Lipidemic Responses in Rats Fed Biscuits Made with Fish Oil[1]

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ABSTRACT

In an eight-week experiment, young hypercholesterolemic rats were fed diets that differed widely in the content of omega-6 (*n*-6) and omega-3 (*n*-3) polyunsaturated fatty acids but contained the same level of fat. Fat provided 30% of the total calories. Omega-3s in the diet originated from biscuits made with fish oil. The objective was to determine the *n*-6:*n*-3 ratio that modifies the blood lipid profile most favorably. This was observed in the diet with an *n*-6:*n*-3 ratio of 1:0.7 (*n*-6, 1.93 g; *n*-3, 1.39 g/100 g). This diet caused a time-dependent reduction of serum total cholesterol. By week 8, serum triglyceride levels were also significantly lower on this diet, whereas high-density lipoprotein cholesterol increased steadily with the level of fish oil in the diet.

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Hyperlipidemia is strongly linked to cardiovascular disease (CVD). There is increasing evidence that dietary omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs) such as those found in fish oil may reduce the development of CVD. Eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the most common *n*-3s found in fish oil. These acids are reported (Herold and Kinsella 1986, Hartog et al 1987) to affect several biochemical and metabolic parameters in man and animals, including the lowering of elevated blood cholesterol (CH) and triglyceride (TG) levels. Epidemiologically, Eskimos' freedom from CVD has been related to their abundant consumption of *n*-3 PUFAs in comparison to *n*-6 PUFAs and saturated fatty acids (Dyerberg and Bang 1982, Herold and Kinsella 1986).

Although the CH- and TG-lowering effect of *n*-3s is generally recognized, their effect on high-density lipoprotein (HDL) CH remains inconsistent (Kobatake et al 1984, Haug and Hostmark 1987). Information on the dietary *n*-6:*n*-3 ratio that affects the blood lipid profile most favorably is also lacking. The time course of lipidemic responses to fish oil is also largely unknown (Hartog et al 1987). The experiment reported here was designed to address these questions. Rats were fed diets that varied greatly in *n*-6:*n*-3 ratios, and their lipidemic responses were measured at two-week intervals for eight weeks. Partially hydrogenated vegetable oils (a good source of *n*-6 PUFAs) and fish oil (a good source of *n*-3 PUFAs) were used separately or in combination to make biscuits that served as the sources of *n*-6 and *n*-3 acids in test diets.

MATERIALS AND METHODS

Biscuits

Baking powder biscuits, using vegetable shortening and fish oil alone or in combination, were made by the following formula: pastry flour, 100 g; baking powder, 3 g; salt (NaCl), 1 g; nonfat dry milk, 3 g; water, 50 g; shortening and/or fish oil, 25 g. Six large batches of biscuits (labeled A through F, corresponding with diets A–F) were made, with ratios of shortening to fish oil as follows: A 25:0, B 20:5, C 15:10, D 10:15, E 5:20, and F 0:25. Whereas shortening contained only a small amount (0.5%) of *n*-3 fatty acid, fish oil was quite high, containing 15.1% EPA and 9.1% DHA (Table I). After the ingredients were mixed, biscuits were baked at 215°C for 24 min.

Diets

Air-dried and finely ground biscuits were used to formulate a control (diet A, no fish oil) and five test diets (diets B–F) that contained fish oil. These diets were frozen until needed. Table II lists the composition and energy distribution of each diet. All diets contained the same level of fat (13.2%), of which about 97% originated from the shortening or fish oil in the biscuits; the remaining 3% was contributed by pastry flour. Whereas diet A was virtually free of *n*-3s, diet F contained about four times as much *n*-3s as *n*-6s. The content of *n*-6 and *n*-3 PUFAs and their ratios in the experimental diets are listed in Table II. Diets were complete in all nutrients required by the growing rats (NRC 1987), including alpha-tocopherol.

Animals

Six groups of male, weanling rats (11 rats/group) of Sprague-Dawley strain (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually in mesh bottom stainless steel cages in a controlled (light and dark cycle, 12 hr) environment. Diets and deionized water were offered to the animals ad libitum for eight weeks. Body weight and diet intake records were kept.

Blood Sampling

At two-week intervals, the rats were fasted for 14 hr. They were then lightly anesthetized and about 2.0 ml of blood was withdrawn by heart puncture. The blood was allowed to clot and then centrifuged prior to obtaining the serum. Separated serum was refrigerated for lipid analysis the following day.

Analytical Methods

Finely ground biscuits were analyzed for protein (Kjeldahl), fat (acid hydrolysis), and ash by the standard AACC methods (AACC 1983). Moisture was determined under vacuum (16 hr, 70°C, 25 mmHg). Total CH and HDL-CH in serum were determined enzymatically using kit no. 351 from Sigma Chemical Co. (St. Louis, MO); the same method was used to determine cholesterol in fish oil. HDL-CH was determined following sodium phosphate-magnesium precipitation of nonHDL-CH. Kit no. 336 from Sigma

TABLE I
Fatty Acid Composition (%) of Fats Used in Biscuits

Fatty Acid[a]	Shortening[b]	Fish Oil[c]
14:0–20:0	25.5	30.6
16:1–16:4	···	17.7
18:1	55.0	11.4
18:2	19.0	1.5
18:3	0.5	1.6
18:4	···	3.5
20:1	···	1.6
20:4	···	2.0
20:5	···	15.5
21:5	···	0.8
22:1	···	0.5
22:5	···	2.4
22:6	···	9.1

[a] Composition is based on information provided by the manufacturers.
[b] Partially hydrogenated soybean and cottonseed oils (All-purpose shortening from PVO Foods, St. Louis, MO).
[c] Specially processed Menhaden oil from Zapata Haynie Corp., Reedville, VA.

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was used to determine serum triglyceride levels. The method involves hydrolysis of TG by lipase. The glycerol produced is then measured by coupled enzyme reactions catalyzed by glycerol kinase, glycerol-1-phosphate dehydrogenase, and diaphorase.

Statistical Analyses

Mean comparisons were made with Duncan's multiple-range test. Quadratic regression analysis was used to predict lipidemic responses. All statistical analyses were computed following the Statistical Analysis System (SAS 1982).

TABLE II
Experimental Diets

Components	Diet A	B	C	D	E	F
Ingredients, g/100 g						
Biscuits[a]	66.84	68.29	68.68	68.61	68.64	67.90
Vitamin mix[b]	2.2	2.2	2.2	2.2	2.2	2.2
Mineral mix[c]	3.5	3.5	3.5	3.5	3.5	3.5
Cholesterol[d]	1.00	0.99	0.97	0.96	0.94	0.93
Cholic acid	0.2	0.2	0.2	0.2	0.2	0.2
Casein[e]	7.32	7.70	7.46	7.33	7.47	7.35
Cornstarch[f]	18.94	17.13	16.99	17.20	17.05	17.92
Composition, %						
Fat	13.2	13.2	13.2	13.2	13.2	13.2
From flour	0.4	0.4	0.4	0.4	0.4	0.4
From shortening	12.8	10.3	7.7	5.1	2.5	···
From fish oil	···	2.5	5.1	7.7	10.3	12.8
Protein	11.5	11.5	11.5	11.5	11.5	11.5
Energy distribution, %						
Fat	29.8	30.2	30.3	30.2	32.2	30.0
Protein	11.5	11.7	11.7	11.7	11.7	11.6
Carbohydrates	58.7	58.1	58.0	58.1	58.1	58.4
Energy content, kJ/100 g	1,669	1,648	1,644	1,648	1,648	1,657
Fatty acid, g/100 g						
Omega-6 (*n*-6)[g]	2.65	2.30	1.93	1.57	1.21	0.86
Omega-3 (*n*-3)[h]	0.07	0.72	1.39	2.06	2.72	3.37
n-6:*n*-3 ratio	1:0.0	1:0.3	1:0.7	1:1.3	1:2.3	1:3.9

[a] Finely ground.
[b] Vitamin diet fortification mixture from ICN, Cleveland, OH.
[c] American Institute of Nutrition mixture 76 from United States Biochemicals, Cleveland, OH.
[d] From Sigma Chemical Co., St. Louis, MO. Fish oil (biscuits B–F) contributed some cholesterol.
[e] Contained 92% crude protein.
[f] Instant cornstarch from American Maize Products, Hammond, IN.
[g] From linoleic (18:2, *n*-6) and arachidonic (18:4, *n*-6) acids.
[h] From linolenic acid (18:3, *n*-3), EPA (20:5, *n*-3) and DHA (22:6, *n*-3).

RESULTS

Body Weight Gain

Body weight gains of rats averaged between 199 ± 21 g (diet F) and 230 ± 23 g (diet C) after eight weeks (Table III). Weight gains were significantly higher ($P < 0.05$) in rats fed moderate levels of fish oil (diets B and C) than higher levels (diets D–F). Reduced gains on diets D–F resulted primarily from lower diet intakes and lower diet utilization (diet/gain ratios) efficiencies (Table III).

Serum Total CH

Irrespective of the dietary *n*-6:*n*-3 ratio (Table II), serum total CH (TCH) levels were significantly lower ($P < 0.01$) in rats fed diets containing fish oil (diets B–F) as compared to the control diet (diet A). This lowering effect due to fish oil was highly pronounced and remained so at all blood sampling intervals even as the serum TCH levels declined with time (Table III).

Diet B contained the lowest level of fish oil (2.5%). Due to a higher diet intake, animals fed diet B also consumed more dietary CH (Table III). Despite this, serum TCH in rats fed diet B was no higher than that in rats fed diets C–F (Table III). A higher level of fish oil, however, appears more hypocholesterolemic when the data are subjected to quadratic regression analysis (Fig. 1). This higher level represents diet C, with an *n*-6:*n*-3 ratio of 1:0.7.

Serum HDL cholesterol

As percent of TCH, serum HDL-CH levels were significantly higher ($P < 0.01$) in rats fed diets containing fish oil (diets B-F) as compared to that with no fish oil (diet A). This effect was highly pronounced and persisted throughout the study (Table III). Based on responses early in the experiment, HDL-CH levels approached the highest levels in rats fed diet C. Afterwards, however, they continued to increase steadily with the level of *n*-3s in the diet.

Serum TG

Initially, only rats fed the lowest level of fish oil showed a TG-lowering effect (diet B vs. diet A). This effect of diet B became significant ($P < 0.05$) by week 4 (Table III). By week 6, rats fed still higher levels of fish oil (diets C and D) also showed a TG-lowering effect. However, it was not until week 8 that a significant ($P < 0.05$)

TABLE III
Physiological Responses in Rats Fed Test Diets for Eight Weeks[a]

Component	Diet A	B	C	D	E	F
Fish oil, %	0	2.5	5.1	7.7	10.3	12.8
Body weight gain,[b] g	215 ± 14 ab	229 ± 19 a	230 ± 23 a	208 ± 16 b	201 ± 15 b	199 ± 21 b
Diet intake, g	679 ± 36 ab	717 ± 43 a	712 ± 56 a	678 ± 41 ab	664 ± 32 b	660 ± 51 b
Cholesterol intake, g	6.8 ± 0.4	7.2 ± 0.4	7.1 ± 0.6	6.8 ± 0.4	6.6 ± 0.3	6.6 ± 0.5
Diet/gain, ratio	3.2 ± 0.1 bc	3.1 ± 0.1 bc	3.1 ± 0.2 c	3.3 ± 0.2 ab	3.3 ± 0.2 a	3.3 ± 0.1 a
Serum total cholesterol[c], mg/dl						
week 2	387 ± 96 a	181 ± 25 b	188 ± 23 b	215 ± 23 b	223 ± 36 b	223 ± 29 b
week 4	489 ± 112 a	165 ± 36 b	185 ± 32 b	176 ± 32 b	158 ± 19 b	183 ± 16 b
week 6	254 ± 33 a	126 ± 26 c	157 ± 36 b	132 ± 23 c	111 ± 14 c	126 ± 18 c
week 8	220 ± 51 a	117 ± 19 b	97 ± 20 b	111 ± 20 b	97 ± 4 b	100 ± 6 b
Serum HDL-cholesterol,[c] % of total						
week 2	10.5 ± 4.1 c	30.5 ± 5.9 b	33.8 ± 5.6 ab	33.0 ± 4.7 ab	34.8 ± 6.5 ab	37.0 ± 7 .5 a
week 4	5.0 ± 1.6 e	26.9 ± 7.1 d	36.9 ± 10.3 bc	32.5 ± 7.7 cd	46.5 ± 5.1 a	40.5 ± 4.5 b
week 6	10.9 ± 3.2 d	18.8 ± 5.6 c	18.0 ± 5.5 c	27.5 ± 6.2 b	25.7 ± 3.6 b	44.0 ± 4.9 a
week 8	14.4 ± 5.2 d	28.0 ± 7.0 c	43.2 ± 13.4 b	31.5 ± 5.1 c	62.2 ± 9.2 a	69.2 ± 10.1 a
Serum triglycerides,[c] mg/dl						
week 2	48.8 ± 8.7 c	40.8 ± 8.3 c	48.5 ± 5.9 c	54.0 ± 6.1 c	76.3 ± 14.3 b	113.6 ± 29.2 a
week 4	58.5 ± 8.6 c	47.8 ± 6.8 d	58.6 ± 12.2 c	54.7 ± 5.2 cd	72.3 ± 11.6 b	88.5 ± 10.5 a
week 6	49.8 ± 5.7 ab	42.6 ± 8.7 b	41.9 ± 8.2 b	47.8 ± 8.1 b	50.4 ± 8.7 ab	57.5 ± 13.3 a
week 8	72.9 ± 11.4 a	60.7 ± 10.1 b	48.9 ± 8.0 c	53.6 ± 5.2 bc	48.8 ± 8.6 c	55.1 ± 4.1 bc

[a] Values are averages (9–10 rats/diet) ± SD. Averages not followed by a common letter in a line are significantly different ($P < 0.05$).
[b] Initial (0-day) body weight averaged 43 g (all groups).
[c] Initial (0-day) serum lipid profile: total cholesterol, 90 ± 7 mg/dl; high-density lipoprotein cholesterol, 55.6 ± 6 (% of total); triglycerides, 50 ± 3 mg/dl.

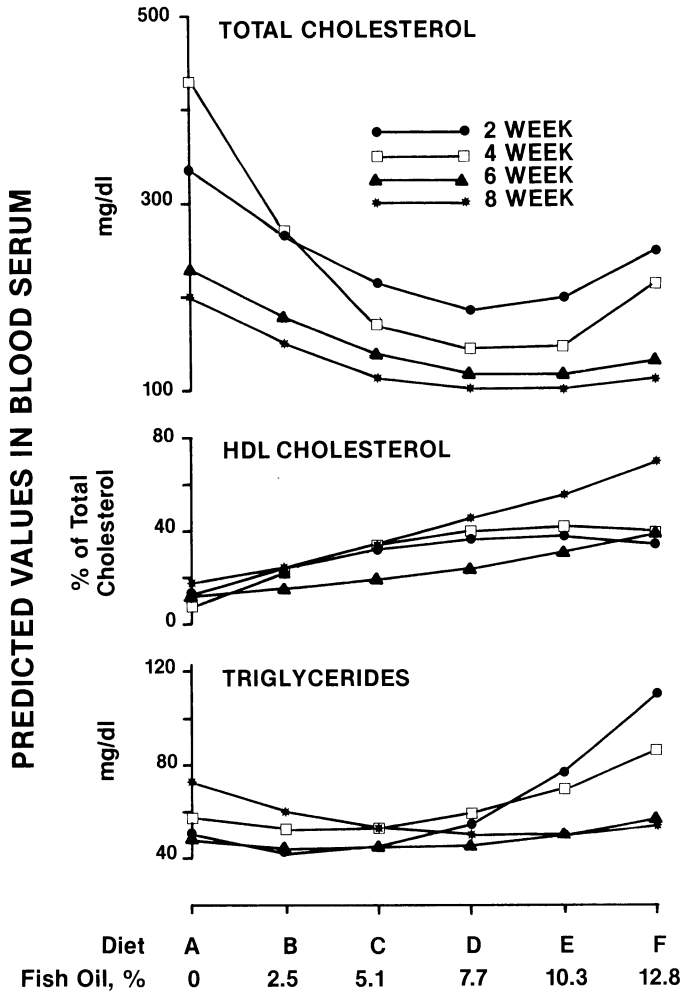

Fig. 1. Quadratic regression for serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides on fish oil in the diet.

hypotriglyceridemic effect was observed in rats fed fish oil diets compared with the control diet. Based on a quadratic regression analysis (Fig. 1), diet C (*n*-6:*n*-3 ratio, 1:0.7) produced a greater hypotriglyceridemic response than did the other fish oil diets.

DISCUSSION

The main purpose of this study was to determine the amount and the ratio of *n*-6:*n*-3 PUFAs that may produce a desired and persistent lipidemic response in rats. Diets fed to these animals were formulated with wide *n*-6:*n*-3 ratios using biscuits where shortening in the formula was increasingly replaced with fish oil.

Fish oil and/or shortening, the major sources of fat in the experimental diets, provided 30% of the total calories, a level now widely recommended (McNutt 1980) to reduce the risk of CVD in the American population. At this level, calories from PUFAs averaged 8% and those from monounsaturated and saturated fatty acids 22%. Rats fed diets containing this lipid profile showed a significant change in their blood lipids when shortening in biscuits was replaced with fish oil. This change was most significant when only 40% of the shortening had been replaced. At this replacement level, the resultant diet (diet C) contained 1.93 g *n*-6 PUFAs and 1.39 g *n*-3 PUFAs/100 g of diet (ratio, 1:0.7).

To be meaningful, the modification of blood lipid profile in rats was examined under a diet-induced hypercholesterolemic condition. This hypercholesterolemia peaked at week 4 (Table III), but at all measurement intervals fish oil reduced serum TCH levels significantly ($P < 0.01$). Several mechanisms, such as reduced CH absorption (Chen et al 1987), transfer of CH into bile (Balasubramaniam et al 1985), hepatic accumulation (perhaps transitory) of CH (Sanders 1986), or even inhibition of hepatic synthesis of CH (Norum and Drevon 1986), have been proposed as being responsible for this effect.

Irrespective of the mechanism(s) involved, the maximum hypocholesterolemic effect observed in rats did not require the highest *n*-6:*n*-3 ratio (1:3.9) studied; a ratio of 1:0.7 appeared to be as effective (Fig. 1) as higher ratios. In several studies of man and animals (Herold and Kinsella 1986), a hypocholesterolemic effect of fish oil has variously been reported. This study differs in that it focuses on the amount and ratio of *n*-6 and *n*-3 acids that most favor the hypocholesterolemic response.

This study also examined the effect of fish oil on serum TG and HDL-CH levels. Unlike TCH, the effect of fish oil in reducing serum TG did not become significant until late in the experiment (Table III, Fig. 1). Experimental diets contained about 58% of the total calories as carbohydrates (starch in pastry flour and cornstarch). Although this level of carbohydrate is now widely recommended (McNutt 1980) in the American diet, some hypertriglyceridemic effect may result. This may have initially nullified the anticipated hypotriglyceridemic effect of fish oil. However, the TG-lowering effect that was observed in rats by week 8 approached a maximum on diet C, as did the CH-lowering effect. Fish oil is reported to reduce serum TG levels by reducing hepatic synthesis of fatty acids (Norum and Drevon 1986), perhaps due to its EPA content (Kobatake et al 1984).

The concentration of the protective (against CVD) CH fraction, namely HDL-HC, also neared a maximum level initially on diet C (Fig. 1). However, later in the experiment it increased steadily with fish oil in the diet. This contrasts with the nonlinearity of the lowering effect observed on serum TCH and TG (Table III). This also contrasts with the results reported by Hartog et al (1987), which showed that whereas fish oil decreases plasma TCH and TG levels in pigs, it also decreases HDL-CH. Our results, however, confirmed the findings of Kobatake et al (1984) who worked with rats.

This study suffers from the limitation that it adds to the controversy surrounding the effect of fish oil on HDL-CH level. However, it is meaningful in suggesting that excessive intake of fish oil is perhaps not necessary to achieve the maximum CH- and TG-lowering effect.

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