also demonstrated that elevated triacylglycerol levels may be a significant independent risk factor for CHD (Jacobson et al. 2007). In addition, we found that the combination of dietary fish protein and fish oil decreased serum triacylglycerol, cholesterol, and LDL-C contents due to the additive effect of both compounds, but not due to any synergistic effect. This suggested that the combined fish protein and fish oil diet might function to prevent the development of CHD as compared with an individual fish protein diet. However, because rats have high amounts of circulating HDL particles and small amounts of circulating LDL particles, these findings did not contribute much to the HDL-

Table 4 Growth parameters and relative organ wt of rats fed the experimental diets for 4 weeks

	Dietary groups			
	Control	FP	FO	FPO
Growth parameters				
Initial BW(g)	128±3	128±2	131±3	129±3
Final BW(g)	312±5	316±1.7	318±7.6	319±5.7
BW gain (g/day)	6.62±0.11	6.68±0.08	6.69±0.19	6.75±0.15
Food consumption (g/day)	17.3±0.5	18.3±0.9	17.0±0.8	17.4±0.8
Food efficiency (g/kcal)	0.0964±0.0011	0.0934±0.0008	0.0994±0.0019	0.0992±0.0015
Organ wt (g/100 g BW)				
Liver wt	3.75±0.09	3.59±0.08	3.83±0.11	3.86±0.13
Epididymal WAT wt	1.50±0.03	1.54±0.05	1.51±0.11	1.32±0.04
Mesentery WAT wt	1.27±0.09	1.43±0.12	1.38±0.11	1.35±0.05
Perirenal and retroperitoneal WAT wt	1.26±0.09	1.35±0.31	1.35±0.28	1.34±0.13
Total WAT wt	4.04±0.16	4.31±0.22	4.24±0.36	4.01±0.13

BW body wt, WAT white adipose tissue

Total WAT wt = sum of wts from epididymis, mesentery, perinephria and retroperitoneal WAT

Data are means ± SE ($n=7$)

elevating and LDL-lowering effect of the FPO diet, but the results could be of importance from a health benefit perspective.

As shown in Table 5, liver triacylglycerol content was lower in rats fed fish oil diets than in rats fed soybean oil diets ($P<0.0001$). Fish protein fed groups exhibited lower liver cholesterol content than did casein fed groups ($P=0.012$). The combination of fish protein and fish oil had no synergistic effect, but it had an independent effect on liver lipid contents. Table 6 shows the fatty acid composition of liver total lipids. The percentages of 18:0 ($P=0.001$), 20:5n-

3 ($P<0.0001$), 22:5n-3 ($P<0.0001$), 22:6n-3 ($P<0.0001$), and Σ n-3 ($P<0.0001$) fatty acids were higher in rats fed fish oil diets than in rats fed soybean oil diets. In particular, 20:5n-3 and 22:6 n-3 fatty acids were significantly higher in rats fed fish oil diets than in rats fed soybean oil diets. Conversely, 18:1n-9 ($P<0.0001$), 18:2n-6 ($P=0.003$), 20:4n-6 ($P=0.004$), 18:1n-9/18:0 ($P=0.0008$), Σ n-6 ($P<0.0001$) and Σ n-6/ Σ n-3 ($P<0.0001$) fatty acids in dietary fish oil groups were lower than in dietary soybean oil groups. Dietary fish oil changed liver total lipid fatty acid composition, and n-3 PUFAs were incorporated into liver

Table 5 Lipids parameters in serum, liver, and feces of rats fed experimental diets

	Control	FP	FO	FPO	ANOVA (<i>P</i> values)		
					Protein (P)	Lipid (L)	P×L
Serum (mg/dl)							
Triacylglycerol	33.6±0.6	31.0±2.2	28.7±1.5	27.9±2.0	0.366	0.044	0.647
Cholesterol	64.2±2.3 ^a	55.9±1.7 ^{ab}	62.5±2.6 ^a	52.7±2.6 ^b	0.001	0.328	0.762
HDL-C	44.0±1.5	47.8±1.9	41.6±1.7	46.6±1.9	0.021	0.347	0.734
LDL-C	7.05±0.32 ^{ab}	5.77±0.29 ^c	7.59±0.41 ^a	6.03±0.26 ^{bc}	0.0004	0.255	0.692
Liver (mg/g Liver)							
Triacylglycerol	34.4±3.0 ^a	35.6±2.3 ^a	17.3±0.5 ^b	18.3±1.5 ^b	0.617	<0.0001	0.964
Cholesterol	7.22±0.66 ^a	6.17±0.31 ^{ab}	6.80±0.56 ^a	4.93±0.32 ^b	0.027	0.154	0.1129
Feces							
Cholesterol (mg/day)	36.0±2.4 ^{ac}	47.4±2.3 ^b	32.2±2.4 ^a	45.4±3.4 ^{bc}	0.0003	0.311	0.746
Bile acid (μmol/day)	60.8±4.7 ^a	106±8 ^c	68.7±6.6 ^{ab}	96.4±8.2 ^{bc}	<0.0001	0.929	0.241

HDL-C high density lipoprotein-cholesterol, LDL-C low density lipoprotein-cholesterol

Data are means ± SE ($n=7$). Values in the same row not sharing a common letter are significantly different ($p<0.05$) according to the Tukey–Kramer test

Table 6 Fatty acid composition of liver lipid of rats fed experimental diets

Fatty acid	Control	FP	FO	FPO	ANOVA (<i>P</i> values)		
					Protein (P)	Lipid (L)	P×L
(wt%)							
16:0	24.1±0.6	25.5±0.7	25.5±0.6	25.7±1.2	0.330	0.351	0.457
18:0	11.2±1.1 ^a	10.6±0.5 ^a	13.5±0.8 ^{ab}	15.0±0.9 ^b	0.568	0.001	0.241
18:1 n-9	15.3±0.8 ^a	16.6±0.3 ^a	12.9±0.7 ^b	12.5±0.7 ^b	0.526	<0.0001	0.182
18:2 n-6	22.0±1.2 ^a	21.5±1.0 ^{ab}	19.4±0.9 ^{ab}	18.1±0.5 ^b	0.346	0.003	0.624
18:3 n-3	0.769±0.072	0.766±0.055	0.677±0.056	0.572±0.028	0.340	0.016	0.366
20:4 n-6	11.8±0.9 ^a	10.4±0.5 ^{ab}	8.84±0.74 ^b	9.03±0.68 ^b	0.378	0.004	0.256
20:5 n-3	0.226±0.020 ^a	0.221±0.019 ^a	1.28±0.17 ^b	1.34±0.13 ^b	0.859	<0.0001	0.834
22:5 n-3	0.477±0.053 ^a	0.361±0.024 ^a	0.792±0.081 ^b	0.785±0.062 ^b	0.306	<0.0001	0.367
22:6 n-3	2.90±0.17 ^a	2.94±0.12 ^a	6.76±0.70 ^b	7.18±0.56 ^b	0.625	<0.0001	0.686
18:1n-9/18:0	1.41±0.08 ^a	1.49±0.19 ^a	0.921±0.081 ^b	0.843±0.092 ^b	0.863	0.001	0.501
Σ n-6	35.6±0.9 ^a	33.2±1.0 ^a	29.1±1.3 ^b	27.9±1.0 ^b	0.092	<0.0001	0.587
Σ n-3	4.79±0.14 ^a	4.75±0.19 ^a	9.45±0.84 ^b	10.8±0.8 ^b	0.263	<0.0001	0.235
Σ n-6/n-3	7.46±0.19 ^a	7.04±0.27 ^a	2.76±0.09 ^b	2.62±0.12 ^b	0.155	<0.0001	0.457

Data are means ± SE (*n*=7). Values in the same row not sharing a common letter are significantly different (*p*<0.05) according to the Tukey–Kramer test

lipids. Dietary protein, such as soybean protein and fish protein hydrolyzate, can modify essential fatty acid metabolism through the suppression of δ -6 desaturase (Madani et al. 1998; Wergedahl et al. 2004). However, dietary fish protein did not affect total liver lipid fatty acid composition. A key enzyme required for the biosynthesis of monounsaturated fatty acid (MUFA) is SCD-1, which catalyzes the δ -9-cis desaturation of fatty acid substrates (Paton and Ntambi 2009). As shown in Table 8, SCD-1 expression levels were decreased by dietary fish oil while dietary fish protein did not affect SCD-1. Increased MUFA levels have been implicated in various disease states, including obesity, diabetes, and cardiovascular disease (Pan et al. 1994). Consequently, dietary fish oil significantly decreased δ -9 desaturation indices, which is the ratio of oleic acid (18:1 n-9) versus stearic acid (18:0), in total liver lipids. Therefore, fish oil has the potential to prevent obesity and diabetes through decreased MUFA levels and δ -9 desaturation indices through the suppression of SCD-1. However, the dietary combination of fish protein and fish oil affected liver total lipid fatty acid composition, although the effect was not different from that of the individual fish oil diet.

Table 7 shows the activities of liver enzymes associated with fatty acid metabolism. CPT-2 activity, a key enzyme of fatty acid β -oxidation in mitochondria, was higher in rats fed fish protein diets than in rats fed casein diets (*P*<0.0001), and it was higher in rats fed fish oil diets than in rats fed soybean oil diets (*P*<0.0001). Furthermore, CPT-2 activity affected the protein plus lipid interaction (*P*=

0.002). ACOX activity, a key enzyme of fatty acid β -oxidation in peroxisomes, was higher in the fish oil fed groups than in the soybean oil fed groups (*P*<0.0001). Interestingly, the combination of fish protein and fish oil diet markedly increased liver CPT-2 activity. Frøyland et al. (1997) suggested that EPA (20:5 n-3) stimulates mitochondrial β -oxidation, whereas DHA (22:6 n-3) is more effective for peroxisomal β -oxidation. In this study, there were no significant differences in liver EPA and DHA contents between the FO and FPO groups. In contrast, the activity of FAS, a key enzyme in the regulation of fatty acid *de novo* synthesis, was not significantly different among the groups. G6PDH activity, a key enzyme in the production of cellular NADPH, and in the biosynthesis of fatty acids and cholesterol, was decreased by dietary fish oil (*P*=0.002) while it was not affected by dietary fish protein. We believe that the decreased liver triacylglycerol content as a result of the fish oil diet may be due to the suppression of G6PDH and the enhancement of fatty acid β -oxidation in mitochondria and peroxisomes, whereas dietary fish protein may not affect liver enzyme activities related to fatty acid metabolism. The synergistic effects on the elevation of liver CPT-2 activity by the combination of fish protein and fish oil are not clear at present.

As shown in Table 5, fecal cholesterol and bile acid excretion levels were higher in rats fed fish protein diets than in rats fed casein diets (*P*=0.0003 and *P*=0.0001, respectively), whereas dietary fish oil did not affect fecal excretions of cholesterol and bile acid. The decrease in serum cholesterol because of dietary protein was related to

Table 7 Liver enzyme activities of fatty acid metabolism in rats fed experimental diets

	Control	FP	FO	FPO	ANOVA (<i>P</i> values)		
					Protein(P)	Lipid(L)	P×L
(nmol/min/mg protein)							
CPT-2	7.52±0.43 ^a	8.51±0.55 ^{ab}	9.74±0.44 ^b	14.4±0.5 ^c	<0.0001	<0.0001	0.002
ACOX	1.50±0.08 ^a	1.58±0.12 ^a	2.93±0.21 ^b	2.62±0.21 ^b	0.542	<0.0001	0.291
FAS	3.47±0.37	3.62±0.21	3.32±0.18	3.68±0.27	0.387	0.878	0.722
G6PDH	21.3±1.7 ^a	20.2±0.7 ^a	16.0±1.0 ^{ab}	14.9±1.7 ^b	0.938	0.002	0.526

CPT-2 carnitine palmitoyltransferase-2, *ACOX* acyl-coenzyme A oxidase, *FAS* fatty acid synthase, *G6PDH* glucose-6-phosphate dehydrogenase
Data are means ± SE (*n*=7). Values in the same row not sharing a common letter are significantly different (*p*<0.05) according to the Tukey–Kramer test

the amino acid composition of the protein, in particular the ratio of lysine to arginine (Spielmann et al. 2008) and the content of specific amino acids i.e. methionine (Sugiyama et al. 1986), cysteine (Serougne et al. 1984), and glycine (Katan et al. 1982). In this study, the half of dietary protein was replaced in fish protein; therefore, the amino acid composition and the lysine : arginine ratios of the casein and fish protein diets (1.55 and 1.76 respectively) were similar. This suggests that the hypocholesterolemic effect of fish protein was not the result of a metabolic effect due to differences in amino acid composition. Protein digestion products are often thought to interrupt the intestinal absorption of cholesterol and bile acid (Sugano et al. 1990). Therefore, it is likely that the decreases in serum cholesterol, LDL-C, and liver cholesterol contents in the fish protein diet were due to the inhibition of cholesterol and bile acid reabsorption by fish protein peptic hydrolysate. In this study, we used Alaska pollock as the source of

dietary protein. Based on the results of previous reports, it is generally thought that the fish meat protein has a hypocholesterolemic effect (Zhang and Beynen 1993; Wergedahl et al. 2004; Shukla et al. 2006; Hosomi et al. 2009).

Cholesterol homeostasis is mainly achieved by the balance between its biosynthesis, storage, catabolism, and export in the liver. Table 8 shows relative mRNA expression levels of genes encoding proteins related to lipid metabolism in the liver of rats. mRNA expression levels of ABCA1, a transporter associated with the production of HDL, were higher in the fish protein diet groups than that in the casein diet groups (*P*=0.048). On the other hand, the mRNA expression level of SRB1, which mediates the selective uptake of HDL-derived lipids into liver, was not changed by the fish protein diet. Accordingly, we speculated that the increases in serum HDL-C by the fish protein diets were, at least in part, due to the regulation

Table 8 Relative mRNA expression levels of enzymes and transporters related to lipid metabolism in rats fed experimental diets

	Control	FP	FO	FPO	ANOVA (<i>P</i> values)		
					Protein(P)	Lipid(L)	P×L
(Relative expression levels)							
ABCA1	1.00±0.08	1.55±0.09	0.91±0.05	1.11±0.09	0.048	0.444	0.754
ABCG5	1.00±0.08 ^{ab}	0.77±0.10 ^a	1.13±0.11 ^b	0.98±0.05 ^{ab}	0.049	0.076	0.689
ABCG8	1.00±0.10 ^{ab}	0.60±0.10 ^a	1.20±0.15 ^b	1.03±0.11 ^{ab}	0.038	0.022	0.378
HMGR	1.00±0.05	1.00±0.07	0.84±0.07	1.09±0.09	0.094	0.639	0.102
CYP7A1	1.00±0.18	1.10±0.27	0.78±0.20	0.73±0.09	0.874	0.169	0.716
LDLR	1.00±0.05	0.98±0.04	0.86±0.06	0.81±0.03	0.524	0.004	0.763
SRB1	1.00±0.10	1.07±0.08	1.01±0.07	0.99±0.06	0.724	0.681	0.559
SCD1	1.00±0.06 ^a	0.92±0.04 ^{ab}	0.66±0.03 ^c	0.70±0.09 ^{bc}	0.769	0.0002	0.288

ABCA1 ATP-binding cassette A1, *ABCG5* ATP-binding cassette G5, *ABCG8* ATP-binding cassette G8, *HMGR* 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *CYP7A1* cholesterol 7 α -hydroxylase, *LDL-R* low density lipoprotein receptor, *SR-BI* scavenger receptor class B type 1, *SCD-1* stearoyl-coenzyme A desaturase-1, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

Data are means ± SE (*n*=7). Relative expression level means the ratio of each mRNA to GAPDH mRNA. Values in the same row not sharing a common letter are significantly different (*p*<0.05) according to the Tukey–Kramer test

of ABCA1. mRNA expression levels of LDLR, a key enzyme for cholesterol uptake by cells, were lower in rats fed fish oil diets than in rats fed soybean oil diets ($P=0.004$). It was thought that the increase of serum LDL-C content by the FO diet may be induced by the decrease in liver LDLR expression levels. Previous studies have shown that fish oil intake suppresses the activity of HMGR, a rate-limiting enzyme in cholesterol synthesis (Frøyland et al. 1996). In this study, FO diet tended to lower HMGR mRNA expression levels, but the combination of fish protein and fish oil diets did not change these levels. mRNA expression levels of CYP7A1 were regulated by the farnesoid X receptor (FXR)/small heterodimer partner (SHP)-dependent pathway, which is negatively regulated by the decreased reabsorption of bile acid. In our previous study, dietary fish protein enhanced liver CYP7A1 levels through the FXR/SHP pathway (Hosomi et al. 2009), although CYP7A1 levels were not increased by the fish protein diets in this study, which is a contradiction that cannot be readily explained.

Recently, it has been observed that ABCG5 and ABCG8 functioned to excrete cholesterol from the liver to the bile (Ikeda et al. 2009; Sabeva et al. 2009). As shown in Table 8, mRNA expression levels of ABCG5 and ABCG8 tended to be low in the FP group while they tended to be higher in the FO group. ABCG5 and ABCG8 expression levels in the FPO diet were not different from those in the control diet, and the FP diet decreased the excretion of cholesterol from the liver to the bile, although fecal cholesterol excretion was increased. In this study, liver ABCG5 and ABCG8 expression levels in the FP group tended to be lower than those in the FPO group, but fecal cholesterol excretion was not found to be different between the FP and FPO groups (47.4 ± 2.3 and 45.4 ± 3.4 mg/day, respectively). Therefore, we suggest that liver ABCG5 and ABCG8 expression levels may not affect the amount of fecal cholesterol excretion.

Conclusion

The important findings in this study were that a fish protein diet decreased serum cholesterol content, while the combined fish protein and fish oil diet decreased serum cholesterol, triacylglycerol, and LDL-C. The results suggest that the combined fish protein and fish oil diet may have beneficial effects on the prevention of life-style related diseases as compared with an individual fish protein diet.

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