Reduction in plasma cholesterol and increase in biliary cholesterol by a diet rich in n–3 fatty acids in the rat

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Abstract Cholesterol and lipoprotein metabolism were investigated in a group of rats fed a fish oil-supplemented diet, a rich source of n-3 fatty acids. For comparison purposes, other groups of rats were fed either safflower oil (n-6 fatty acids) or coconut oil (saturated fatty acids). Diets were isocaloric and contained identical amounts of cholesterol. Rats fed fish oils for 2 weeks showed a 35% lower plasma cholesterol level than rats fed safflower oil, who in turn showed a 14% lower plasma cholesterol level than those fed coconut oil. The fall in plasma cholesterol level with fish oils was associated with significant falls in low density and high density lipoprotein cholesterol levels, but with no significant change in the ratio of low density to high density lipoprotein cholesterol. The fatty acid compositions of plasma, hepatic, and biliary lipids showed relative enrichment with n-3 fatty acids, reflecting the composition of the diet. The fish oil diet increased the basal secretion rate of cholesterol into bile, but the bile acid secretion rate remained unchanged. It is suggested that n-3 fatty acids reduce the plasma cholesterol level in rats by increasing the transfer of cholesterol into bile. — **Balasubramaniam, S., L. A. Simons, S. Chang, and J. B. Hickie.** Reduction in plasma cholesterol and increase in biliary cholesterol by a diet rich in n-3 fatty acids in the rat. *J. Lipid Res.* 1985. **26:** 684–689.

Supplementary key words fish oils • lipoproteins • n-6 fatty acids

There is evidence to suggest that n-3 fatty acids in the diet have protective effects against cardiovascular disease (1, 2). These fatty acids contain 20–22 carbon atoms, multiple double bonds, and are found in large quantities in marine oils. It has been shown that enrichment of the diet with n-3 fatty acids, amongst other effects, lowers the plasma concentration of cholesterol and triglycerides in man (3–6). To gain further understanding of the effects of n-3 fatty acids on cholesterol and lipoprotein metabolism, we have undertaken studies in the rat. To identify the specificity of action of n-3 fatty acids, comparisons have been made between isocaloric diets supplemented with n-3 fatty acids, n-6 fatty acids, or saturated fatty acids. The results indicate that a diet enriched with n-3 fatty acids significantly lowers plasma cholesterol levels, in association with an increase in biliary cholesterol excretion.

MATERIALS AND METHODS

Animal treatment

Immediately after weaning, rats were given normal rat chow for 1 week prior to being placed on experimental diets. These diets were prepared every 3 days by powdering rat chow, thoroughly mixing in the various supplementary oils, and then repelleting. The rats were fed isocaloric diets supplemented with either 10% (w/w) coconut oil, safflower oil, or fish oil (Maxepa R, R. P. Scherer, Melbourne, Australia). Since the fish oil contained 0.6% (w/v) cholesterol, the other experimental diets were supplemented with cholesterol such that all three diets contained the same amount of cholesterol (0.09% w/w). Each diet provided 30% of energy from fat, and contained vitamin E as anti-oxidant. Feeding continued for a period of 2 weeks, unless otherwise specified. All animals were kept under conditions of controlled lighting with alternate dark (1800–0600 hours) and light (0600–1800 hours) cycles, and had free access to food and water. At the end of an experimental period, blood was collected through the abdominal aorta under ether anesthesia and the livers were removed promptly.

Collection and analysis of bile

Bile duct cannulation was performed under Nembutal® anesthesia. Immediately after cannulation rats were returned to their restraining cages. Hourly collections of bile were made over a period of 24 hr and the volume of bile in each fraction was recorded. Bile steroids were extracted with methanol (1:10 v/v), the sample was centrifuged to remove protein, and aliquots were taken for assay of cholesterol, bile acids, and phospholipids. Total bile

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; n-3 fatty acids, fatty acids of the α-linolenic acid (18:3 $\Delta^{9,12,15}$) family; n-6 fatty acids, fatty acids of the linoleic acid (18:2 $\Delta^{9,12}$) family.

acids were assayed enzymatically by the method of Talalay (7), as modified by Turley and Dietschy (8). Cholesterol was measured by gas-liquid chromatography after hydrolysis, using 5α-cholestane as internal standard (2% SE-30 on 80–100 mesh Chromosorb-W, 1.5-meter glass column, N_2 carrier gas, 250°C isothermal). Phospholipids were assayed according to Bartlett (9).

Lipoprotein fractionation; liver microsomal preparation; assay of cholesterol and triglycerides in plasma, lipoproteins and liver

Plasma containing EDTA (0.01%, w/v) was separated into very low density lipoproteins (VLDL, d < 1.006 g/ml), low density lipoproteins (LDL, d 1.006–1.063 g/ml), and high density lipoproteins (HDL, d 1.063–1.21 g/ml) by sequential density adjustment in the preparative ultracentrifuge (10). Liver microsomes were prepared as previously described (11). Plasma cholesterol and triglycerides were assayed by automated enzymatic methods (Cholesterol Liquid Stable Reagent Set, Medical Analysis Systems, Camarillo, CA; Triglycerides Reagent Set, Worthington Diagnostic Systems, Freehold, NJ). Triglyceride assay incorporated a correction for unesterified glycerol. Cholesterol concentrations in lipoproteins and liver were assayed by gas-liquid chromatography, after alkaline hydrolysis and extraction with chloroform–methanol 2:1 (v/v).

Fatty acid analyses in plasma, liver, and bile

Total lipids were extracted with chloroform–methanol 2:1 (v/v). The dried extract was transmethylated with 0.25 ml of boron trifluoride–methanol reagent (14% w/v). The reaction was carried out in screw-capped tubes in a N_2 atmosphere at 85°C for 10 min. The fatty acid methyl esters were extracted with hexane and analyzed by gas-liquid chromatography using a flame ionization detector (10% SP 2300 on 80–100 mesh Supelcoport R, 2-meter glass column, N_2 carrier gas, 225°C isothermal). For fatty acid analysis of phospholipids, triglycerides, and cholesteryl esters, the various lipid classes were first separated by thin-layer chromatography prior to transesterification.

Statistical analysis

Differences between means for different diets were tested by analysis of variance using the Statistical Package For the Social Sciences (12).

RESULTS

In all comparisons presented in this study, rats were offered and consumed similar amounts of food irrespective of the dietary regimen. The fatty acid compositions of the various experimental diets are presented in **Table 1**. The safflower oil diet had at least three times the degree of polyunsaturation of the fish oil diet [(n-6) + (n-3)/saturated]. n-3 Fatty acids accounted for 21% of the total fatty acids in the fish oil diet, as compared with minor quantities in the other two diets. During the second, third, and fourth week of feeding, the group fed fish oils showed 9–17% lower body weights than the other groups. The differences in weight were statistically significant after 2 weeks feeding ($P < 0.05$), 3 weeks feeding ($P < 0.02$), and 4 weeks feeding ($P < 0.04$).

TABLE 1. Fatty acid composition of diets supplemented with coconut, safflower, or maxepa oils

	Coconut Oil Diet	Safflower Oil Diet	Fish Oil Diet
	percent of total		
Saturated			
12:0	29		
14:0	10		6
16:0	13	12	19
18:0	4	3	4
Monounsaturated			
16:1	1	2	8
18:1	21	20	19
(n-6) Group			
18:2	21	62	21
20:4	trace	trace	trace
(n-3) Group			
20:5	trace	trace	12
22:6	trace	trace	9
(n-6) + (n-3)/Saturated	0.4	4.2	1.4

The plasma lipid data are presented in **Table 2**. Rats fed fish oil for 2 weeks showed a 35% lower mean plasma cholesterol concentration than the group fed safflower oil ($P < 0.001$). Rats fed safflower oil showed a 14% lower mean plasma cholesterol level than those fed a coconut oil diet ($P < 0.001$). These findings persisted even when the duration of feeding was extended to 4 weeks. Plasma triglyceride concentrations were significantly lower in rats fed the fish oil diet, compared with the other two groups after 2 weeks ($P < 0.01$). Although the triglyceride concentrations remained lower in the fish oil-fed group when feeding was extended to 4 weeks, differences between the groups were not statistically significant.

To examine the possibility that the reduction in plasma cholesterol in rats fed fish oil was a consequence of the 9–17% lower body weights experienced by these animals, a weight control experiment was carried out. One group of five rats had free access to normal chow, while a companion group of five rats had restricted access to the same chow over a 2-week period. Rats on the restricted diet showed 15% lower body weights (229 ± 9 vs. 191 ± 10 g; $P < 0.001$), but plasma cholesterol concentrations in both groups were similar (68 ± 2 vs. 66 ± 5 mg/dl).

Lipoprotein cholesterol concentrations are presented in Table 2. The group fed fish oil had a significantly lower cholesterol concentration in LDL compared with the

TABLE 2. Summary of lipid and lipoprotein data[a]

	Coconut Oil Diet	Safflower Oil Diet	Fish Oil Diet
		mg/dl	
Plasma cholesterol (2W)	82 ± 9[b]	72 ± 8[b]	47 ± 4[b]
Plasma cholesterol (4W)	81 ± 7[b]	69 ± 5[b]	51 ± 5[b]
Plasma triglycerides (2W)	91 ± 10	93 ± 18	65 ± 7[c]
Plasma triglycerides (4W)	85 ± 11	89 ± 8	73 ± 19
Lipoprotein cholesterol (2W)			
VLDL	5.0 ± 1.1	6.5 ± 2.6	4.8 ± 0.8
LDL	7.9 ± 1.9[d]	6.9 ± 1.9	5.2 ± 0.5[d]
HDL	67.0 ± 10.1[b]	54.0 ± 4.8[b]	36.2 ± 4.7[b]
LDL/HDL	0.13 ± 0.05	0.13 ± 0.04	0.14 ± 0.03

[a]Mean ± SD (n = 6); 2W, 2 weeks; 4W, 4 weeks.
Comparing different diets:
[b]Significantly different at $P < 0.001$ from all other groups.
[c]Significantly different at $P < 0.01$ from all other groups.
[d]Significantly different at $P < 0.05$.

group fed coconut oil ($P < 0.05$). In rats fed fish oil, HDL cholesterol was significantly lower than in rats fed the other diets ($P < 0.001$). The variations in lipoprotein cholesterol concentration with the different diets were not associated with a significant change in the ratio of LDL to HDL cholesterol.

The fatty acid composition of total lipids of plasma is presented in **Table 3**. In rats fed fish oil, n-3 fatty acids accounted for 21% of total fatty acids. This proportion was 4% with safflower oil and 8% with coconut oil diets. In rats fed safflower oil, n-6 fatty acids accounted for 51% of total fatty acids. This proportion was 35% with coconut oil and 27% with fish oil diets. Similar trends were observed in fatty acid composition of the individual plasma lipid classes (data not presented). The fatty acid pattern of total lipids derived from individual lipoprotein fractions reflected the pattern found in total plasma lipids (see **Table 4**).

The excretion patterns of biliary lipid in bile duct-cannulated rats fed the various dietary regimens are presented in **Table 5**. The amount of lipid excreted during the first 10 hr was arbitrarily considered to represent the biliary pool and the rate of secretion for the next 14 hr was considered to represent basal lipid synthesis. There were no significant differences in the bile pool sizes or synthetic rates between the groups of rats fed the various diets. The biliary secretion rate of cholesterol in rats fed fish oil was significantly higher than that in rats fed safflower oil, while the cholesterol secretion rate in rats fed safflower oil was higher than that in rats fed coconut oil. The biliary cholesterol pool was highest in rats fed fish oil, next highest in those fed safflower oil, and lowest in those fed coconut oil. The biliary pool size of phospholipids was highest in rats fed fish oil. There were no significant differences in the secretion rates of phospholipids into bile between the groups of rats fed the various diets.

TABLE 3. Fatty acid composition of total plasma lipids by diet[a]

	Coconut Oil Diet	Safflower Oil Diet	Maxepa Oil Diet
		percent of total	
16:0	24.2 ± 0.1	20.0 ± 1.4[b]	26.0 ± 2.0
16:1	2.5 ± 0.8	3.0 ± 0.5	3.8 ± 0.9
18:0	12.1 ± 1.3	12.8 ± 2.8	9.2 ± 1.0[c]
18:1	16.4 ± 1.4	10.9 ± 0.9[b]	15.8 ± 1.5
18:2	15.4 ± 1.3	27.0 ± 4.2[b]	14.5 ± 1.4
20:4	19.2 ± 2.1[d]	24.0 ± 3.2[d]	12.0 ± 1.9[b]
20:5	trace	trace	8.4 ± 1.3
22:6	8.0 ± 0.6[d]	4.2 ± 0.7[d]	12.2 ± 2.3[b]
(n-6) + (n-3)/Saturated	1.2 ± 0.1	1.7 ± 0.2[b]	1.3 ± 0.1

[a]Mean ± SD (n = 5).
Comparing different diets:
[b]Significantly different at $P < 0.001$ from all other groups.
[c]Significantly different at $P < 0.02$ from all other groups.
[d]Significantly different at $P < 0.001$.

TABLE 4. Fatty acid composition of individual plasma lipoprotein fractions by diet[a]

	Coconut Oil Diet			Safflower Oil Diet			Fish Oil Diet		
	VLDL	LDL	HDL	VLDL	LDL	HDL	VLDL	LDL	HDL
	percent of total								
16:0	25	25	23	23	24	22	25	26	25
18:0	6	9	12	6	8	12	5	12	9
16:1	2			10			2	2	2
18:1	25	17	11	14	13	9	18	20	12
18:2	17	12	12	33	27	19	12	10	10
20:4	7	8	28	13	16	28	4	4	14
20:5	tr	tr	tr	tr	tr	tr	9	8	9
22:6	9	7	6	5	4	4	20	12	12
Unidentified	7	12	4	5	6	3	3	4	4

[a]Analysis of one composite plasma pool derived from four rats on each diet; tr, trace.

The fatty acid compositions of total lipids derived from plasma, liver, and bile are presented in **Fig. 1** for rats fed the three diets. In every sampling the proportion of n-3 fatty acids was greater in rats fed fish oil than in rats fed safflower oil or coconut oil. Similarly, safflower oil-fed rats had the highest proportion of n-6 fatty acids in each of the tissue samplings. The fatty acid composition of phospholipids, triglycerides, and cholesteryl esters of plasma, and that of phospholipids in liver and bile reflected the pattern seen in the fatty composition of total lipids in those tissue fractions in relation to the different diets (data not shown). The fatty acid composition of the phospholipids of liver microsomal membranes is presented in **Table 6**. The proportion of n-3 fatty acids was much higher in fish oil-fed rats than in rats fed the other diets. There was a relative reduction in the proportion of n-6 fatty acids in microsomal phospholipids of rats fed fish oil.

DISCUSSION

Dietary supplementation with polyunsatured fats rich in n-6 fatty acids is associated with a reduction in plasma cholesterol concentration in man and other species (reviewed in Ref. 13). The present studies indicate that dietary supplementation with polyunsaturated fats rich in n-3 fatty acids may produce a very marked reduction in plasma cholesterol concentration in rats, even greater than that observed with n-6 fatty acids. This effect is independent of small differences occurring in body weight during dietary supplementation. The same general findings were previously observed in hypercholesterolemic rats (14). As in the case of man, enrichment of diet with n-3 fatty acids also reduces plasma triglyceride concentrations in the rat, although in the present studies this change was less marked.

The lower plasma cholesterol level observed in rats fed n-3 fatty acids essentially reflected a reduced amount of cholesterol carried in both LDL and HDL fractions. However, the proportional decrease in cholesterol content was similar in LDL and HDL, there being no significant change in the ratio of LDL to HDL. This ratio remained unchanged across all dietary regimens. The constancy of this ratio in the rat has also been observed during the administration of specific cholesterol-lowering drugs (11).

TABLE 5. Effects of diet on hepatobiliary metabolism[a]

	Coconut Oil Diet	Safflower Oil Diet	Fish Oil Diet
Plasma cholesterol (mg/dl)	79 ± 4[b]	70 ± 3[b]	49 ± 5[b]
Liver cholesterol (mg/g)	1.5 ± 0.1[b]	2.4 ± 0.3[b]	1.8 ± 0.1[b]
Biliary pool size (μmol)			
Cholesterol	2.0 ± 0.4[b]	4.5 ± 0.9[b]	10.5 ± 2.1[b]
Bile acids	137 ± 8	142 ± 11	135 ± 13
Phospholipids	20.8 ± 4.2[c]	23.1 ± 3.1	26.7 ± 3.5[c]
Biliary secretion rate (μmol/hr)			
Cholesterol	0.16 ± 0.03[b]	0.25 ± 0.02[b]	0.51 ± 0.04[b]
Bile acids	6.8 ± 0.9	6.9 ± 0.5	7.1 ± 0.6
Phospholipids	1.43 ± 0.22	1.55 ± 0.24	1.65 ± 0.23

[a]Mean ± SD (n = 6).
Comparing different diets:
[b]Significantly different at $P < 0.001$ from all other groups.
[c]Significantly different at $P < 0.05$ from all other groups.

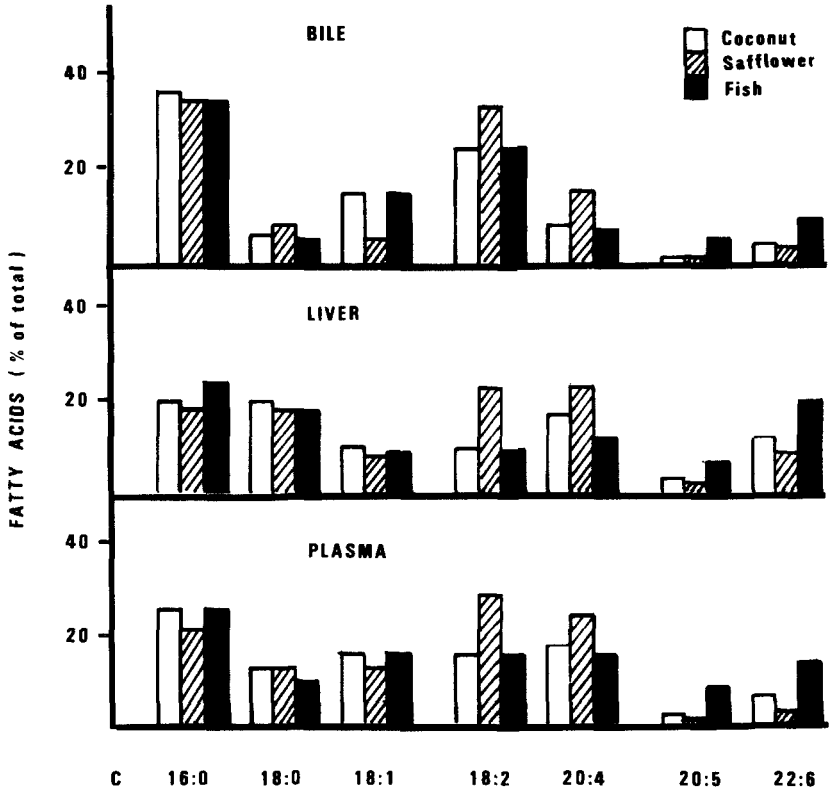

Fig. 1. Fatty acid compositions of total lipids derived from plasma, liver, and bile in rats fed the indicated dietary regimens.

In man it has been shown that the reduction in plasma cholesterol by a fish oil diet is mainly due to reduction in plasma LDL concentration (15).

The composition of plasma lipid fatty acids closely reflected the type of diet consumed by the animals. Rats fed n-3 fatty acids had relatively higher proportions of these fatty acids compared with those fed other diets. The fatty acid patterns of individual classes of lipids in plasma and of individual classes of lipoprotein clearly reflected the diet. Moreover, the total lipids and phospholipids of liver membranes and bile also showed enrichment in fatty acids that were unique to the individual diet.

An important observation in the current studies is the significant increase in the secretion of cholesterol into bile of rats fed fish oil. The biliary cholesterol pool (representing the amount excreted in the first 10 hr after cannulation) and the basal secretion rate of cholesterol (representing the amount secreted after 10 hr) in this group were markedly increased over groups fed the other diets. Enrichment of the diet with n-6 fatty acids has previously been shown to stimulate cholesterol secretion in bile (16), and this is in agreement with the present findings: a twofold increase both in bile cholesterol pool and basal rate of cholesterol secretion occurred when n-6 fatty acids replaced saturated fatty acids. Enrichment of the diet with n-3 fatty acids leads to still greater increases in the biliary secretion of cholesterol. In the absence of an increase in bile acid secretion, the decrease in LDL and HDL cholesterol concentration in the rat fed n-3 fatty acids seems to be related to the increase in cholesterol secretion into bile. Since there is no reason to suspect a change in the percentage absorption of cholesterol with this diet, there is probably a net increase in cholesterol excretion from the animal during fish oil feeding.

The fall in VLDL and LDL levels in man with fish oil

TABLE 6. Fatty acid composition of liver microsomal phospholipids in rats fed various diets[a]

	Coconut Oil Diet	Safflower Oil Diet	Fish Oil Diet
		percent of total	
16:0	17.5 ± 3.2	16.3 ± 4.1	16.0 ± 3.4
16:1	0.6 ± 0.3	0.8 ± 0.4	1.0 ± 0.5
18:0	26.0 ± 3.1	24.1 ± 2.0	23.5 ± 2.5
18:1	10.5 ± 1.6	9.4 ± 2.1	8.5 ± 1.8
18:2	7.5 ± 1.5[b]	12.5 ± 2.0[b]	5.0 ± 1.3[b]
20:4	26.1 ± 2.4[b]	32.8 ± 1.9[b]	12.8 ± 3.6[b]
20:5			7.5 ± 2.1
22:5			2.1 ± 0.5
22:6	10.3 ± 2.4[b]	6.5 ± 1.9[b]	23.3 ± 3.1[b]
n-3/n-6	0.33 ± 0.05[b]	0.15 ± 0.03[b]	2.00 ± 0.11[b]

[a] Mean ± SD (n = 5).
[b] Significantly different at $P < 0.001$ from all other diet groups.

feeding has been attributed to a reduction in lipoprotein synthetic rates (15, 17). The present data suggest that increased catabolism of lipoproteins may also play a role. However, the biochemical mechanism underlying the increased secretion of cholesterol into bile during fish oil feeding is difficult to explain. One possibility is accelerated uptake of lipoproteins by the liver due to alteration in the plasma membrane of the liver cells (see Table 6).

The increased amount of cholesterol transferred to bile during ingestion of a diet rich in n-3 fatty acids might increase the lithogenic properties of bile. In man this could increase the chance of gallstone formation. However, the absence of an excess incidence of gallstones in populations habitually consuming quantities of n-3 fatty acids suggests increased solubility of cholesterol in bile, perhaps due to a change in the fatty acid composition of biliary phospholipids (18) or an increase in phospholipid concentration in the bile.

We acknowledge technical assistance provided by Mr. J. Ruys, statistical analysis by Mrs. J. Simons, and secretarial assistance by Ms. L. Denty.

Manuscript received 5 December 1983.

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