

Dietary fish protein alters blood lipid concentrations and hepatic genes involved in cholesterol homeostasis in the rat model

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It is known that various dietary plant proteins are capable of influencing the lipid metabolism of human subjects and animals when compared with casein. Less, however, is known about the effects of fish protein on the cholesterol and triacylglycerol metabolism. Therefore, two experiments were conducted in which rats were fed diets containing 200 g of either fish protein, prepared from Alaska pollack fillets, or casein, which served as control, per kilogram, over 20 and 22 d, respectively. As parameters of lipid metabolism, the concentrations of cholesterol and triacylglycerols in the plasma and liver, the faecal excretion of bile acids and the hepatic expression of genes encoding proteins involved in lipid homeostasis were determined. In both experiments, rats fed fish protein had higher concentrations of cholesteryl esters in the liver, a lower concentration of cholesterol in the HDL fraction ($p > 1.063 \text{ kg/l}$) and lower plasma triacylglycerol concentrations than rats fed casein ($P < 0.05$). The gene expression analysis performed in experiment 2 showed that rats fed fish protein had higher relative mRNA concentrations of sterol regulatory element-binding protein (SREBP)-2, 3-hydroxy-3-methylglutaryl coenzyme A reductase, LDL receptor, apo AI, scavenger receptor B1 and lecithin-cholesterol-acyltransferase in their liver than did rats fed casein ($P < 0.05$). The faecal excretion of bile acids and the mRNA concentrations of cholesterol 7 α -hydroxylase, SREBP-1c and corresponding target genes were not altered. These findings show that fish protein had multiple effects on plasma and liver lipids that were at least in part caused by an altered expression of the hepatic genes involved in lipid homeostasis.

Fish protein: Plasma lipids: Hepatic genes: Rat

A growing body of research offers insight into the influence of different dietary proteins on CVD risk through their impact on blood lipids. For example, plant proteins such as soyabean protein or lupin protein in the diet have been shown to reduce the cholesterol and/or triacylglycerol concentrations of plasma in human subjects (Bakhit *et al.* 1994; Anderson *et al.* 1995) and animals (Sirtori *et al.* 1998, 2004; Tachibana *et al.* 2005) when compared with casein. Furthermore, protein isolated from fish fillets also seems to influence blood lipid concentrations in experimental animals (Kritchevsky *et al.* 1982; Zhang & Beynen, 1993; Wergedahl *et al.* 2004; Ait Yahia *et al.* 2005). However, the few studies that have been conducted in this field include conflicting results. Recent studies with rats (Murata *et al.* 2004; Ait Yahia *et al.* 2005) and a previous experiment with rabbits (Bergeron *et al.* 1992) have found a reduction in plasma and VLDL triacylglycerol concentrations in groups fed fish protein compared with groups fed casein and soyabean protein, respectively. Other experiments with rats, however, have not found any effect of fish protein on the concentrations of triacylglycerol in the plasma and VLDL (Ait Yahia *et al.* 2003; Wergedahl *et al.* 2004). Additionally, the effects of isolated fish protein on HDL-cholesterol also include conflicting

results (Kritchevsky *et al.* 1982; Zhang & Beynen, 1993; Wergedahl *et al.* 2004; Ait Yahia *et al.* 2005), which are suggested to be caused by the different fish protein sources used for the feed studies (Zhang & Beynen, 1993).

To our knowledge, no study has analysed the effects of protein isolated from Alaska pollack fillets on the metabolism of cholesterol and triacylglycerol, although Alaska pollack is widely used for human nutrition. The aim of our first experiment was to investigate the possible effects of a protein isolated from Alaska pollack, compared with casein, on the concentrations of plasma and liver lipids. In order to search for a possible mechanism responsible for the numerous fish protein-induced alterations in lipids in the liver and plasma observed in experiment 1, a second experiment on rats was performed. Rats from experiment 2 were not fasted overnight at the end of the experimental period because overnight fasting leads to a significant downregulation of the genes involved in cholesterol and fatty acid metabolism (see, for example, Horton *et al.* 1998; Shimano *et al.* 1999).

The liver is important in maintaining lipid homeostasis, and sterol regulatory element-binding proteins (SREBP) are proteins that regulate the transcription of genes encoding enzymes in the

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CYP7A1, cholesterol 7 α -hydroxylase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LCAT, lecithin-cholesterol acyltransferase; SR-B1, scavenger receptor B1; SREBP, sterol regulatory element-binding protein.

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biosynthetic pathways of cholesterol and triacylglycerol. To investigate whether the observed effects of fish protein on lipid concentrations in the plasma and liver were induced by an altered activity level of SREBP, the mRNA concentrations of SREBP target genes were determined in a second experiment. To search for a possible mechanism underlying the fish protein-induced increase in cholesterol concentration in the liver, the relative mRNA concentration of SREBP-2 in the liver was determined, together with the mRNA concentrations of its target genes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and LDL receptor, the key enzymes required for cholesterol synthesis and cholesterol uptake into cells (Vallett *et al.* 1996; Horton *et al.* 2002). Cholesterol homeostasis is achieved by a balance of uptake, biosynthesis, storage, catabolism and export. To gain further insights into cholesterol catabolism, the mRNA concentration of cholesterol 7α -hydroxylase (CYP7A1), the key enzyme in the synthesis of bile acids from cholesterol, and the total amounts of bile acids in the faeces would provide information about the possible effects of fish protein on cholesterol excretion via the bile acids.

The determination of the relative mRNA concentrations of the genes involved in HDL metabolism should provide an insight into the effects observed with fish protein on the HDL fraction in experiment 1. These were apo AI, scavenger receptor B1 (SR-B1) and lecithin-cholesterol acyltransferase (LCAT). Apo AI is one of the two major protein components of HDL and SR-B1, otherwise known as HDL receptor, and serves as a docking platform for the selective uptake of HDL and unloading of the HDL cholesterol in the liver (Acton *et al.* 1996), whereas the esterification of cholesterol by LCAT is critical for optimal cholesterol uptake and the maturation of HDL (Genest *et al.* 1999).

The measurement of SREBP-1c, an important determinant of lipogenic gene transcription in the liver (Shimano *et al.* 1999; Osborne, 2000; Horton *et al.* 2002), should provide additional information about possible mechanisms by which the fish protein modulates triacylglycerol concentrations in the plasma. Besides the relative mRNA concentration of SREBP-1c, the SREBP-1c-activated target genes such as glucose-6-phosphate dehydrogenase, fatty acid synthase and $\Delta 9$ -desaturase involved in the *de novo* synthesis of fatty acids were also determined.

Data obtained from previous studies on the effects of different dietary proteins suggest that alterations in lipid metabolism could be mediated by differences in the amino-acid patterns of the dietary proteins (Ait Yahia *et al.* 2003; Wergedahl *et al.* 2004). To search for constituents that could be responsible for the effects of fish protein on circulating cholesterol and triacylglycerol, we measured the concentrations of amino acids in the diets and in the plasma.

Materials and methods

Animals

Two experiments were conducted with a total of forty-eight male Sprague–Dawley rats (twenty-four in each experiment) supplied by Charles River (Sulzfeld, Germany). The rats were randomly assigned to two groups of twelve rats each in each experiment, with an initial body weight of 72 (SD 6) g in experiment 1 and 77 (SD 5) g in experiment 2. Young growing rats were chosen for both experiments according to

recent experiments on the effects of fish protein on plasma lipids (Wergedahl *et al.* 2004; Ait Yahia *et al.* 2005). For the measurement of plasma lipid concentration, rats from experiment 1 were fasted 12 h before being killed. Rats from experiment 2 were not fasted before being killed because food deprivation before killing leads to a significant downregulation of the genes involved in fatty acid synthesis and cholesterol metabolism, which were to be measured in this study (Horton *et al.* 1998; Shimano *et al.* 1999).

All rats were kept individually in Macrolon cages in a room maintained at a temperature of 23°C and 50–60% relative humidity with lighting from 06.00 to 18.00 hours. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt, Germany.

Experimental diets

All rats were fed a semi-synthetic diet containing 200 g of either casein or fish protein per kilogram. The amount of experimental protein in the diet was chosen according to a recent experiment with rats on the effects of fish protein on blood pressure and lipid metabolism (Ait Yahia *et al.* 2005). The diets contained the following ingredients (g/kg): fish protein or casein, 200; sucrose, 200; lard, 100; cellulose, 50; corn starch, 390; vitamin and mineral mixture, 60; DL-methionine, 2; cholesterol 0.5. Lard as the type of dietary fat and cholesterol used for the experimental diets were added to the diet not primarily in order to increase the plasma cholesterol concentration but to mimic Western diets, which are commonly rich in saturated fats and cholesterol. Vitamins and minerals were supplemented according to recommendations of the American Institute of Nutrition for rat diets (Reeves *et al.* 1993).

In both experiments, food intake was controlled. The amount of diet was slightly below that of similar diets consumed *ad libitum* by rats in preliminary studies. The amount of food offered daily was increased continuously during experiment 1 from 8.0 to 14.0 g and during experiment 2 from 6.0 to 14.0 g. Thus, all rats within one experiment were fed equal amounts of diet daily. The amount of diet was slightly below that consumed *ad libitum* by the rats and was administered once daily at 08.00 hours. Water was freely available from nipple-drinkers. The experimental diets were fed for 20 d (experiment 1) and 22 d (experiment 2), respectively. The differences between the time schedules of the two experiments were caused by technical reasons.

Preparation and characterisation of the dietary proteins

Fish protein was isolated from fresh Alaska pollack fillets obtained from a local supermarket. After all visible fat and connective tissues had been removed, the fillets were cut into small pieces, boiled for 30 min and then chilled overnight at 4°C. The fish mash was homogenized, freeze-dried and ground. The resulting fish powder was treated twice with acetone and once with acetone–ethanol (1:1, v/v) to remove the protein-associated lipids. After evaporation of the remaining acetone and ethanol, the crude fish protein was ground again and stored at –20°C. Casein was obtained from Meggle (Wasserburg, Germany) and underwent no further processing.

The crude components of the dietary proteins, determined by the official methods of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (Naumann & Basler, 1993), are given in Table 1. The concentrations of crude protein and crude ash were similar in the two proteins. The concentration of crude fat was low in both proteins. The amino acid concentrations of the diets are shown in Table 2, and the fatty acid composition of the diets is shown in Table 3. The fatty acid composition was essentially identical in diets containing casein and diets containing fish protein. Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2n-6) were quantitatively the most important fatty acids, whereas the concentrations of *n*-3 fatty acids were low in both types of diet.

Sample collection

Rats fasted overnight from experiment 1 and non-fasted rats from experiment 2 were decapitated under light anaesthesia with diethyl ether after overnight fasting. Plasma was separated from heparinised whole blood by centrifugation at 1500g for 10 min at 4°C. Liver was excised, weighed and immediately snap-frozen in liquid N₂. Aliquots of liver for

RNA isolation were stored at -80°C; other samples were stored at -20°C. During the second week of the experimental period, faeces were collected, dried, weighed and stored at -20°C until analysis.

Amino acid analysis

To determine the amino acid concentrations in the diet, samples were oxidised and then hydrolysed with 6 M-HCl (Bassler & Buchholz, 1993). Separation and quantification of the amino acids were performed by ion-exchange chromatography following post-column derivatisation in an amino-acid analyser (Biotronic LC 3000; Eppendorf, Hamburg, Germany). Tryptophan was determined by digesting the diet with Ba(OH)₂ (Fontaine *et al.* 1998). The tryptophan concentration in the diet was measured by reversed-phase HPLC (Eder *et al.* 2001). The concentrations of free amino acids in the plasma of the rats were measured as isoindole derivatives by HPLC (1100 series; Agilent Technologies, Waldbronn, Germany) according to Schuster (1988) after pre-column derivatisation. Isoindole derivatives were detected at an excitation wavelength of 337 nm and an emission wavelength of 454 nm. Homocysteine and cysteine concentrations were also determined by HPLC (Vester & Rasmussen, 1991).

Plasma and liver lipids

Plasma lipoproteins were separated by stepwise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900 000g at 4°C for 1.5 h by appropriate density cuts commonly used for the measure of rat lipoproteins (Sparks *et al.* 1998; Giudetti *et al.* 2003; Sirtori *et al.* 2004). Plasma densities were adjusted by NaCl and KBr, and the lipoprotein fractions $\rho < 1.006$ kg/l (defined as VLDL in experiment 1 and VLDL plus chylomicrons in experiment 2), 1.006 kg/l $< \rho < 1.063$ kg/l (defined as LDL) and $\rho > 1.063$ kg/l (defined as HDL) were removed by suction. Lipids were extracted from liver with a mixture of *n*-hexane and isopropanol (3:2, v/v; Hara & Radin, 1978). For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids dissolved using Triton X-100 (De Hoff *et al.* 1978). Concentrations of triacylglycerols and cholesterol in the plasma and lipoproteins and those of liver were determined using enzymatic reagent kits (1.14 830, 1.14 856; VWR International, Darmstadt, Germany). The fractions of free and esterified cholesterol in liver were separated by TLC and determined densitometrically (Hojnacki *et al.* 1976).

Faecal bile acids

The total bile acid content of the faeces of rats in experiment 2 was determined enzymatically (DiaSys Diagnostic Systems, Holzheim, Germany) by a modified method of Marlett & Fischer (2002) using taurocholic acid as a standard. For that purpose, the bile acids were extracted from freeze-dried faeces with ethanol and NaOH as described recently (Dongowski *et al.* 2002).

Table 1. Crude nutrients in the dietary proteins used in experiments 1 and 2

Components	Casein† Experiments 1 and 2 (g/100 g DM)	Fish protein Experiment 1 (g/100 g DM)	Fish protein Experiment 2 (g/100 g DM)
Crude protein	93.0	92.6	91.8
Crude fat	0.9	0.8	0.9
Ash	4.7	4.2	4.7

† The same batch of casein was used in experiments 1 and 2.

Table 2. Concentrations of amino acids in the diets used in experiments 1 and 2

Amino acid	Casein diet† Experiments 1 and 2 (g/kg diet)	Fish protein diet Experiment 1 (g/kg diet)	Fish protein diet Experiment 2 (g/kg diet)
Methionine	5.3	5.7	5.0
Cysteine	0.8	1.7	1.6
Glycine	3.4	7.9	7.5
Lysine	14.7	17.4	15.7
Arginine	6.3	10.7	11.0
Valine	12.1	9.3	8.7
Leucine	17.7	15.4	14.2
Isoleucine	8.8	7.9	7.7
Histidine	6.4	6.0	5.7
Tyrosine	8.6	5.1	5.1
Phenylalanine	9.7	6.6	7.1
Threonine	7.9	8.1	7.7
Serine	10.8	7.8	7.4
Alanine	5.5	11.4	10.0
Proline	20.2	5.6	5.7
Tryptophan	2.3	1.9	1.8
Glutamic acid	42.2	30.6	27.5
Aspartamic acid	13.2	20.0	17.4

† The same batch of casein was used in experiments 1 and 2.

Table 3. Concentrations of fatty acids in the diets used in experiments 1 and 2

Fatty acid	Casein diet Experiment 1 (g/kg diet)	Casein diet Experiment 2 (g/kg diet)	Fish protein diet Experiment 1 (g/kg diet)	Fish protein diet Experiment 2 (g/kg diet)
16:0	25.6	25.3	25.4	25.4
16:1	2.4	2.3	2.4	2.3
18:0	16.2	15.1	16.0	15.0
18:1 <i>n</i> -9	37.4	39.6	37.1	39.3
18:2 <i>n</i> -6	10.8	10.4	10.7	10.4
18:3 <i>n</i> -3	0.9	0.9	0.9	0.9
20:5 <i>n</i> -3	0.1	0.1	0.1	0.1
22:6 <i>n</i> -3	< 0.1	< 0.1	< 0.1	< 0.1

Relative mRNA concentrations of hepatic genes

The relative mRNA concentrations of hepatic genes involved in lipid homeostasis were only measured in rats from experiment 2 that were not fasted before decapitation. For analysis of gene expression, total RNA was extracted from frozen liver samples using Trizol reagent (Invitrogen, Karlsruhe, Germany). RNA was quantified by A_{260} and its integrity verified by agarose gel electrophoresis using ethidium bromide for visualisation. Total RNA and oligo dT primer (Amersham Pharmacia, Freiburg, Germany) were used for cDNA synthesis (Omniscript RT Kit, Qiagen, Hilden, Germany; Mastercycler Personal, Eppendorf). The concentration of cDNA was analysed by real-time detection PCR (Rotorgene 2000; Corbett Research, Mortlake, Australia) using Sybr Green I (Sigma-Aldrich, Taufkirchen, Germany). The amplification efficiency and the take-off point calculated by the Rotorgene software 4.6 (Corbett Research, Mortlake, Australia) were used for comparative quantification. The expression signal of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for normalisation. PCR was carried out using 100 μ l PCR tubes with a final volume of 15 μ l reaction mixture containing 500 μ mol/l dNTP (Roth, Karlsruhe, Germany), 3.5 mmol/l $MgCl_2$, 5 U Taq DNA polymerase and 1.5 μ l 10 \times buffer (all from Promega, Mannheim, Germany), 0.38 μ l 10 \times Sybr Green I, 2 μ l of the first-strand cDNA and 2 μ l of the primer mix (Roth). The primer oligonucleotides were selected using the Primerselect software (DNA-Star Inc., Madison, WI, USA) and the Abi-Prism Primerpairs software (Applied Biosystems, Weiterstadt, Germany) from database sequences.

The primer sequences used for RT-PCR were as follows: 5'-GCA-TGG-CCT-TCC-GTG-TTC-C-3' (forward) and 5'-GGG-TGG-TCC-AGG-GTT-TCT-TAC-TC-3' (reverse) for rat GAPDH (*EC* 1.2.1.12); 5'-TGC-CCT-CAT-GAC-CGA-CCC-CAA-GTT-3' (forward) and 5'-GAC-AGG-ACC-AGC-GCC-CAT-CTC-AGG-3' (reverse) for rat apo AI; 5'-GGT-TTG-GTG-CGC-CCC-TGT-TTC-TC-3' (forward) and 5'-CGA-CGC-CCT-TGA-CGG-ATT-TGA-TG-3' (reverse) for rat SR-B1; 5'-AGC-TGG-CAG-GAC-TGG-TAG-AGG-AG-3' (forward) and 5'-CAG-GGG-GAA-GTT-GTG-GTT-ATG-C-3' (reverse) for rat LCAT (*EC* 2.3.1.43); 5'-AAG-GGG-CGT-GCA-AAG-ACA-ATC-3' (forward) and 5'-ATA-CGG-CAC-GGA-AAG-AAC-CAT-AGT-3' (reverse) for rat HMG-CoA reductase (*EC* 1.1.1.34); 5'-AGA-ACT-GCC-GGG-CCG-AAG-ACA-C-3' (forward) and 5'-AAA-CCG-CTG-GGA-CAT-AGG-CAC-TCA-3' (reverse) for rat LDL receptor; 5'-CCG-GTA-ATG-ATG-GGC-CAA-GAG-AAA-G-3' (forward)

and 5'-AGG-CCG-GGG-GAGACA-TCA-GAA-G-3' (reverse) for SREBP-2; 5'-CAA-GAC-GCA-CCT-CGC-TAT-CC-3' (forward) and 5'-CCG-GCA-GGT-CAT-TCA-GTT-G-3' (reverse) for CYP7A1 (*EC* 1.14.13.17); 5'-GGC-CGC-CTC-TGT-GTC-CCT-GTC-3' (forward) and 5'-CCC-CTC-GAC-CCT-GCC-TTC-CTA-AG-3' (reverse) for ACAT-2 (*EC* 2.3.1.26); 5'-GGA-GCC-ATG-GAT-TGC-ACA-TT-3' (forward); 5'-AGG-AAG-GCT-TCC-AGA-GAG-GA-3' (reverse) for rat SREBP-1c; 5'-CCA-GCC-TCC-ACA-AGC-ACC-TCA-AC-3' (forward) and 5'-AAT-TAG-CCC-CCA-CGA-CCC-TCA-GTA-3' (reverse) for rat glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49); 5'-CCT-CTT-CCC-TGG-CAC-TGG-CTA-CCT-3' (forward) and 5'-ACT-CGG-CGG-GGA-TCG-GGA-CTT-3' (reverse) for rat fatty acid synthase (*EC* 2.3.1.85); 5'-CCG-TGG-CTT-TTT-CTT-CTC-TCA-3' (forward) and 5'-CTT-TCC-GCC-CTT-CTC-TTT-GA-AGT-3' (reverse) for rat Δ 9-desaturase (*EC* 1.14.99.5).

The cDNA of GAPDH, apo AI, SR-B1, LCAT, HMG-CoA reductase, LDL receptor, SREBP-2, CYP7A1, ACAT, SREBP-1c, glucose-6-phosphate dehydrogenase, fatty acid synthase and Δ 9-desaturase was amplified in cycles of 20 s denaturation at 95°C, 30 s annealing at an appropriate temperature and 40 s elongation at 72°C. Fluorescence was measured at 72°C. A final melting curve guaranteed the authenticity of the target product.

Statistics

Means of the two groups were compared by Student's *t* test for each experiment. Values in the text are means and standard deviations. Means were considered significantly different at $P < 0.05$.

Results

Experiment 1

Food intake throughout the feeding period was the same for each rat, averaging 13.5 g/d. The body weight gains did not differ between the two groups of rats (casein group 6.15 (SD 0.35) g/d, fish protein group 6.10 (SD 0.65) g/d; n 12). Rats fed the fish protein had lower plasma concentrations of homocysteine, serine, histidine, threonine, tyrosine and valine, and higher concentrations of plasma glycine, arginine and taurine than rats fed casein (Table 4). The plasma concentrations of cysteine, glutamic acid, asparagine, glutamine, alanine, methionine, tryptophan, isoleucine, phenylalanine, leucine, and lysine did not differ between the two groups of rats (Table 4).

Rats fed fish protein had lower concentrations of cholesterol in plasma and HDL (Fig. 1), and a higher ratio of LDL:HDL

Table 4. Concentrations of amino acids in the plasma of rats fed a diet containing casein or fish protein in experiments 1 and 2 (Mean values and standard deviations for twelve determinations)

Amino acid	Experiment 1				Experiment 2			
	Casein ($\mu\text{mol/l}$)		Fish protein ($\mu\text{mol/l}$)		Casein ($\mu\text{mol/l}$)		Fish protein ($\mu\text{mol/l}$)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Methionine	41	5	42	10	56	10	54	9
Homocysteine	11	3	7	1*	11	3	8	1*
Cysteine	156	23	147	27	132	42	118	18
Taurine	50	10	171	30*	60	20	159	24*
Glycine	246	25	354	53*	158	27	349	58*
Lysine	476	81	517	84	474	61	614	150*
Arginine	112	18	146	22*	89	17	148	22*
Valine	130	21	106	16*	186	56	108	17*
Leucine	105	17	94	17	119	32	86	19*
Isoleucine	67	11	68	12	83	20	70	11
Histidine	51	8	41	6*	43	12	29	9*
Tyrosine	94	21	65	10*	89	21	55	9*
Phenylalanine	49	9	46	9	42	7	41	8
Tryptophan	56	9	61	11	67	11	70	14
Threonine	527	117	313	50*	533	140	408	76*
Serine	331	33	276	26*	276	37	258	30
Alanine	351	67	464	197	533	61	566	134
Glutamic acid	121	27	125	16	93	12	95	17
Glutamine	689	66	614	82	665	88	547	81*

* Mean values were significantly different from those of rats fed the casein-based diet: $P < 0.05$.

cholesterol than rats fed casein (casein group 0.54 (SD 0.11) mmol/mmol; fish protein group 0.69 (SD 0.17) mmol/mmol; n 12; $P < 0.05$). Concentrations of cholesterol in VLDL and LDL did not differ between the two groups of rats (Fig. 1).

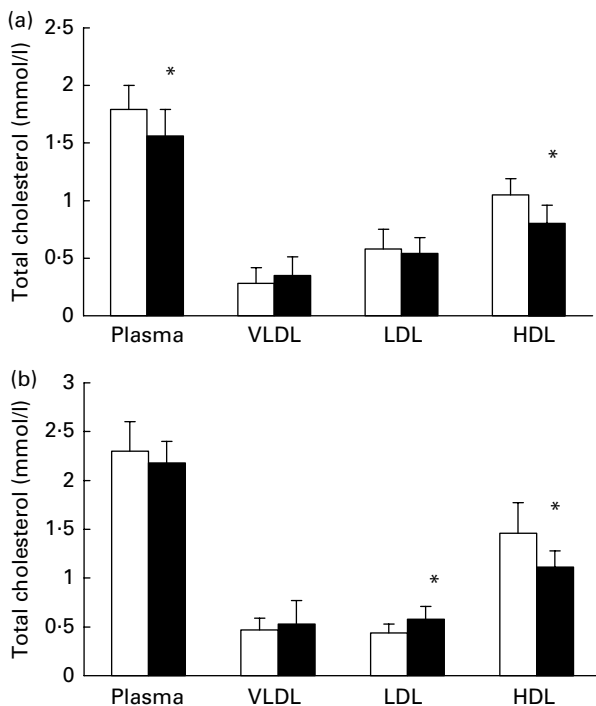


Fig. 1. Concentration of total cholesterol in the plasma and lipoproteins of rats fed a diet containing casein (□) or fish protein (■) in (a) experiment 1 and (b) experiment 2. Values are means with their standard deviations shown by vertical bars, for twelve determinations. *Mean values were significantly different from those of rats fed the casein-based diet: Student's *t* test, $P < 0.05$.

Concentrations of triacylglycerols in plasma, VLDL and HDL were lower in rats fed the fish protein than in rats fed casein (Fig. 2). The concentration of triacylglycerols in the LDL fraction did not differ between the two groups of rats (Fig. 2).

The liver weight/100 g body weight did not differ between the two groups of rats (casein group 3.54 (SD 0.91) g/100 g body weight; fish protein group 3.78 (SD 0.61) g/100 g body weight; n 12). The concentrations of total cholesterol and cholesteryl esters in the liver were higher in rats fed fish protein than in rats fed casein (Fig. 3). The concentration of free cholesterol in the liver did not differ between the two groups of rats (Fig. 3). The concentrations of triacylglycerols in liver were no different between the two groups of rats (Fig. 3).

Experiment 2

Food intake throughout the feeding period was the same for each rat, averaging 10 g/d. The body weight gains of rats fed fish protein were higher than those of rats fed casein (casein group 5.45 (SD 0.22) g/d; fish protein group 5.97 (SD 0.28) g/d; n 12; $P < 0.05$). Rats fed the fish protein had lower plasma concentrations of homocysteine, histidine, threonine, tyrosine, valine, leucine, glutamine and asparagine, and higher plasma concentrations of glycine, arginine and taurine than rats fed casein (Table 4). The plasma concentrations of cysteine, glutamic acid, alanine, methionine, tryptophan, isoleucine, phenylalanine and lysine were no different between the two groups of rats (Table 4).

Rats fed fish protein had a higher concentration of LDL cholesterol, a lower concentration of HDL cholesterol (Fig. 1) and a higher ratio of LDL:HDL cholesterol than rats fed casein (casein group 0.31 (SD 0.02) mmol/mmol; fish

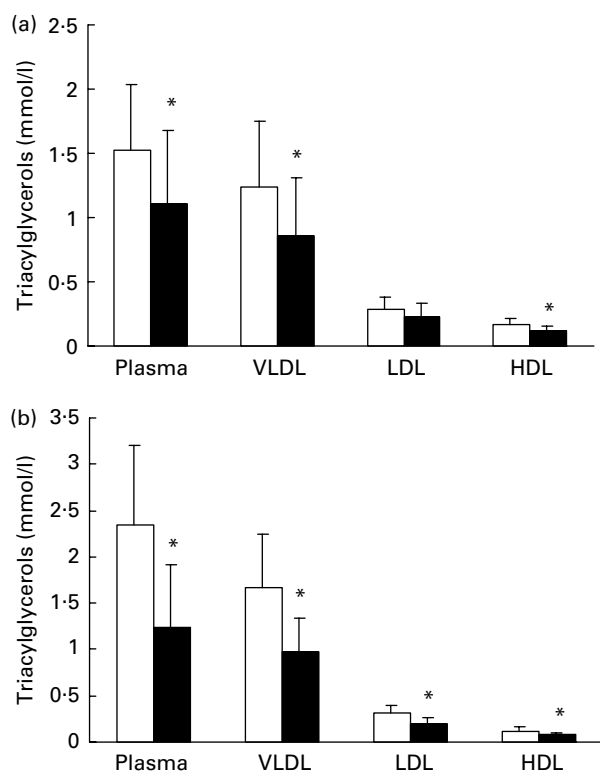


Fig. 2. Concentration of triacylglycerol in the plasma and lipoproteins of rats fed diets containing casein (□) or fish protein (■) in (a) experiment 1 and (b) experiment 2. Values are means with their standard deviations shown by vertical bars, for twelve determinations. *Mean values were significantly different from those of rats fed the casein-based diet: Student's *t* test, $P < 0.05$.

protein group 0.54 (SD 0.10) mmol/mmol; n 12; $P < 0.05$). Concentrations of cholesterol in the plasma and the triacylglycerol-rich lipoprotein fraction, including VLDL and chylomicrons, did not differ between the two groups of rats (Fig. 1). Rats fed fish protein had lower concentrations of triacylglycerols in their plasma, in the triacylglycerol-rich lipoprotein fraction (VLDL plus chylomicrons), in LDL and in HDL (Fig. 2).

The liver weight per 100 g body weight was lower in rats fed fish protein than in rats fed casein (casein group 4.51 (SD 0.37) g/100 g body weight; fish protein group 4.08 (SD 0.37) g/100 g body weight; n 12; $P < 0.05$). The concentrations of total cholesterol and cholesteryl esters in the liver were higher in rats fed fish protein than in rats fed casein (Fig. 3). The concentration of the free cholesterol in liver remained unaffected by the diet (Fig. 3). The concentration of triacylglycerol in the liver was no different between the two groups of rats (Fig. 3).

The excretion of bile acids via the faeces did not differ between the two groups of rats (casein group 7.48 (SD 3.78) μ mol/d; fish protein group 8.22 (SD 1.99) μ mol/d; n 12).

Rats fed fish protein had higher relative mRNA concentrations of apo AI, LCAT and SR-B1 (Fig. 4), and higher relative mRNA concentrations of SREBP-2, HMG-CoA reductase and LDL receptor in the liver (Fig. 5), than did rats fed casein. The relative mRNA concentration of ACAT (casein group 1.00 (SD 0.43); fish protein group 1.19 (SD 0.31); n 12) and the relative gene expression of CYP7A1 (casein group 1.00 (SD 0.56); fish protein group 0.96 (SD 0.67); n 12) were not different between the two groups of rats. The relative

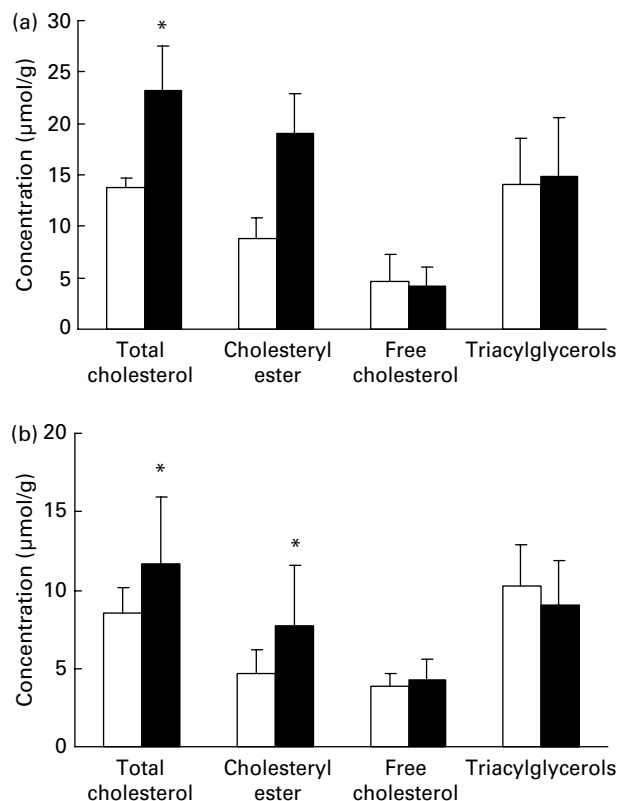


Fig. 3. Concentrations of total cholesterol, cholesteryl esters, free cholesterol and triacylglycerol in the livers of rats fed diets containing casein (□) or fish protein (■) in (a) experiment 1 and (b) experiment 2. Values are means with their standard deviations shown by vertical bars, for twelve determinations. *Mean values were significantly different from those of rats fed the casein-based diet: Student's *t* test, $P < 0.05$.

mRNA concentration of SREBP-1c (casein group 1.00 (SD 0.56); fish protein group 1.03 (SD 0.53); n 12), and the relative mRNA concentrations of the corresponding downstream genes glucose-6-phosphate dehydrogenase (casein group 1.00 (SD 0.85); fish protein group 1.54 (SD 1.18); n 12), fatty acid synthase (casein group 1.00 (SD 0.98); fish protein group 2.18

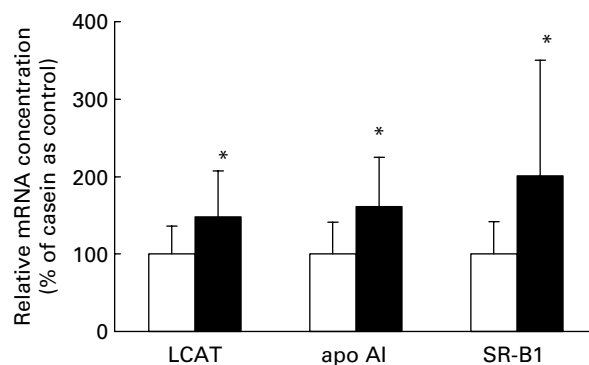


Fig. 4. Relative mRNA concentrations of lecithin-cholesterol acyltransferase (LCAT), apolipoprotein (apo) AI, and scavenger receptor B1 (SR-B1) in the liver of male rats fed a diet containing 200 g/casein kg (□) or fish protein (■) for 22 d (experiment 2). Values were related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Values are means with their standard deviations shown by vertical bars, for twelve determinations. *Mean values were significantly different from those of rats fed the casein-based diet (expressed as 100): Student's *t* test, $P < 0.05$.

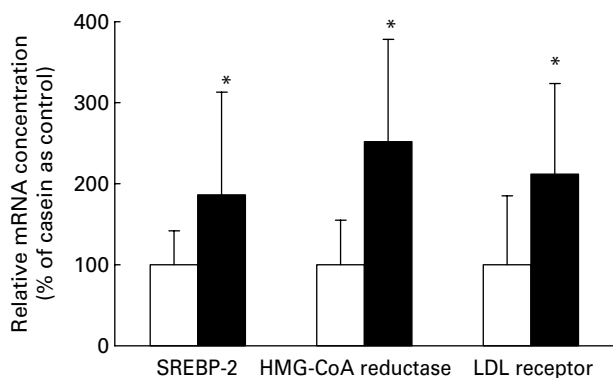


Fig. 5. Relative mRNA concentrations of sterol regulatory element-binding protein 2 (SREBP-2), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and LDL receptor in the liver of male rats fed diets containing 200 g/kg casein (□) or fish protein (■) for 22 d (experiment 2). Values were related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Values are means with their standard deviations shown by vertical bars, for twelve determinations. *Mean values were significantly different from those of rats fed the casein-based diet (expressed as 100); Student's *t* test, $P < 0.05$.

(SD 0.99); n 12) and $\Delta 9$ -desaturase (casein group 1.00 (SD 0.65); fish protein group 0.78 (SD 0.35; n 12) were not different between the two groups of rats.

Discussion

The current experiments on the effects of isolated fish protein from Alaska pollack fillets on the cholesterol and triacylglycerol concentration of lipoproteins and liver, and the genes involved in lipid homeostasis, show a variety of significant fish protein-associated alterations in lipid metabolism. The main effects of the fish protein on cholesterol metabolism compared with casein were an increase in liver cholesterol concentration, a reduction in HDL ($\rho > 1.063$ kg/l) and a stimulated gene expression of the genes for apo AI, SR-B1, LCAT, SREBP-2, HMG-CoA reductase and LDL receptor.

The finding that the relative mRNA concentrations of SREBP-2 and HMG-CoA reductase were higher in rats fed fish protein compared with rats fed casein suggests that cholesterol synthesis might possibly be stimulated by the fish protein, which could in turn be responsible for the observed accumulation of cholesterol in the liver. This assumption is supported by the findings of Wergedahl *et al.* (2004), who observed a higher activity of HMG-CoA reductase in rats fed fish protein compared with those fed casein, although this effect was observed only in genetically hyperlipidaemic obese Zucker rats but not in normal Wistar rats.

As well as an increase in HMG-CoA reductase expression, mRNA levels of LDL receptor were also increased in rats fed fish protein. Both are target genes of SREBP-2 and indicate an activation of this transcription factor by fish protein. The increase in cholesteryl esters in the livers of rats fed fish protein supports the fact that greater amounts of cellular cholesterol are normally associated with a higher concentration of esterified cholesterol (Field *et al.* 1987). The failing response of the expression of ACAT, which catalyses the formation of cholesteryl esters from cholesterol and long chain fatty acyl-Co-A, thus modulating the potential toxic effects of excess cholesterol in cell membranes (Chang *et al.* 1997), is

most likely explained by the fact that an enlarged amount of cellular cholesterol stimulates primarily the activity rather than the transcription of ACAT (Chang *et al.* 2001; Lange *et al.* 2004). The observed difference in the liver cholesterol concentrations of rats from experiments 1 and 2 can possibly be explained by the differences in the amounts of food that were ingested during experiments 1 and 2.

As hepatic cholesterol homeostasis is achieved by a balance of biosynthesis, storage, catabolism and export, processes that influence cholesterol excretion may have contributed to the observed cholesterol accumulation in the livers of rats fed fish protein. Excess cholesterol is normally eliminated from liver mainly via bile acids and CYP7A1 is the key enzyme of synthesis of bile acids from cholesterol (Vlahecevic *et al.* 1999). As the relative mRNA concentration of CYP7A1 in the liver and the amounts of bile acids excreted via the faeces did not, however, differ between the two groups of rats, it is suggested that the cholesterol accumulation in the livers of rats fed fish protein might not be due to a reduced excretion of the cholesterol via the bile acids.

However, despite the observed cholesterol accumulation in the liver of rats fed protein from Alaska pollack, the concentration of cholesterol in the plasma was not increased compared with that in rats fed casein. By measuring the different density fractions in plasma from rats of both experiments, we found a lower cholesterol concentration in the HDL fraction ($\rho > 1.063$ kg/l) in those fed fish protein compared with those fed casein. The cut-off chosen for lipoprotein separation was typical for human subjects but also matched that used in previous rat studies (Sparks *et al.* 1998; Giudetti *et al.* 2003; Sirtori *et al.* 2004). This means the $1.006 < \rho < 1.063$ lipoprotein fraction contained, besides IDL, LDL and some HDL particles as well; this may be the reason for the inconsistent effects of fish protein on this density fraction in experiments 1 and 2. A major regulator of circulating LDL-cholesterol is the LDL receptor (Meddings *et al.* 1986). The higher mRNA concentration of LDL receptor in the liver of rats fed fish protein could have possibly contributed to an increased removal of LDL from the circulation, thereby preventing a distinct LDL accumulation in plasma, albeit with an increased expression of HMG-CoA reductase.

The HDL-lowering effect observed with fish protein feeding is in agreement with recent findings in hyperlipidaemic obese Zucker rats fed fish protein from salmon (Wergedahl *et al.* 2004). HDL is the principal vehicle for the removal of surplus cholesterol from the peripheral tissues for disposal in the liver. Several genes are involved in HDL metabolism, such as LCAT, critical for optimal cholesterol uptake and maturation of HDL-3 to HDL-2 (Genest *et al.* 1999), apo AI, a structural component of HDL, and SR-B1, otherwise known as HDL receptor, which is responsible for the selective uptake of HDL. The increased gene expression of LCAT and apo AI would normally contribute to an increase in HDL cholesterol concentration. Although, the reduced concentration of HDL cholesterol observed in the rats fed fish protein could possibly be related to an increased expression of SR-B1, which mediates the selective uptake of HDL-derived lipids into the liver, further investigation is necessary to clarify the mechanisms.

Another remarkable effect of fish protein from Alaska pollack compared with casein was a distinct reduction in the triacylglycerol concentration in the plasma, by about 27% in experiment 1 and 48% in experiment 2. The hypotriacylglycerolaemia

primarily resulted from reduced amounts of circulating triacylglycerol-carrying lipoproteins ($\rho < 1.006 \text{ kg/l}$) and confirms other reports on the effects of fish protein in spontaneously hypertensive rats (Ait Yahia *et al.* 2005), rabbits (Bergeron *et al.* 1992) and premenopausal women (Gascon *et al.* 1996). Hypotriacylglycerolaemia could possibly be caused by a diminished synthesis of triacylglycerols in the liver, an increased catabolism of fatty acids or a diminished secretion of triacylglycerols from the liver via VLDL. The measurements of SREBP-1c mRNA concentration, along with the mRNA concentrations of the SREBP-1c target genes such as fatty acid synthase, glucose-6-phosphate dehydrogenase and $\Delta 9$ -desaturase, were not indicative of an inhibition of lipogenesis. Thus, in contrast to SREBP-2, the SREBP-1c-mediated pathway seems not to be altered by fish protein. A former study with rabbits has found a higher lipoprotein lipase activity in animals fed fish protein compared with those fed soyabean protein (Bergeron *et al.* 1992). We therefore suggest that hypotriacylglycerolaemia observed in the rats fed fish protein could be caused by a stimulation of plasma triacylglycerol clearance, although this needs to be proved in a further study.

Studies dealing with the effects of different dietary proteins from plant and animal sources on lipid metabolism suggest that specific amino acids could be responsible for the various effects observed (Kritchevsky *et al.* 1982; Sugiyama *et al.* 1986; Morita *et al.* 1997; Wergedahl *et al.* 2004). Methionine is assumed to be one of the most important amino acid that contributes to changes in lipid metabolism. Recent data also indicate that dietary methionine exerts its effects on cholesterol metabolism by an elevation of circulating homocysteine, which in turn is suggested to be an activator of the transcription factor SREBP-2 and thereby stimulates the gene expression of cholesterol-synthesising enzyme HMG-CoA reductase (Woo *et al.* 2005). However, the concentrations of the two amino acids did not differ between the treatment groups and did not provide any explanation for the observed activation of SREBP-2.

In conclusion, the present findings suggest that the fish protein from Alaska pollack exerted distinct effects on plasma and liver lipids, which were, at least in part, caused by an altered expression of the hepatic genes involved in lipid homeostasis.

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