Lipid Lowering Effects of High Linoleate and High α-Linolenate Diets in Rats and Mice. Consequence of Long-Term Feedings

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Diets high in linoleate (safflower oil) or high in α-linolenate (perilla oil) were fed to rats for 11 months, and the effects of the diets on plasma and tissue lipids were compared. The plasma levels of total cholesterol (Cho), phospholipids (PL) and triacylglycerol (TG) were significantly lower in the high α-linolenate group than in the high linoleate group, the differences being more than 30% in the levels of total Cho and TG. The diets had differential effects on the lipid contents of major tissues: the TG level in muscle was higher but both the TG level in depot fat and the PL level in muscle were lower in the high α-linolenate group than in the high linoleate group. In order to clarify whether or not the hypolipidemic effect of the high α-linolenate diet was due to changes in the distribution of lipids among tissues, whole body lipids were estimated in mice fed these diets for 5 months. The whole body Cho content was significantly lower, by 28%, in the high α-linolenate group compared with the high linoleate group, but the total lipid content, PL and neutral lipids were similar between the groups. Our results indicate that the high α-linolenate diet has a more potent cholesterol lowering effect in plasma and body tissue than the high linoleate diet; interestingly, whole body TG levels are similar but tissue distributions of TG are different between the two dietary groups.

Keywords hypolipidemic effect; hypocholesterolemic effect; linoleic adid; α-linolenic acid; safflower oil; perilla oil; essential fatty acid; vegetable oil; cholesterol; long-term feeding

Introduction

Safflower oil rich in linoleic acid (18:2n-6) and perilla oil rich in α-linolenic acid (18:3n-3) were shown in our laboratory to have different effects on the severity of chronic diseases and on general behavioral patterns in rats and mice. Beneficial effects of perilla oil as compared with safflower oil have been shown in the prevention of tumorigenesis,[1,2] tumor metastases,[3] stroke,[4] thrombosis,[5] allergic hyperreactivity[6] and aging.[7]

Dietary marine oils rich in the n-3 fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have beneficial effects against cardiovascular disorders[8-10] as well as on some chronic diseases.[11] As one of the mechanisms for the prophylactic effects of fish oils on chronic diseases, the hypolipidemic effects of the oils have been noted. However, the comparative hypolipidemic effects of safflower oil and perilla oil have not been directly examined.

In the present study, rats were fed either a high α-linolenate diet or a high linoleate diet for a relatively long time to compare their effects on lipid levels in major tissues. In order to clarify whether the observed hypolipidemic effect is due to changes in the distribution of lipids among tissues, whole body lipids were analyzed from a macroscopic point of view using a smaller animal model, mice, which were fed these diets for 5 months. The results indicated that the high α-linolenate diet had a more potent cholesterol lowering effect than the high linoleate diet.

Experimental Procedures

Animal and Diets Male Sprague-Dawley rats (Shizuoka Laboratory Animals Co., Ltd., Shizuoka, Japan) at 3 weeks of age were fed the test diets for 11 months. A conventional diet (CE-2, Nippon Clea Co., Ltd., Tokyo) was extracted with *n*-hexane to remove endogenous lipids and was then supplemented with 5% vegetable oils and a 2% vitamin mixture.[12] Perilla seed oil from *Perilla frutescens* var. *crispa* (Ohta Oil Co., Ltd., Okazaki, Japan) and safflower seed oil, both prepared for human use, were used. ICR mice were fed a semi-purified diet containing either 10% perilla oil or 10% safflower oil for 5 months after weaning. Animals were kept at 23±2 °C. The diets and water were given *ad libitum*. Diets with peroxide values below 30 meq/kg were served. The fatty acid compositions of the dietary oils are shown in Table I.

Lipid Analysis After being fasted overnight, rats (4 to 6 rats per group) were sacrificed by decapitation, and livers, brains, hearts, skeletal muscles of the high thigh, mesentery depot fats and blood samples were taken. Plasma was obtained by centrifugation (3500 rpm for 10 min) of the blood samples with ethylenediaminetetraacetic acid (EDTA) supplemented as an anticoagulant. Each preparation was kept frozen at −80 °C until lipid analyses. Total lipids were extracted according to the method of Bligh and Dyer.[13] Neutral lipids and phospholipids (PL) were separated by thin layer chromatography (Silica Gel H, E. Merck) with developing solvents of petroleum ether/diethyl ether/acetic acid (80:30:1, v/v) and chloroform/methanol/water (70:30:5, v/v), respectively. Spots were visualized by spraying first with a Rhodamine 6G solution and then with 28% NH_4OH. Lipids were extracted from the corresponding spots twice with chloroform/methanol/3% NH_4OH (6:5:1, v/v) and then once with chloroform/methanol (2:1, v/v). Fatty acids were converted to methyl ethers by treatment with 5% HCl in methanol and then analyzed by gas liquid chromatography (GLC) using a packed column (EGSS-X). Heptadecanoic acid was added as an internal standard. Free cholesterol was quantitated by GLC as trimethylsilyl ether using 5α-cholestane as an internal standard. In the calculation of lipid contents per animal, the amounts of plasma, muscle, and depot fat were considered to be 2.6, 40.0 and 31.1% of the body weight, respectively.

For the estimation of lipids of the whole body, mice (3 mice per group) fasted overnight were killed by decapitation and whole bodies were minced. The total lipids were extracted with chloroform/methanol (2:1, v/v) by the method of Bligh and Dyer.[13] PL and neutral lipids were separated by acetone precipitation.[14] Lipids soluble in acetone at 30 °C were designated neutral lipids. Fatty acids and cholesterol (Cho) were de-

TABLE I. Fatty Acid Compositions of Dietary Oils

Fatty acid[a]	High linoleate oil (Safflower oil)	High α-linolenate oil (Perilla oil)
	(wt% of total fatty acids)	
16:0	8.5	9.2
18:0	2.6	2.2
18:1	13.6	18.3
18:2n-6	71.3	19.7
18:3n-3	1.2	49.9
Others	2.9	0.9

a) Fatty acids are designated by chain length: number of double bonds and the position of the first double bond numbered from the methyl end of the molecule.

termined by GLC.

Statistical Analysis Data are expressed as means ± S. E. and statistical analyses were performed using a Student's *t*-test.

Results

Growth and Tissue Weights of Rats and Mice No significant differences were observed in the growth rates, body weights, or tissue weights (liver, brain and heart) of the two dietary groups (data not shown).

Exp. I. Lipid Contents of Various Tissues of Rats Most Cho in plasma exists in an esterified form (ChoE). The total plasma Cho level was significantly lower (by 28%) in the high α-linolenate (perilla oil) group than in the high linoleate (safflower oil) group (Fig. 1). The total Cho levels in the liver, heart, brain, and muscle were relatively similar between the two dietary groups. By contrast, the depot fat fraction appeared to contain *ca.* 10 times more Cho. Because of a large variation in ChoE content in the depot fat fraction, the difference in the total Cho content was not statistically significant between the two dietary groups (Fig. 1).

Plasma triacylglycerol (TG) levels were significantly lower (by 33%) in the high α-linolenate group than in the high linoleate group (Fig. 2). However, the contribution of plasma TG to the whole body TG pool was minor because muscle and depot fat fractions contained 50-fold and 8000-fold more TG, respectively. Interestingly, the TG level in muscle was significantly higher but that of depot fat was significantly lower in the high α-linolenate group than in the high linoleate group.

The plasma PL level was significantly lower in the high α-linolenate group than in the high linoleate group, but no statistically significant differences were observed in the PL levels of the liver, heart, and brain (Fig. 3). Again, the contribution of plasma PL to the total body PL pool was very small. For example, musle contained 80-fold more PL than plasma.

It should be noted that the TG/PL ratios of muscle showed a 2-fold difference, 0.92 in the high α-linolenate group as compared to 0.45 in the high linoleate group (Figs. 2 and 3).

These results suggest that the Cho lowering effect of the

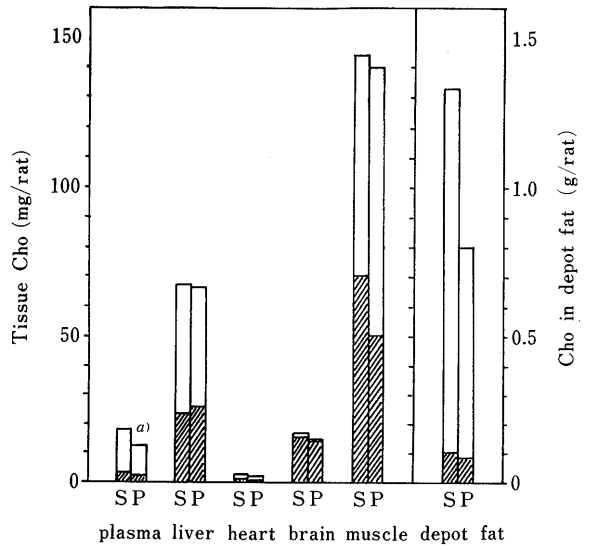

Fig. 1. Effect of High α-Linolenate and High Linoleate Diets on Cho Contents of Rat Tissues

Sprague-Dawley rats were fed a high linoleate (safflower oil) diet (S) or a high α-linolenate (perilla oil) diet (P) for 11 months. Free Cho and ChoE were determined by GLC. Values are means for 3 to 6 rats. *a)* $p<0.05$ in Student's *t*-test. Note the difference in the scales of the ordinate. □, ChoE; ▨, free Cho.

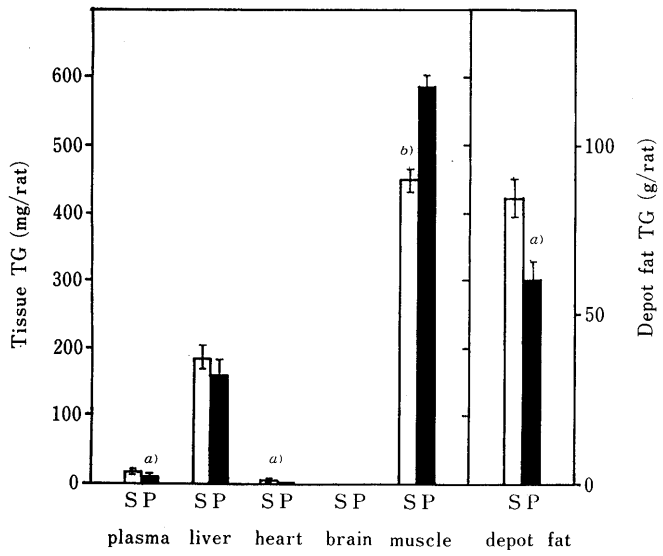

Fig. 2. Effects of High α-Linolenate (P) and High Linoleate (S) Diets on TG Contents of Rat Tissues

Statistically significant differences determined using Student's *t*-test are shown as *a)* $p<0.05$; *b)* $p<0.01$.

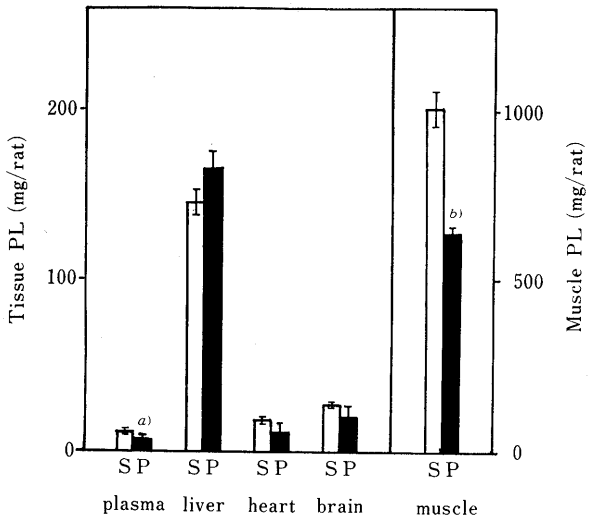

Fig. 3. Effects of High α-Linolenate (P) and High Linoelate (S) Diets on the PL Contents of Rat Tissues

Averages of determinations for 3 to 6 rats ±S.E. are presented. *a)* $p<0.05$; *b)* $p<0.01$.

TABLE II. Effects of High α-Linolenate and High Linoleate Diets on Whole Body Lipid Contents of Mice

Lipids	High linoleate (Safflower oil) group	High α-linolenate (Perilla oil) group
Total lipids (g)	6.1±1.8	5.5± 0.6
PL (g)	0.3±0.0	0.4± 0.0
Neutral lipids (g)	5.5±0.2	4.5± 0.4
Total Cho (mg)	167.6±6.6	120.2± 6.7 ($p<0.01$)
Cho E (mg)	59.1±6.0	44.8± 4.3
Free Cho (mg)	108.4±2.3	75.4±10.9 ($p<0.05$)

Values (g or mg/mouse) are means ± S.E. for three mice. Student's *t*-test was used for the statistical analysis.

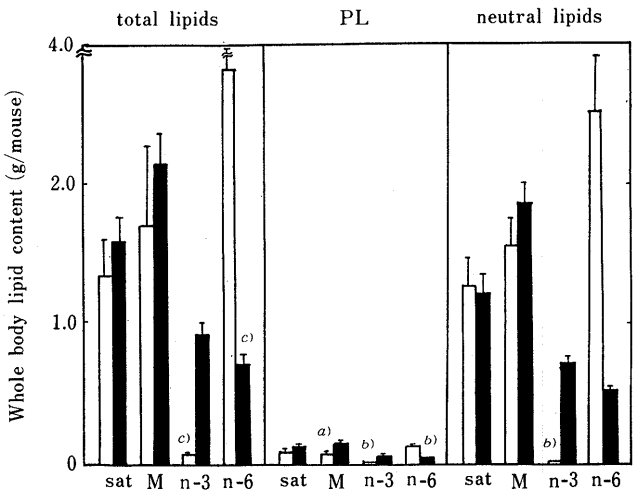

Fig. 4. Effects of High α-Linolenate and High Linoleate Diets on the Levels of Various Types of Fatty Acids in Whole Bodies of Mice

Mice were fed a high linoleate (□) or a high α-linolenate (■) diet for 5 months after weaning. Fatty acids in total lipid, PL and neutral lipid fractions were quantitated by GLC. The amounts of saturated (sat), monounsaturated (M), n-3 fatty acids (n-3) and n-6 fatty acids (n-6) were determined, and the values presented are means ± S.E. for 3 mice in each group. *a)* $p<0.05$; *b)* $p<0.01$; *c)* $p<0.001$.

high α-linolenate diet is greater than that of the high linoleate diet in the whole body of rats; but the TG and PL lowering effects might be due to changes in the distributions of these lipids among the tissues. Furthermore, the samples of muscle and depot fat taken for lipid analyses may not be representative. These uncertainties led us to analyze whole body lipid contents using a smaller animal model, mice.

Exp. II. Lipids of Mouse Whole Body Mice were fed similar diets for 5 months, and the lipid contents of whole bodies were measured (Table II). The contents of total lipids, PL, and neutral lipids, all measured as fatty acids, were not significantly different between the high α-linolenate group and the high linoleate group. However, total Cho (both ChoE and free Cho) was significantly lower (by 28%) in the high α-linolenate group than in the high linoleate group, consistent with the results obtained with rats. Thus, the greater hypocholesterolemic effect of the high α-linolenate diet is apparently not attributable to redistribution of Cho among the tissues; rather, the effect is mirrored in whole body Cho levels.

As shown in Fig. 4, the PL fatty acids comprise a minor fraction and the neutral lipid fatty acids a major fraction of the total fatty acids in whole bodies of mice under our nutritional conditions. The proportion of α-linolenate in the high α-linolenate diet was 50% while that of linoleate in the high linoleate diet was 71%—a 1.4-fold difference. However, relatively much more n-6 fatty acids accumulated in the high linoleate diet group as compared with the n-3 fatty acids accumulating in the high α-linolenate diet group; the difference was 3-fold. Similarly, in the depot fat of rats, 2.5-fold more n-6 was found in the high-linoleate group than n-3 fatty acids found in the same fraction of the high α-linolenate group (data not shown) suggesting preferential catabolism of n-3 fatty acids as compared with n-6 fatty acids by mice. The major n-6 fatty acids of whole bodies of mice fed the high linoleate diet were 18 : 2n-6 (42.9% of the total), 20 : 3n-6 (0.2%), 20 : 4n-6 (1.3%), and 22 : 5n-6 (0.1%). The major n-3 fatty acids of whole bodies of mice fed the high α-linolenate diet were 18 : 3n-3 (15.6%), 20 : 5n-3 (0.3%), and 22 : 5n-3 (0.1%). All other components of n-3 and n-6 fatty acids were below 0.1% of the total (data not shown).

Discussion

Vegetable oils rich in linoleic acid are known to be hypocholesterolemic, but the biochemical mechanisms underlying this effect remain to be clarified. Decreases in plasma Cho levels are often accompanied by the accumulation of Cho in liver,[15-18] *e. g.* the hypocholesterolemic effect of drugs[19] and fish oil supplements,[15] although hepatic Cho accumulation depends on the experimental conditions.[20,21] It is possible that differences in treatment periods with drugs or fish oils induce such apparent discrepancies. In fact, Garg *et al.*[15] observed a slight decrease in plasma Cho by feeding rats safflower oil for 4 weeks as compared with beef tallow, but this decrease was accompanied by increases in hepatic Cho levels. Accordingly, it seems important to evaluate the consequences of relatively long-term administration of drugs or oils.

Here, we found that relatively long-term feeding of a high α-linolenate diet resulted in decreased plasma Cho level as compared with a high linoleate diet. Although minor components other than fatty acids in these oils may have affected the plasma Cho level, these changes occurred without a significant accumulation of Cho in various tissues of rats or in the whole bodies of mice. This observation is in agreement with that of Garg *et al.*[15] who reported that a safflower oil diet shifted Cho from plasma to liver pools but that a linseed oil diet (rich in α-linolenic acid) did not. The n-3 fatty acids may inhibit Cho synthesis, may stimulate Cho catabolism to bile acids and to neutral sterols excreted in feces, or may stimulate the excretion of Cho and its metabolites as dermal lipids. Fish oil supplements have been shown to increase biliary secretion of Cho,[17,22] and affect the activities of acyl-coenzyme A (CoA): Cho acyltransferase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase.[23]

Cho synthesis and TG synthesis are metabolically interrelated *via* acetyl-CoA. Dietary polyunsaturated fatty acids, both linoleate and α-linolenate, but not saturated and monounsaturated fatty acids, are known to suppress fatty acid synthetase. Therefore, under conditions in which significant amounts of dietary polyunsaturates are supplied continuously, excess carbohydrate or protein catabolized to acetyl-CoA may not be easily converted to fatty acids and subsequently to TG. Under such conditions, acetyl-CoA may be utilized preferentially for Cho and ketone body syntheses, leading to elevated levels of Cho. However, α-linolenate appears to be β-oxidized preferentially to linoleate by mitochondria and peroxisomes,[24] resulting in a smaller pool size of n-3 fatty acids as compared with that of linoleate (n-6)[25] (Fig. 4). Accordingly, suppressive effects on fatty acid synthetase may be less with high α-linolenate diets as compared with high linoleate diets; thus, high α-linolenate diets may allow acetyl-CoA to be converted preferentially to saturated and monounsaturated fatty acids through fatty acid synthetase rather than being used for cholesterol synthesis. In fact, a trend toward increased proportions of saturated and monounsaturated fatty acids was observed in the high α-linolenate group as compared

to the high linoleate group (Fig. 4). A decreased suppression of fatty acid synthetase activity may account indirectly for decreased Cho levels in the whole bodies of mice fed the high α-linolenate diet.

Our explanation for the greater Cho lowering effect of α-linolenate as compared with linoleate, is obviously speculative and does not apply to the difference in muscle TG/PL ratios of the two dietary groups. However, nutritionally it is important to note that the Cho lowering effect of α-linolenate is significantly greater than that of linoleate. In other experiments, we have shown[26] that excess linoleate stimulates the incidence or severity of many kinds of chronic diseases including cancer; in contrast, increasing α-linolenate levels in diets is useful in suppressing or preventing such diseases. Currently, people in industrialized countries are ingesting linoleate in amounts averaging almost 10 times more than required (below 1 energy %). The data presented here strengthen our proposal[26] that the intake of linoleic acid should be decreased and that of n-3 fatty acids be increased in order to prevent chronic diseases prevailing in industrialized countries.

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