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ORIGINAL CONTRIBUTION

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Fish protein improves blood pressure but alters HDL_2 and HDL_3 composition and tissue lipoprotein lipase activities in spontaneously hypertensive rats

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Summary The two-month effects of dietary fish protein and casein on VLDL, HDL_2 and HDL_3 compositions and hepatic lipase (HTGL) and tissue lipoprotein lipase (LPL) activities were examined in spontaneously hypertensive rats (SHR) at 4 wk of age. After 2 mo of experiment, the fish protein diet induced lower blood pressure (–14 %) as compared to casein. Liver triacylglycerol and total cholesterol concentrations were 1.37- and 1.71-fold lower in the fish protein group than in the casein group, respectively. Total cholesterol concentration in plasma was also diminished by fish protein (–21 %) and was reflected in HDL_2 fraction (–44 %). SHR fed the fish protein diet as compared with those fed casein, showed a significantly low HDL_3 particle number, as measured by diminished HDL_3 mass and apo A-I. The consumption of fish protein did not affect VLDL particle number, but significantly decreased VLDL-triacylglycerol (–32 %) and adipose tissue total lipid concentrations as compared to casein. This was accompanied by diminished HTGL and adipose tissue LPL activities (–10 %, –91 %, respectively). These data demonstrate that fish protein plays an antihypertensive role and reduces plasma and tissue lipid concentrations. Thus, a fish protein intake might be beneficial for patients with hypertension.

Key words hypertension – fish protein – liver – plasma – lipoproteins – lipids – lipoprotein lipase – spontaneously hypertensive rats

Introduction

Epidemiological data have shown an inverse relationship between fish consumption and the mortality rate due to coronary heart disease (CHD) in Western and Asian countries [1, 2]. The study of Pauletto et al. [3] indicates a significant decrease in systolic blood pressure, plasma concentrations of triglycerides and total cholesterol, and an increase in plasma n-3 polyunsaturated fatty acids in the fish-consuming group compared with the vegetarian group. Cardiovascular protection resulting from fish consumption could result from elevated eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) levels. In particular, these fatty acids have been reported to lower blood pressure and prevent the development of hypertension [4, 5], which is one of the most critical factors involved in cardiovascular pathogenesis such as atherosclerosis or stroke. However, another nutrient in fish, such as protein, might influence CHD risk factors, by lowering blood pressure. In humans, the estimated protein intake is inversely related to blood pressure [6, 7]. Feeding fish protein to stroke-prone spontaneously hypertensive rats (SHR-SP) led to lower blood pressure [8] and resulted in higher HDL-cholesterol which is associated with a fall in VLDL-cholesterol and triglycerides in rabbits, compared to casein [9]. The synthesis of HDL particles is

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linked to the metabolism of triglyceride-rich lipoprotein [10]. Indeed, the surface components released during VLDL catabolism are transferred to HDL. Lipoprotein lipases (LPL, EC 3.1.1.34 and HTGL, EC 3.1.1.3) regulate lipoprotein catabolism through the activity of lipolytic enzymes and ligands for lipoprotein receptors [11, 12]. In human, LPL deficiency results in massive hypertriglyceridaemia due to the accumulation of both chylomicrons and large VLDL [13, 14], while HTGL deficiency leads to typical type III hyperlipoproteinaemia with impaired clearance of chylomicron remnants [15]. Because the hydrolysis of triglyceride-rich particles is dependent upon lipoprotein lipase activity, the extent of LPL-mediated breakdown of circulating VLDL is a major determinant in the formation of HDL [10]. Thus, LPL and hepatic lipase are possible targets for the effects of dietary proteins on plasma lipoproteins. To our knowledge, very few studies have analysed the relationship between fish protein and hypertension [16] and no experiment has investigated the effect of changes in lipoprotein lipase activity on the development of hypertension. We have therefore explored the influence of fish protein on blood pressure and on VLDL, HDL_2 and HDL_3 lipid and apolipoprotein compositions and their relationship to lipoprotein lipase and hepatic lipase activities in spontaneously hypertensive rats.

Material and methods

Animals and diets

Male spontaneously hypertensive rats (SHR, n = 20) 4 weeks old, weighing 80 ± 5 g (IFFA Credo, l'Arbresle, France), were housed in stainless-steel cages under controlled temperature (24°C) and lighting (12-h light: dark cycle) and at constant humidity (60 %). The rats were fed a standard commercial chow diet for 7 d. After this adaptation period, animals were randomly divided into two groups of ten rats and were subjected to diets containing either 20 % casein [containing 95 % proteins] or highly purified fish protein [containing 94 % proteins] (Seah-International, Boulogne-sur-Mer, France) combined with 5 % Isio 4 oil (Lesieur, Neuilly-sur-Seine, France), 5 % cellulose, 2 % vitamins, 4 % minerals, 5 % sucrose, with starch to 100 %. The composition of mineral, vitamin and Isio 4 oil has been previously reported by Frenoux et al. [17]. Diets were isoenergetic (16.28 MJ/kg). The amino acid composition of fish protein and casein is described in Table 1. The amino acid composition of both proteins was determined after separation by HPLC in the Biochemistry Laboratory from the Central Hospital of Nancy, France by F. NABET and P. NABET. Free access to fresh food and water was provided for a total of 2 mo. Food intakes were recorded daily and body weights were measured weekly. The general guidelines for the care and use of laboratory animals, recommended by the Council of European Communities [18], were followed.

Table 1 Amino acid composition of dietary proteins (g/kg protein)

	Casein	Fish Protein
Isoleucine	42	28
Leucine	92	55
Lysine	74	70
Methionine + cystine	25	37
Phenylalanine + tyrosine	102	63
Threonine	46	41
Tryptophane	–	9
Valine	57	39
Arginine	36	65
Histidine	34	14
Alanine	30	69
Aspartic Acid	66	80
Glutamic Acid	207	130
Glycine	18	123
Proline	116	58
Serine	55	53

Measurement of blood pressure

Systolic blood pressure of prewarmed, conscious rats was measured weekly by a tail cuff plethysmographic method as advised by the "Committee on the care and use of spontaneously hypertensive rat" (BP recorder 8005, W + W Electronic Inc, Basel-Munchenstein, Switzerland) [19]. The reported blood pressure values are means of five measurements taken at each time point.

Blood and tissue samples

After 2 mo of experiment and an overnight fasting, ten rats from each group were anasthetized with sodium pentobarbital (60 mg/kg body weight) and then bled from the abdominal aorta into tubes containing EDTA. Plasma was prepared by low speed centrifugation (1000 × g, 20 min, 10 °C). Liver, gastrocnemius muscle, fat tissue surrounding the kidney and epididymal areas, heart and kidneys were removed, washed with cold saline, quickly blotted and weighed. An aliquot of 100 mg of each tissue were diluted with ice-cold water (1 ml), homogenized at 4 °C in a Potter-Elvejhem homogenizer and used for protein determination. To measure lipolytic activity, tissue homogenates in 0.9 % (w/v) NaCl containing heparin (Sigma, St Louis, MO) were prepared as described by Mathe et al. [20] for liver, and

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as described by Inadera et al. [21] for adipose tissue, heart, kidneys and gastrocnemius.

Plasma, lipoprotein and hepatic lipid concentrations

Plasma VLDL($d < 1.006$ g/ml) and HDL ($1.12 < d < 1.21$ g/ml) were separated by discontinuous gradient ultracentifugation [22]. HDL_2 and HDL_3 subfractions were separated by precipitation using $MgCl_2$ and dextran sulfate wt 500000 (Sigma Chemical Company, France) [23].

Total cholesterol (TC) and triglyceride (TG) contents in plasma, liver lipid extracts [24] and lipoprotein fractions were determined using the enzymatic method (Boehringer kits, Meylan, France) using cholesterol and glycerol as standards, respectively. VLDL, HDL_2 and HDL_3 phospholipids (PL) were estimated by their phosphorus content, using Boehringer enzyme kits (Meylan, France), multiplied by 25. Protein contents of tissue homogenates, plasma and lipoproteins were determined by the method of Lowry et al. [25], using bovine serum albumin as a standard. Liver, heart, kidneys, gastrocnemius and adipose tissue total lipids were analysed according to Folch et al. [24].

Apolipoprotein separation and quantification

After concentration and partial lyophylisation and rapid diethyl ether delipidation of VLDL, HDL_2 and HDL_3, the apolipoproteins of each fraction were estimated by electrophoresis (SDS-PAGE), with 2.5–20 % acrylamide according to the method of Irwin et al. [26]. Electrophoresis was performed in a LKB 2001.001 vertical electrophoresis unit (LKB produkte, Bromme, Sweden) at 4 °C for 18 h with 20 mA/gel slab. Gels were then stained with Coomassie Brillant blue G250. The stained gels were scanned at 600 nm with a densitometer (Model Profil 26, Sebia, Issy les Moulineaux, France). To estimate the concentration of each apolipoprotein, the percentage of the area relative to each apolipoprotein was multiplied by total apolipoprotein concentration of each plasma sample. When 50–200 µg of total apolipoprotein were applied, chromogenicity of each major band varied linearly with the amount of total protein applied to the gel. Data were expressed as arbitrary units (AU)/L plasma.

Determination of lipolytic activities

Tissue homogenates were centrifuged at 1500 g for 5 min, and the supernatants containing heparin-releasable lipase were assayed for LPL and HTGL activities as described by Nilsson-Ehle & Ekman [27]. For the HTGL assay, 100 µl of liver supernatant (the enzyme source) was adjusted to 1 mol/L NaCl, and was incubated at 37 °C for 1 h with 100 µl of [^{3}H]triolein emulsion substrate [final concentrations: 1.42 mmol/L triolein, 0.1 mmol/L lysophosphatidylcholine, 0.2 % (w/v) albumin, 0.1 mmol/L Tris/HCl, pH 9.0, 0.5 mol/NaCl]. For adipose tissue, heart, kidneys and gastrocnemius LPL determination, the incubation medium contained 1.42 mmol/L triolein, 0.1 mmol/L lysophosphatidylcholine, 0.2 % (w/v) albumin, 5 % (v/v) heart-inactivated serum (providing apolipoprotein C-II, an activator of LPL), 0.1 mol/L Tris/HCl (pH 8) and 0.15 mol/L NaCl. At the end of incubation, the fatty acids released were extracted with choloroform/methanol/heptane (1.25/1.41/1, by vol) followed by 0.1 mol/L potassium carbonate/borate buffer, pH 10.5. [^{3}H] radioactivity in 1.5 ml aliquots of the methanol/water upper phase was measured in 10 ml of scintillation liquid (Ready Solv HP/6; Beckman) in a 7500 LS scintillation counter (Beckman, Palo Alto, CA). Enzyme activity was expressed as nmoles of fatty acids released$\cdot min^{-1}\cdot mg^{-1}$ protein.

Statistical analysis

Data were subjected to ANOVA followed by Fisher's test through the STATISTICA analysis system (version 4.1, statsoft, Tulsa, OK). Values are expressed as means ± SEM for ten rats per group. A difference of $P < 0.05$ was considered significant between fish protein group and casein group.

Results

Body weight, food intake, blood pressure and liver, heart and kidney weights

In spite of similar food intake, SHR fed the fish protein diet exhibited lower body weights as compared to those fed the casein diet (Table 2). Moreover, absolute liver

Table 2 Body weight, food intake, blood pressure and absolute weights of liver, heart and kidney in SHR fed the fish protein or casein diet[1, 2]

	Diet	
	Casein	Fish protein
Body weight (g)	332.8 ± 12.11	291.8 ± 26.37*
Food intake (g/d)	17.11 ± 1.25	16.33 ± 0.94
Blood pressure (mmHg)	220.7 ± 8.77	189.79 ± 10.51*
Liver weight (g)	10.08 ± 0.58	7.81 ± 1.19*
Heart weight (g)	1.48 ± 0.13	1.42 ± 0.11
Kidney weight (g)	2.47 ± 0.18	2.30 ± 0.28

[1] Values represent means ± SEM for ten rats
[2] *$P < 0.05$, fish protein group vs casein group

weights were significantly reduced (–23 %) in rats fed the fish protein diet, when compared to those fed the casein diet, while heart and kidney weights were similar. Systolic blood pressure was also significantly diminished in the fish protein fed group and represented only 86 % of the casein group value.

Plasma and liver total cholesterol and triacylglycerol concentrations

Dietary fish protein exerted a significant ($P < 0.05$) decrease in liver total cholesterol and triacylglycerol concentrations as compared to casein (Table 3). Plasma total cholesterol concentrations were also diminished while triacylglycerol contents remained unchanged in either group.

Concentrations and compositions of plasma VLDL, HDL_2, HDL_3

Plasma VLDL, HDL_2 and HDL_3 concentrations and compositions are presented in Fig. 1. Compared with the casein group, fish protein exhibited a lower HDL_3 mass reflected mainly by low apolipoprotein concentrations. However, VLDL and HDL_2 amounts were similar in both groups. Feeding fish protein compared with casein decreased triacylglycerol concentrations (–32 %) in VLDL, and total cholesterol (–44 %) and phospholipids contents (–45 %) in HDL_2.

Profiles of VLDL, HDL_2 and HDL_3 apolipoproteins

The profiles of VLDL, HDL_2 and HDL_3 apolipoproteins are described in Fig. 2. Fish protein consumption decreased VLDL apo B-48 and enhanced apo C values compared with the values obtained with casein. No differences were reported in apo B-100, A-I, A-IV and E. In the HDL_2 fraction, apo A-II and A-IV were significantly

Table 3 Plasma and liver total cholesterol and triacylglycerol concentrations in SHR fed the fish protein or casein diet[1, 2]

	Diet	
	Casein	Fish protein
Plasma		
Cholesterol (mmol/L)	1.56 ± 0.18	1.24 ± 0.42*
Triacylglycerol (mmol/L)	0.39 ± 0.12	0.39 ± 0.15
Liver		
Cholesterol (µmol/g liver)	21.66 ± 5.05	12.62 ± 3.42*
Triacylglycerol (µmol/g liver)	52.46 ± 11.93	38.15 ± 9.33*

[1] Values represent means ± SEM for ten rats
[2] * $P < 0.05$, fish protein group vs casein group

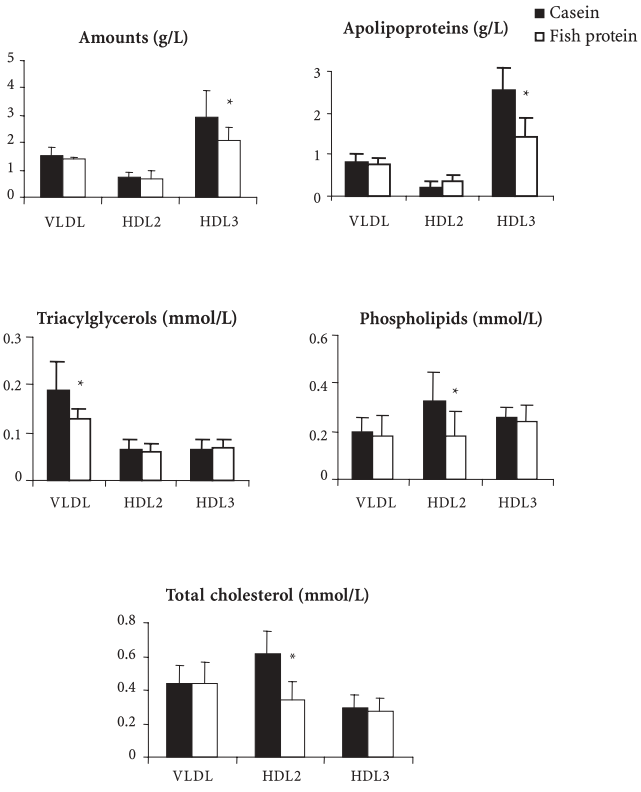

Fig. 1 Plasma VLDL, HDL_2 and HDL_3 amounts and compositions. Values are means ± SEM for ten rats per group. * $P < 0.05$, fish protein group vs casein group

higher (71 %, 41 %, respectively) in the fish protein group than in the casein group, whereas apo A-I and C did not differ in either group. HDL_3 apo A-I, A-II and A-IVwere significantly lower (–38 %, –55 %, –41 %, respectively) in rats fed the fish protein diet than in those fed the casein diet.

Lipoprotein lipase activity and total lipids of tissues

After 2 mo of experiment, consumption of fish protein resulted in significantly lower HTGL and adipose tissue LPL activities (–10 % and –91 %, respectively) as compared to casein (Table 4). LPL activity in gastrocnemius, kidneys and heart remained, however, unchanged with either dietary protein. Feeding fish protein to rats led to a 1.36-fold drop in total lipid concentrations in adipose tissue as compared to casein; liver total lipid concentrations were also lower, but not to a significant extent, due to large inter-individual variation. No difference was found in kidney, gastrocnemius and heart total lipid contents in either group.

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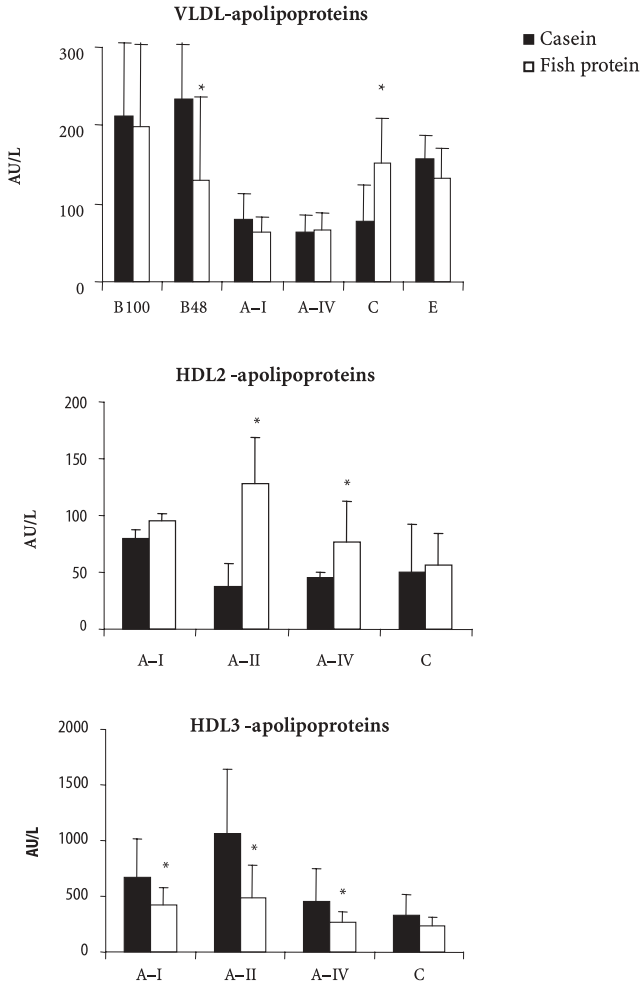

Fig. 2 Plasma VLDL, HDL_2 and HDL_3 apolipoproteins. Values are means ± SEM for ten rats per group. * $P < 0.05$, fish protein group vs casein group

Discussion

This study in spontaneously hypertensive rats (SHR) was conducted to test the action of fish protein on blood pressure and on VLDL, HDL_2 and HDL_3 composition and to relate their changes to lipolytic enzyme activities. The influence of dietary fish protein on the development of hypertension and on the lipoprotein metabolism has not been thoroughly investigated.

Although the food intake was similar, rats fed the fish protein diet exhibited significantly lower body and liver weights as compared to those fed the casein diet. The lowered body weight with fish protein consumption could be related to lower fat deposition. Indeed, rats fed the fish protein diet instead of the casein diet had lower total lipids in adipose tissue (Table 4).

A decrease in systolic blood pressure in response to a fish protein intake has been observed by Yamori et al. [28, 29]. Our results show that the values of blood pressure in the animals receiving highly purified fish proteins were significantly lowered as compared with the values obtained in the rats fed the casein diet. Gutierrez et al. [16] have also reported a diminution in blood pressure following the feeding of a high fish protein diet in normotensive rats made hypertensive by NG-nitro-L-arginine. Moreover, lower blood pressure was observed in SHR-SP fed a fish protein diet compared with those fed a chow diet [8]. As previously reported, the lowered blood pressure observed in rats fed fish protein could result from increased PGI_2 synthesis as a negative correlation between blood pressure and the rate of the urinary excretion of PGI_2 observed in rats fed fish protein instead of casein [30]. PGI_2 acts as a vasodilator and was shown to determine the severity of the hypertensive state. A negative correlation between urinary PGI_2 excretion and blood pressure has been reported by several studies in hypertensive subjects and animal models [31, 32]. The amino acid composition of proteins may be also relevant to account for the effects on blood pressure [33]. Fish proteins are rich in arginine (Table 1) which are involved in the biosynthesis and the release of the vasodilatator NO [34]. Nitric oxide, the metabolic product of arginine through the activity nitric oxide synthase plays a pivotal role as a vasorelaxant and lowers blood pressure [35, 36]. Soybean protein also contains a higher arginine level than casein and has been shown to exert antihypertensive effect in SHR [37].

In the present work, feeding fish protein as compared with casein significantly diminished liver and VLDL triacylglycerol concentrations. The reduced triacylglycerol contents in VLDL fraction might reflect a triacylglycerol

Table 4 Tissue lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) activities (nmol fatty acid released · min^{-1} · mg protein) and total lipids (mg/g tissue) in SHR fed the fish protein or casein diet[1, 2, 3]

	Diet	
	Casein	Fish protein
Liver		
Total lipids	96 ± 27.16	72.5 ± 33.26
HTGL	37.10 ± 3.65	33.37 ± 2.77*
Heart		
Total lipids	58.67 ± 14.14	66.67 ± 16.51
LPL	42.43 ± 8.41	44.37 ± 12.71
Kidney		
Total lipids	41.25 ± 12.46	38.92 ± 12.40
LPL	7.79 ± 1.83	9.68 ± 2.51
Gastrocnemius		
Total lipids	24 ± 11.73	17 ± 4.83
LPL	9.98 ± 3.32	10.72 ± 2.10
Adipose tissue		
Total lipids	851.9 ± 89.85	625 ± 21.21*
LPL	47.09 ± 7.28	4.37 ± 1.50*

[1] Values represent means ± SEM for ten rats
[2] *$P < 0.05$, fish protein group vs casein group

poor VLDL hepatic synthesis. Indeed, plasma VLDL mass and apo B-100 level (the major VLDL apolipoprotein) were not modified in either group, indicating a similar number of VLDL particles. Moreover, HTGL and adipose tissue LPL activities were found to be significantly reduced in rats fed fish protein as compared to those fed casein. This might explain the lowered total lipids in adipose tissue in the fish protein fed group compared with casein. Indeed, increased adipose tissue LPL activity was shown to enhance fat storage and accumulation [38]. Low LPL and HTGL activities are normally associated with enhanced lipoprotein concentrations and decreased remnant catabolism, resulting especially in higher plasma triacylglycerol concentrations [13]. This inverse correlation between lipolytic activities and plasma lipids was not found in SHR fed fish protein. Taken together, these results suggest that a reduced rate of triacylglycerol synthesis combined to the lowered triacylglycerol catabolism by lipoprotein lipase in fish protein fed rats might contribute to maintain a normal VLDL lipid profile. As compared to soybean protein, fish protein was also shown to diminish the VLDL triacylglycerol concentration, though associated with similar HTGL activity [39]. In summary, feeding fish protein decreased triacylglycerol levels in VLDL, but did not affect either those of plasma or the number of VLDL particles. Feeding rats with fish oils, however, was shown to exert hypotriglyceridemic action. This resulted from a reduction in apo B-100 and VLDL secretion by liver [17, 40, 41].

On the other hand, HDL lipid and apolipoprotein constituents have been shown to result from the LPL-dependent lipolysis of VLDL. In rat, during VLDL lipolysis by LPL action, surface components are transferred from VLDL to HDL_3 [42]. This process might be diminished in rats fed fish protein as a result of low LPL activity leading to decreased VLDL catabolism. Indeed, rats fed fish protein as compared with those fed casein showed a significantly low HDL_3 particle number, as measured by diminished HDL_3 mass and apo AI (the major HDL apolipoprotein). Furthermore, the lowered HDL particle number observed after feeding fish protein could also result from reduced HDL_3 synthesis by liver. Surprisingly, in the present work, the HDL_2 particle number, as measured by HDL_2 mass and apo AI, was similar with both dietary proteins. This finding indicates a higher conversion of HDL_3 to HDL_2 in rats fed fish protein, probably related to lecithin:cholesterol acyltransferase (LCAT) activity enhancement (result not shown). This enzyme, located on HDL, catalyses cholesterol esterification, transferring a fatty acid from phosphatidylcholine to unesterified cholesterol [43, 44]. The production rate of large HDL (HDL_2) from small HDL (HDL_3) particles was shown to be dependent on LCAT activity [45]. The underlying process involves the transport of excess free cholesterol from peripheral tissues by HDL_2 to the liver, and subsequently its release into bile as cholesterol and bile acids [46]. In our study, in spite of a similar HDL_2 particle number, HDL_2 particles from rats fed fish protein were depleted in total cholesterol, especially cholesteryl ester (data not shown) leading to reduced plasma total cholesterol concentrations. This is in accordance with other reports investigating fish protein in normotensive rats [47] and in rabbits [9] and indicates a general hypolipidaemic effect of fish protein as compared to casein. In rats with low cholesterol ester transfer protein (CETP) activity, HDL cholesterol concentrations would not be directly affected by interactions between HDL and triacylglycerol-rich lipoproteins [48]. A possible mechanism would be the enhanced HDL_2 cholesterol return to the liver for further excretion as free cholesterol and bile acids. Indeed, feeding fish protein, as compared with casein, was shown to increase faecal excretion of cholesterol and bile acids in rat [49]. Hence, further analysis on faecal steroid excretion under our experimental conditions is required to check our hypotheses.

In conclusion, the results from the present study show that fish protein plays an antihypertensive role and also exerts a cholesterol lowering effect reflected mainly in the HDL_2 fraction. Fish protein intake also decreases VLDL triacylglycerol concentrations. Similar findings have been reported with fish oil diets [17]. Thus, such results transposed to humans would indicate that fish consumption might be beneficial for patients with hypertension.

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