Mem. Fac. Fish., Kagoshima Univ.
Vol. 33, No. 1, pp. 73~78 (1984)

Hypocholesterolemic Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid in Rats

Akio KANAZAWA*1, Shin-ichi TESHIMA*1, Sigeru TOKIWA*2
and Nobuya IMATANAKA*3

Abstract

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) methylesters (ME) were prepared from a squid-liver oil and their hypocholesterolemic activities examined with rats. The supplement of 0.3% EPA-ME to the diet containing 1.0% cholesterol and 4.0% butter as lipids reduced a serum-cholesterol level markedly, whereas DHA-ME gave almost no effect on the serum-cholesterol level. Both EPA-ME and DHA-ME reduced the liver-cholesterol level as effectively as linoleic acid did. The supplement of small amounts of EPA-ME was also effective in lowering the serum-cholesterol level; a dose of 0.03% EPA-ME suppressed by 60.7% the elevation of serum-cholesterol level.

The relationship between elevated serum-cholesterol levels and the incidence of coronary artery diseases is well established[1]. In general, animal fats increase serum-cholesterol levels, whereas vegetable oils have a hypocholesterolemic effect in experimental animals and man. On the other hand, fish oils have been shown to reduce serum-cholesterol levels in man[2-4] and animals[5-7]. STANSBY[8,9] has also revealed that fish oil rich in highly unsaturated fatty acids (HUFA) are possibly effective in preventing the heart diseases relating to atheriosclerosis in man.

The hypocholesterolemic effects of fish oils have been supposed to be due to HUFA, especially eicosapentaenoic acid (EPA: 20:5ω3)*4 and docosahexaenoic acid (DHA: 22:6ω3)[10,11]. Futhermore, several investigations have demonstrated that EPA is probably effective in preventing the formation of thrombi.[12-14] However, further detailed studies have not been carried out probably due to the difficulty in preparing large quantities of HUFA such as EPA and DHA for biological and clinical tests.

Previously, we have prepared large amounts of EPA and DHA methylesters (ME) by column chromatography on $AgNO_3$-impregnated silica gel.[15] In the present study, hence, we intend to examine the effects of EPA-ME and DHA-ME on the serum- and liver-cholesterol levels in rats. This paper deals with these results and discussion.

Materials and Methods

Preparation of Fatty Acids

EPA-ME and DHA-ME were prepared from a squid-liver oil were converted to the methylesters with hydrogen chloride in methanol, and then EPA-ME and DHA-ME were isolated from the

*1 Faculty of Fisheries, Kagoshima University, 50-20 Shimoarata-4, Kagoshima 890, Japan.
*2 Taito-Faizer Co. Ltd., 1139-7 Shimokakido, Kubo-cho, Matsusaka, Mie 515, Japan.
*3 Kagakuhin-Anzen-Center, Kagakuhin-Kensa-Kyokai, 800-9 Kizan-cho, Hita, 877 Oita, Japan.
*4 The IUPAC Rule recommended the use of icosapentaenoic acid instead of eicosapentaenoic acid. In this paper, however, the name of eicosapentaenoic acid was used according to the traditional designation.

mixture of fatty acid methlesters by column chromatography on 5%(w/w) $AgNO_3$-Kieselgel 60. The EPA-ME and DHA-ME so prepared had the purities of 98.5% and 99.0% by gas-liquid chromatography (GLC) on 10% DEGS[16], respectively. Linoleic acid (18:2ω6; purity 99.8%) was obtained from Sigma Chemical Co. (USA).

Feeding Experiments

Two feeding trials were carried out. Male Wistar-strain rats were obtained from a commercial breeder and maintained on diet 2 containing 1.0% cholesterol and 4.0% butter as lipids (Table 1) for 1 week. Then, the rats, 180-200 g in body weight, were grouped (each group, 6 rats) and reared on the test diets (diets 1 to 9) for 2 weeks. The rats were fed the diets daily at a level of 10% of body weight and water *ad lib.* The basal diet (diet 1) was the essentially same as that reported previously[17] and contained the following ingredients (%): Sucrose 60, casein 18.5, gelatin 5, starch 5, α-cellulose 3, minerals 4, sodium cholate 0.25, vitamins 0.071, choline hydrochloride 0.25, and butter 4.0.

Analytical Methods

The content of liver lipids was determined by the method of BRAGDON[18]. The content of cholesterol in the liver and serum was measured by the method of SPERRY and WEBB[19].

Results and Discussion

Table 1 shows the body weight gain, liver weight, hepatosomatic index, and liver lipid content of the rats fed the test diets in experiments I and II. Table 2 indicates the effects of dietary EPA-ME, DHA-ME, or linoleic acid on cholesterol levels of the serum and liver in rats.

Table 1. Effects of EPA and DHA methylesters on the hepatosomatic index and liver lipid content in rats.

Exptl.	Diet No.	Composition of diet	Body wt. gain (%)	Liver wt. (g)	Hepatosomatic index (%)*1	Liver lipid (mg/g tissue)
I	1	Basal diet (BD)*2	53.5 ± 8.4*5	11.3 ± 1.1*5	4.78	36.1 ± 2.1*5
	2	BD + 1.0% CH (cholesterol)	66.2 ± 2.9	14.1 ± 0.8	5.68	61.3 ± 1.7
	3	BD + 1.0% CH + 0.30% EPA-ME*3	70.7 ± 5.3	12.8 ± 0.4	5.60	51.3 ± 1.2
	4	BD + 1.0% CH + 0.30% DHA-ME*4	60.3 ± 5.2	11.6 ± 0.9	4.83	51.6 ± 0.9
	5	BD + 1.0% CH + 0.30% Linoleic acid	65.0 ± 7.7	12.8 ± 1.0	5.21	54.7 ± 1.6
II	1	BD	48.3 ± 3.3	11.7 ± 0.5	5.98	56.6 ± 4.9
	2	BD + 1.0% CH	43.3 ± 3.1	11.5 ± 0.5	6.06	86.7 ± 4.6
	6	BD + 1.0% CH + 0.03% EPA-ME	36.7 ± 6.2	10.5 ± 0.4	5.69	86.0 ± 3.3
	7	BD + 1.0% CH + 0.06% EPA-ME	67.2 ± 3.2	14.1 ± 0.4	5.67	52.6 ± 3.0
	8	BD + 1.0% CH + 0.09% EPA-ME	38.3 ± 2.5	10.7 ± 0.1	5.73	72.7 ± 5.2
	3	BD + 1.0% CH + 0.30% EPA-ME	70.7 ± 5.3	14.1 ± 0.4	5.63	51.3 ± 1.2
	9	BD + 1.0% CH + 0.60% EPA-ME	54.0 ± 6.1	11.3 ± 0.6	4.83	60.4 ± 1.3

*1 Liver wt./Body wt. × 100

*2 Basal diet contained 4% butter as lipid sources

*3 Eicosapentaenoic acid (20:5ω3) methylester

*4 Docosahexaenoic acid (22:6ω3) methylester

*5 S.E.

Table 2. Effects of EPA and DHA methylesters on cholesterol levels of the liver and serum in rats

Exptl.	Diet No.	Composition of diet	Serum cholesterol		Liver cholesterol	
			Total (mg/dl)	Free (mg/dl)	Total (mg/g)	Free (mg/g)
I	1	Basal diet (BD)	132.0 ± 15.9*	15.0 ± 2.5*	10.2 ± 1.3*	1.1 ± 0.1*
	2	BD + 1.0% CH (cholesterol)	225.6 ± 15.1	53.6 ± 8.3	41.1 ± 3.4	3.3 ± 0.3
	3	BD + 1.0% CH + 0.30% EPA-ME	145.9 ± 7.9	31.9 ± 4.1	16.6 ± 2.1	2.5 ± 0.1
	4	BD + 1.0% CH + 0.30% DHA-ME	226.3 ± 24.4	47.5 ± 9.9	16.4 ± 1.5	2.4 ± 0.2
	5	BD + 1.0% CH + 0.30% Linoleic acid	242.4 ± 19.9	62.2 ± 9.3	15.6 ± 2.3	2.8 ± 0.3
II	1	BD	136.8 ± 9.5	28.1 ± 5.8	11.5 ± 1.0	0.8 ± 0.1
	2	BD + 1.0% CH	241.7 ± 38.0	80.2 ± 9.9	41.1 ± 3.4	3.3 ± 0.3
	6	BD + 1.0% CH + 0.03% EPA-ME	178.0 ± 19.2	40.0 ± 7.7	23.0 ± 3.4	1.5 ± 0.1
	7	BD + 1.0% CH + 0.06% EPA-ME	173.5 ± 12.8	41.9 ± 4.2	19.4 ± 2.5	2.2 ± 0.2
	8	BD + 1.0% CH + 0.09% EPA-ME	188.9 ± 17.9	43.4 ± 4.6	15.3 ± 1.0	0.9 ± 0.1
	3	BD + 1.0% CH + 0.30% EPA-ME	145.9 ± 7.9	31.9 ± 4.1	16.6 ± 2.1	2.5 ± 0.1
	9	BD + 1.0% CH + 0.60% EPA-ME	140.9 ± 10.9	47.5 ± 9.9	35.7 ± 1.5	3.2 ± 0.2

* S.E.

In experiment I, the hypocholesterolemic effects of EPA-ME, DHA-ME, and linoleic acid were examined by the supplement of 3% level of respective compounds to the diet containing 1.0% cholesterol and 4.0% butter as lipids. The addition of 1.0% cholesterol to the basal diet containing 4.0% butter elevated the content of liver lipids markedly and the hepatosomatic index slightly (Table 1). The increase in the liver lipid content was suppressed significantly ($P \leqq 0.01$) by the supplement of 0.3% levels of EPA-ME (diet 3), DHA-ME (diet 4), and linoleic acid (diet 5). However, EPA-ME and linoleic acid had no effect on the hepatosomatic index. Interestingly, the addition of DHA-ME lowered slightly the hepatosomatic index. The cholesterol levels of serum and liver also rose when 1.0% cholesterol was added to the basal diet. The supplement of 0.3% EPA-ME reduced the serum-cholesterol and liver-cholesterol levels significantly ($P \leqq 0.01$). Whereas, neither DHA-ME nor linoleic acid was effective in lowering the serum-cholesterol level, but both compounds reduced the liver-cholesterol level.

Since EPA-ME was found to be hypocholesterolemic in experiment I, the minimum effective dose of EPA-ME was investigated with the diets containing various levels of EPA-ME in experiment II. As for both the serum and liver, a cholesterol-lowering activity was revealed on the diets supplemented with small amounts of EPA-ME (diets 6, 7, and 8), although the activity was high on the inclusions of 0.3% and 0.6% EPA-ME than on those of 0.03%, 0.06%, and 0.09% EPA-ME. When the hypocholesterolemic activity was expressed as a value of suppression (%) (Table 3), the values on 0.03% and 0.6% EPA-ME were 60.7 and 96.1, respectively, as for the serum-cholesterol level. It is noteworthy that EPA-ME was effective in lowering the cholesterol levels of both the serum and liver in spite of small dosage.

Although linoleic acid is hypocholesterolemic, a large dosage is generally required for the reduction of serum-cholesterol level. A large dosage of linoleic acid sometimes gives rise to the disorder of digestive organs such as loss of appetite and loose bowels. In the present study, the inclusion of 0.3% linoleic acid did not merely lower the serum-cholesterol level but also elevated it.

Table 3. Effects of EPA-ME, DHA-ME, and linoleic acid on the suppression (%) of total cholesterol level in the serum and liver of rats

Experiment	Compound added*1	Suppression (%) of cholesterol level*2	
		Serum	Liver
I	0.3% EPA-ME	85.1	79.0
	0.3% DHA-ME	−0.7	79.7
	0.3% Linoleic acid	−17.9	82.3
II	0.03% EPA-ME	60.7	61.1
	0.06% EPA-ME	65.0	73.3
	0.09% EPA-ME	50.3	87.2
	0.3% EPA-ME	91.3	82.8
	0.6% EPA-ME	96.1	18.2

*1 The compounds were added to the diet containing 1.0% cholesterol and 4.0% butter as lipids (diet 2).

*2 Suppression (%) of total cholesterol level was calculated as follows:

$$\text{Suppression (\%)} = \frac{B - C}{B - A} \times 100$$

A: Cholesterol level in the rats fed diet 1 (basal diet)

B: Cholesterol level in the rats fed diet 2 containing 1.0% cholesterol and 4.0% butter as lipids

C: Cholesterol level in the rats fed the diets containing additives examined besides 1.0% cholesterol and 4.0% butter as lipids

KINGSBURY *et al.*[10] have examined the influence of a pentaene- and hexaene-rich fraction prepared from cod-liver oil in man and found that this HUFA fraction lowered plasma cholesterol level. KANEDA *et al.*[11] have also prepared the EPA-ME and DHA-ME rich fractions from a cuttlefish-liver oil; the former contained EPA-ME (51.7%), DHA-ME (37.2%), and other fatty acid ME (11.1%), and the latter involved DHA-ME (77%), EPA-ME (21%), and arachidonic acid ME (2%). They have indicated that EPA-ME fraction reduced the serum-cholesterol level of rats more effectively than DHA-ME fraction, suggesting that the cholesterol-lowering mechanism of each of these fatty acid ME differs from that of linoleic acid. These findings suggest that the hypocholesterolemic effect of fish oils rich in HUFA is due mainly to EPA and/or DHA. The results of the present study shows that EPA-ME has a cholesterol-lowering activity on the serum of rats but DHA-ME has no effect. In contrast with the results obtained by KANEDA *et al.*[11], the present study shows that EPA-ME is effective in lowering cholesterol levels of not only the serum but also the liver. We imagine that the apparent discrepancy of the investigations is due to the difference in the dietary status, especially the purities of EPA-ME and DHA-ME used, besides the physiological conditions such as the age of rats.

The minimum effective dose of EPA-ME for lowering the cholesterol level of serum and liver was estimated to be about 0.03% in the diet under the conditions adopted, and this corresponds to a dose of about 30 mg EPA-ME/day/100 g rat. Thus, EPA-ME was shown to be a substance with a high hypocholesterolemic activity. Several views have been presented as to the mechanism by which polyunsaturated fats and fatty acids reduce serum-cholesterol concentrations. Some workers[20-23] have supposed that unsaturated fatty acids cause an increase in fecal excretion of

neutral and acidic sterols. On the other hand, other workers[24-26] have thought that polyunsaturated fats are merely effective in a redistribution of cholesterol within the body with a shift of cholesterol from the serum or plasma to tissue pools. KURODA and ENDO[27] have pointed out by using a rat liver enzyme preparation that unsaturated fatty acids, arachidonate and linoleate, reduced cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA synthase. The results of the present study do not give a definite explanation for the cholesterol-lowering mechanism of EPA-ME. But, we postulate that the cholesterol-lowering mechanism by EPA-ME is different with that by diunsaturated fatty acids such as linoleic acid, because EPA-ME exerted a hypocholesterolemic effect even in a very small dose in contrast with linoleic acid. Due to a high efficacy, EPA-ME and the compounds containing EPA are expected to be one of promising drugs for cure and prevention of hypocholesterolemia and related diseases.

References

1) KEYS, A., N. KIMURA, A. KUSUKAWA, B. BRONTE-STEWART, N. LARSON and M.H. KEYS (1958): Serum cholesterol studies in Japan, Hawaii, and Los Angeles. *Ann. Int. Med.*, **48**, 83-94.
2) BRONTE-STEWART, B., A. ANTONIS, L. EALES and J.F. BROCK (1956): Effects of feeding different fats on serum-cholesterol level. *Lancet*, **i**, 521-524.
3) ANDERSON, J.T., A. KEYS and F. GRANDE (1957): The effects of different food fats on serum cholesterol concentration in man. *J. Nutr.*, **62**, 421-424.
4) AHRENS, E.H., Jr., W. INSULL, Jr., J. HIRSCH, W. STOFFEL, B.L. PETERSON, J.W. FARQUHAR, T. MILLER and H.J. THOMASSON (1959): The effect on human serum lipids of a dietary fat, highly unsaturated but poor in essential fatty acids. *Lancet*, **i**, 115-119.
5) DE GROOT, A.P. and S.A. REED (1959): Influence of dietary cod-liver oil and some fractions of cod-liver oil on serum cholesterol level of rat. *Nature*, **183**, 1191.
6) PEIFER, J.J., F. JANSSEN, P.A.W. COX and W.O. LUNDBERG (1960): Studies on the distribution of lipids in hypercholesteremic rats-I. The effect of feeding palmitate, oleate, linoleate, linolenate, and menhaden and tuna oils. *Arch. Biochem. Biophys.*, **86**, 302-308.
7) KANEDA, T. and R.B. ALFIN-SLATER (1963): A comparison of the effects of polyunsaturated fatty acids of cuttle fish liver oil and cotton seed oil on cholesterol metabolism. *J. Am. Oil Chem. Soc.*, **40**, 336-338.
8) STANSBY, M.E. (1978): Development of fish oil industry in the United States. *J. Am. Oil Chem. Soc.*, **55**, 238-243.
9) STANSBY, M.E. (1979): Marine-derived fatty acids or fish oils as raw material for fatty acid manufacture. *J. Am. Oil Chem. Soc.*, **56**, 793-795.
10) KINGSBURY, K.J., D.M. MORGAN, C. ALYOTT and E. EMMERSON (1961): Effect of dietary arachidonate, cod-liver oil, and corn oil on the plasma-cholesterol level. A comparison in normal volunteers. *Lancet*, **i**, 739-741.
11) KANEDA, T., K. ARAI and S. TOKUDA (1964): Studies on the effects of marine products on cholesterol metabolism in rats - II. The comparison of the effects of eicosapentaenoic and of docosahexaenoic acid (In Japanese). *Bull. Japan. Soc. Sci. Fish.*, **30**, 419-423.
12) DYERBERG, J. and H.O. BANG (1979): Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet*, **ii**, 433-435.
13) DYERBERG, J. and H.O. BANG (1978): Dietary fat and thrombiosis. *Lancet*, **i**, 152.
14) DYERBERG, J., H.O. BANG, E. STOFFERSON, S. MONCADA and J.R. VANE (1978): Eicosapentaenoic acid and prevention of thrombiosis and atherosclerosis? *Lancet*, **ii**, 117-119.
15) TESHIMA, S., A. KANAZAWA and S. TOKIWA (1978): Separation of polyunsaturated fatty acids by column chromatography on a silver nitrate-impregnated silica gel. *Bull. Japan. Soc. Sci. Fish.*, **44**, 927.
16) TESHIMA, S., A. KANAZAWA and H. OKAMOTO (1976): Analysis of fatty acids of some crustaceans. *Mem.*

Fac. Fish. Kagoshima Univ., **25**, 41-46.

17) Teshima, S., A. Kanazawa, M. Yoshioka and K. Kitahara (1974): Hypocholesterolemic effect of 24-methylenecholesterol and 7-cholestenol in the rat. *J. Steroid Biochem.*, **5**, 69-72.

18) Bragdon, J.H. (1951): Colorimetic determination of blood lipids. *J. Biol. Chem.*, **190**, 513-517.

19) Sperry, W.M. and M. Webb (1950): A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.*, **187**, 97-106.

20) Moore, R.B., J.T. Anderson, H.L. Taylor, A. Keys and I.D. Frantz (1968): Effect of dietary fat on the fecal excretion of cholesterol and its degradation in man. *J. Clin. Invest.*, **47**, 1517-1534.

21) Connor, W.E., D.T. Witiak, D.B. Stone and M.L. Armstrong (1969): Cholesterol balance and fecal neutral steroid and bile acid excretion in normal man fed dietaryfats of different fatty acid composition. *J. Clin. Invest.*, **48**, 1363-1375.

22) Nestel, P.J., N. Havenstein, H.M. Whyte, T.W. Scott and L.J. Cook (1973): Lowering of plasma cholesterol and enriched sterol excretion with the consumption of polyunsaturated ruminant fats. *New Eng. J. Med.*, **288**, 279-282.

23) Nestel, P.F., N. Havenstein, Y. Homma, T.W. Scott and L.J. Cook (1975): Increased sterol excretion with polyunsaturated-fat-high-cholesterol diets. *Metabolism*, **24**, 189-198.

24) Bloomfield, D.K. (1964): Cholesterol metabolism - III. Enhancement of cholesterol absorption and accumulation in safflower oil-fed rats. *J. Lab. Clin. Med.*, **64**, 613-623.

25) Grundy, S.M. (1975): Effects of polyunsaturated fat on lipid metabolism in patients with hypertriglyceridemia. *J. Clin. Invest.* **55**, 269-281.

26) Grundy, S.M. and E.G. Ahrens, Jr. (1970): The effect of unsaturated dietary fats on absorption, excretion, synthesis, and distribution of cholesterol in man. *J. Clin. Invest.*, **49**, 1135-1152.

27) Kuroda, M. and A. Endo (1977): Inhibition of *in vitro* cholesterol synthesis by fatty acids. *Biochim. Biophys. Acta*, **486**, 70-81.