Effects of Various Polyunsaturated Fatty Acids on Blood Cholesterol and Eicosanoids in Rats

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The hypocholesterolemic efficacy of various polyunsaturated fatty acids was compared in rats given cholesterol-enriched diets with (0.004%) or without indomethacin, the cyclooxygenase inhibitor. Evening primrose oil (EPO, linoleic + γ -linolenic), safflower oil (SFO, linoleic) or soybean oil (SBO, linoleic + α -linolenic) were added to diets at the 10% level. The serum cholesterol level of the EPO group was consistently lower than the other groups and after 3 weeks, it was significantly different from the SFO group without indomethacin and the SBO group with indomethacin. In rats fed EPO, the aorta tended to produce more prostacyclin whereas the concentration of plasma thromboxane B₂ was much lower than in rats fed SFO or SBO. The effects of indomethacin on these eicosanoids were less evident in rats fed EPO. Thus, in addition to the hypocholesterolemic action of γ -linolenic acid (GLA) in preference to linoleic and possibly α -linolenic acid, GLA appears to cultivate an environment suitable for the prevention of cardiovascular disease even in the presence of excess cholesterol in the diet.

Available evidence suggests that the hypocholesterolemic effect of linoleic acid is partly, if not entirely, exerted through its conversion to γ -linolenic acid (GLA), dihomo- γ -linolenic acid (DGLA), or arachidonic acid (AA). Thus, these metabolites have been found to have cholesterol-lowering action much greater than the parent molecule, linoleic acid, both in humans^{1~3)} and experimental animals.^{4~6)} As the two desaturase systems but not chain elongation steps leading from linoleic acid to AA are largely insufficient in man as compared with rats and mice⁷⁾ and as the physiological functions of prostaglandins produced from DGLA are shown to be different from those from AA,⁸⁾ the mechanism by which GLA and DGLA manifest the hypocholesterolemic effect may be different from that of AA. The efficacy of the linoleic acid metabolites as a natural protective agent against coronary heart disease may confer benefits by reducing the consumption of linoleic acid, because linoleic acid and hence some prostaglandins of the 2-series are known to act as potent promoters of colon and breast

tumors.^{9~11)}

Evening primrose oil (EPO) is practically the sole natural source of GLA and is attracting considerable attention as a hypocholesterolemic agent.¹²⁾ However, it is still not apparent how GLA manifests its desirable propensity either by itself or through the conversion to specific prostaglandin(s). We have previously observed that the hypocholesterolemic action of EPO is not attributable to its relatively high phytosterol contents but possibly depends on GLA itself.¹³⁾ In this study, the cholesterol-lowering ability and the levels of several prostaglandins were compared in rats fed high cholesterol diets containing EPO (linoleic + γ -linolenic), safflower oil (SFO, linoleic), or soybean oil (SBO, linoleic + α -linolenic). Because of the possible contribution of prostaglandins to the hypocholesterolemic action of polyunsaturated fatty acids, the effects of an inhibitor of cyclooxygenase, indomethacin, were also studied.

TABLE I. FATTY ACID COMPOSITIONS AND STEROL CONTENTS OF DIETARY FATS

Dietary fats	Fatty acids (%)						Phytosterols (mg/100 g)
	16:0	18:0	18:1	18:2	18:3n3	18:3n6	
Safflower oil	6.8	1.5	13.4	75.1	2.0	—	183
Soybean oil	10.8	3.8	23.3	54.0	7.7	—	321
Evening primrose oil	6.1	1.5	11.7	72.0	—	8.2	4.3

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka) were housed individually in the animal room (20~23°C, lights on 0800 to 2000 hours) and given experimental diets for 23 days. The composition of the diet was in weight percent; casein 20, fat 10, mineral mixture 4, vitamin mixture 1, choline chloride 0.2, cellulose 2, cholesterol 0.5, Na-cholate 0.125, and sucrose to 100. The mineral and vitamin mixtures defined by Harper¹⁴⁾ were purchased from Oriental Yeast Co., Tokyo. There were three groups for dietary fats, EPO, SFO, and SBO and each group was composed of two sub-groups with or without 0.004% indomethacin. The level of indomethacin adopted has been shown to be effective in inhibiting the synthesis of eicosanoid.^{15,16)} EPO was a gift from Eisai Co., Tokyo and phytosterols were largely removed by silicic acid chromatography¹³⁾ to prevent their effects on the serum cholesterol level. SBO and SFO were obtained commercially. Fatty acid compositions of dietary fats are shown in Table I. Food intake and body weight were recorded every other day. At times week apart, the rats were deprived of diets at 0830 hours and a small volume of blood was withdrawn from the tail vein at about 1330~1430 hours for enzymatic measurement of serum cholesterol (Cholesterol C-Test, Wako Pure Chemicals Co., Osaka). On the 24th day, rats fasted overnight (1700~0900 hours) were anesthetized with diethyl ether and blood was collected from the abdominal aorta in a syringe containing 3.8% Na₃-citrate (1 ml for 9 ml blood) and indomethacin (final concentration 10⁻⁴ M), and the plasma was separated by centrifugation. The liver, epididymal adipose tissue, and abdominal aorta were immediately excised.

Lipid analyses. Lipids extracted by a mixture of chloroform-methanol (2:1, v/v) were analyzed for cholesterol, triglyceride, and phospholipid.¹⁷⁾ Liver phosphatidylcholine was separated by thin-layer chromatography¹⁸⁾ and the fatty acid composition was assayed by gas-liquid chromatography on a DEGS column.¹⁹⁾

Measurement of prostaglandins. Blood plasma was acidified to pH 3.0 with 0.5 N HCl and put on a SEP-PAK C₁₈ cartridge (Waters Associates, Milford MA) which had been washed with ethanol and then water. The cartridge

was washed successively with water, 15% ethanol, petroleum ether, and a mixture of petroleum ether-chloroform (65:35, v/v), and eicosanoids were then eluted with diethyl ether. The solvent was removed under a stream of N₂ and the residue dissolved in ethanol.²⁰⁾ The concentration of thromboxane B₂ was measured in this alcoholic solution using a commercial radioimmunoassay kit (New England Nuclear NEK-007, Boston MA). Prostaglandins E₁ and E₂ were determined using RIA kits (SG 6013 and SG 6001, respectively, Seragen Inc., Boston MA). Immediately after blood withdrawal, the abdominal aorta was excised and the adhering fatty tissues were removed. The aorta (about 1.5 cm length) was incubated with gentle shaking in 1 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, at 25°C for 30 min. The pH of the incubation medium was adjusted to 5.0 with 3% formic acid and eicosanoids were extracted twice with ethyl acetate.²¹⁾ The extract was dried under a stream of N₂ and dissolved in ethanol. 6-Keto-PGF_{1α} was measured using a New England Nuclear RIA kit (NEK-008). Prostaglandins were all measured in the range around 50% of the normalized percent bound (B/B₀) for each standard by referring to instruction manuals provided by the suppliers.

Determination of Δ^6 -desaturase. The Δ^6 -desaturase activity was measured on liver microsomes using [1-¹⁴C]linoleic acid (Amersham International plc, Buckinghamshire, England) by the method of Svensson.²²⁾

Statistical analysis. Differences between group means were evaluated by two-way analyses of variance and subsequent *t* test to confirm the interaction of dietary fats and/or indomethacin.²³⁾

RESULTS

Growth parameters

Rats weighing on average of 95 g, 8 to 9 rats per group, consumed 17.9 to 20.1 g/day of diets (average for 23 days) and gained 180 to 203 g of body weight for 23 days. Neither the type of dietary fats nor indomethacin influenced these parameters.

FIG. 1. Effects of Dietary Fats and Indomethacin on Serum Cholesterol Levels.

For experimental details, see MATERIALS AND METHODS. Each point represents mean $\pm$ SE of 8 to 9 rats.

*Significantly different from the corresponding evening primrose oil group at $p < 0.05$.

TABLE II. EFFECTS OF DIETARY FATS AND INDOMETHACIN ON THE PRODUCTION OF PROSTACYCLIN BY ABDOMINAL AORTA AND CONCENTRATIONS OF PLASMA EICOSANOIDS

Dietary fats	Prostacyclin		Thromboxane B ₂		Prostaglandin E ₁		Prostaglandin E ₂	
	Indomethacin		Indomethacin		Indomethacin		Indomethacin	
	None	Added	None	Added	None	Added	None	Added
	(pg/mg aorta) ^a		(pg/ml)		(ng/ml)		(ng/ml)	
Safflower oil	471 $\pm$ 50	381 $\pm$ 109 ^{b,c}	512 $\pm$ 143	382 $\pm$ 63	2.7 $\pm$ 0.4	1.2 $\pm$ 0.2	3.4 $\pm$ 0.4 ^b	3.4 $\pm$ 0.3
Soybean oil	460 $\pm$ 77	284 $\pm$ 46 ^c	514 $\pm$ 62	336 $\pm$ 92	1.3 $\pm$ 0.2	1.6 $\pm$ 0.2	1.6 $\pm$ 0.2 ^c	2.4 $\pm$ 0.3
Evening primrose oil	635 $\pm$ 54	569 $\pm$ 87 ^b	321 $\pm$ 67	379 $\pm$ 76	1.4 $\pm$ 0.2	1.7 $\pm$ 0.3	2.4 $\pm$ 0.3 ^c	2.4 $\pm$ 0.3

Means $\pm$ SE of 8 to 9 rats per group.

^a Measured as 6-keto-PGF_{1 α} .

^{b,c} Values in the same column not sharing common superscript letters are significantly different at $p < 0.05$.

Serum cholesterol levels

As shown in Fig. 1, in all groups of rats with or without indomethacin, serum cholesterol levels were initially high and decreased gradually. In general, cholesterol levels were lower in the EPO group than in the SFO or SBO groups, and the difference became statistically significant after 3 weeks; in rats fed diets free of indomethacin EPO showed significantly lower serum cholesterol than those fed SFO, and in rats fed diets containing indomethacin the difference between the EPO and SBO

groups was significant.

Liver lipid levels

Concentrations of liver cholesterol and phospholipid were comparable among the different groups. In contrast, liver triglyceride tended to be higher in rats fed EPO than in those fed SFO or SBO, and the difference observed with diets containing indomethacin was statistically significant, $p < 0.05$ (78.2 ± 9.6 , 49.0 ± 6.9 , and 49.7 ± 7.3 mg/g (mean $\pm$ SE) for the EPO, SFO, and SBO groups,

and the corresponding values for the indomethacin-free groups were 67.3 ± 7.4 , 49.3 ± 4.4 , and 58.3 ± 8.3 mg/g, respectively).

Concentration of eicosanoids

The data for eicosanoids are shown in Table II. In rats fed diets free of indomethacin, the production by aorta of PGI_2 , measured as 6-keto- $\text{PGF}_{1\alpha}$, tended to be greater in the EPO group. In contrast, the level of plasma TXB_2 was somewhat lower in the EPO than in the SFO or SBO groups. Concentrations of plasma PGE_1 and PGE_2 were considerably higher in rats fed SFO than in those fed SBO or EPO, the difference in PGE_2 being significant.

Indomethacin caused a moderate reduction of the aortic production of PGI_2 in the SFO and SBO but not EPO groups. Consequently, the difference was magnified and became significant between the EPO and SBO groups. The plasma level of TXB_2 was also decreased by this inhibitor except for the EPO group and hence the difference among the groups disappeared. The effects of indomethacin on the level of PGE_1 and PGE_2

were variable and the reduction was observed only in the PGE_1 level of the SFO group, while the PGE_2 level, though the difference was not significant, rather tended to increase in the SBO group. Dietary fat-dependent differences in concentrations of plasma PGE_1 and PGE_2 were not seen when indomethacin was fed.

TABLE III. EFFECTS OF DIETARY FATS AND INDOMETHACIN ON ACTIVITY OF Δ^6 -DESATURASE OF LIVER MICROSOMES

Dietary fats	Δ^6 -Desaturase activity	
	Indomethacin	
	None	Added
	(pmol/min/mg protein)	
Safflower oil	44.9 ± 3.7^a	59.1 ± 5.5^a
Soybean oil	69.2 ± 9.3^b	82.8 ± 7.8^b
Evening primrose oil	49.2 ± 3.6^a	52.7 ± 5.8^a

Means $\pm$ SE of 8 to 9 rats per group.

^{a, b} Values in the same column not sharing common superscript letters are significantly different at $p < 0.05$.

TABLE IV. EFFECTS OF DIETARY FATS AND INDOMETHACIN ON FATTY ACID COMPOSITIONS OF LIVER PHOSPHATIDYLCHOLINE AND ADIPOSE TISSUE TRIGLYCERIDE

Dietary fats	Indomethacin	Fatty acids (%)									
		16:0	16:1	18:0	18:1	18:2	18:3n3	18:3n6	20:3n6	20:4n6	22:6n3
Liver phosphatidylcholine											
Safflower oil	None	20.7	1.8	13.1	10.4 ^a	22.6 ^a	0.5	—	2.9 ^a	21.3 ^a	1.4
	Added	20.3	1.4	13.6	10.1 ^a	23.1 ^a	0.5	—	3.1 ^a	21.1 ^a	1.4
Soybean oil	None	20.3	1.8	13.5	11.8 ^b	22.4 ^a	0.7	—	3.4 ^a	16.7 ^b	3.1
	Added	21.3	1.6	13.1	12.4 ^b	21.7 ^a	0.7	—	3.6 ^a	17.9 ^b	3.5
Evening primrose oil	None	21.6	1.2	12.5	9.5 ^a	18.6 ^b	—	0.4	5.3 ^b	24.4 ^a	1.1
	Added	21.6	1.7	12.5	9.3 ^a	19.1 ^b	—	0.5	5.9 ^b	23.1 ^a	0.9
Adipose tissue triglyceride											
Safflower oil	None	14.0	4.2	2.8	20.1 ^a	52.0 ^a	1.0 ^a	—	0.5 ^a	1.0	0.2
	Added	15.0	4.3	2.5	19.8 ^a	52.7 ^a	1.0 ^a	—	0.4 ^a	1.0	0.1
Soybean oil	None	15.1	4.8	2.8	27.8 ^b	39.1 ^b	5.0 ^b	—	0.5 ^a	1.1	0.4
	Added	15.8	4.8	2.8	27.9 ^b	39.8 ^b	4.9 ^b	—	0.3 ^a	0.8	0.2
Evening primrose oil	None	13.7	4.1	2.2	17.2 ^a	50.9 ^a	—	3.6	1.8 ^b	2.0	0.2
	Added	13.2	3.7	2.2	17.2 ^a	51.9 ^a	—	3.7	1.8 ^b	1.8	0.2

Means of 8 to 9 rats per group.

^{a, b} Values in the same column not sharing common superscript letters are significantly different at $p < 0.05$.

Δ^6 -Desaturase activity

As shown in Table III, the Δ^6 -desaturase specific activity in the liver microsomes was significantly higher in rats fed SBO than in those fed EPO or SFO regardless of the presence or absence of indomethacin in the diet.

Fatty acid compositions of tissue lipids

The data shown in Table IV indicated that liver phosphatidylcholine from rats fed EPO contained more DGLA and AA and less linoleic acid than those fed SFO or SBO, thus the ratio of (20:3+20:4)/18:2 was considerably higher in the EPO group. The SBO group contained less AA than the SFO group, and the ratio was somewhat low. Indomethacin had no apparent effects on the fatty acid patterns.

Fatty acid compositions of adipose tissue lipids reflected those of dietary fats. The extent of deposition of α -linolenic acid in the adipose tissue appeared to be more greater than that of GLA. The percentage of DGLA and AA tended to be higher in the EPO group.

DISCUSSION

In our previous study where the phytosterol content of dietary fat was made comparable, the hypocholesterolemic effect of EPO could be ascribed to GLA exclusively in this oil.¹³⁾ For further confirmation of the hypocholesterolemic action of GLA in EPO, EPO essentially free from phytosterols was used currently. This study, in agreement with experiments done under different conditions,^{4~6)} showed a hypocholesterolemic effect of GLA in preference to linoleic acid and presumably α -linolenic acid, although the effect seemed somewhat more moderate than the results observed elsewhere.¹³⁾ It seems that the cholesterol-lowering action of linoleate can be magnified partly through its conversion to GLA and/or further to AA *via* DGLA.^{1,24)} It is also probable that GLA lowers serum cholesterol faster than does linoleic acid, since the rate of the reduction of cholesterol levels during 1 and 2 weeks was considerably greater in the EPO

than in the SFO and SBO groups.

The possibility also exists that a specific prostaglandin of the monounsaturated series (the 1-series PGs), rather than the 2-series PGs, contributes to the cholesterol-lowering action of GLA. Indeed, liver phosphatidylcholine from rats fed EPO contained, in addition to AA, almost twice as much DGLA than in those fed SFO, even though the rat can convert linoleic acid to AA efficiently.⁷⁾ It thus seems likely that when GLA is fed to man, relatively more DGLA may be available since the Δ^5 -desaturase activity is low in this species.^{7,25)} However, these assumptions could not be sustained from our data, since the level of plasma PGE₁, one of the possible candidates for the beneficial effect of GLA, was not higher in the EPO group than in the SFO group. It is, however, plausible that the plasma concentration of PGE₁ does not necessarily reflect its effect on cholesterol dynamics due to its rapid degradation.

Alternatively, GLA stimulated the aortic production of PGI₂ which may be a potent regulator of cholesterol metabolism.^{26,27)} In addition, the suppressive effect of indomethacin on PGI₂ production was much lower in the EPO group, suggesting that GLA cancels the inhibitory action of this inhibitor.

In addition to the beneficial hypocholesterolemic effect, GLA caused a reduction of the concentration of plasma TXB₂. From the diverse effects of TXB₂ and PGI₂ in blood clotting, it seems that EPO, like eicosapentaenoic acid,²⁸⁾ plays a role in the prevention of cardiovascular disease, insofar as the effect of GLA on these eicosanoids may not simply be the reflection of the reduced serum cholesterol levels. Again, the effect of indomethacin on the plasma TXB₂ was seen only in rats fed SFO or SBO (Table II). The effects of cyclooxygenase inhibitor on plasma concentrations of PGE₁ and PGE₂ were variable. There is, however, no preferential reason to rule out the possibility that the effect of indomethacin becomes marginal or existing concentrations of prostaglandins already represent the nadir levels as influenced by dietary chole-

terol. Thus, a similar type of studies using a cholesterol-free diet is required before making definite conclusions.

The specific activity of Δ^6 -desaturase in hepatic microsomes was significantly higher in rats fed SBO than in any other groups. The result is in contrast with the observation that α -linolenic acid is a more preferable substrate for the Δ^6 -desaturation than linoleic acid²⁵⁾ and it competes with the desaturation of linoleic acid and vice versa.²⁹⁾ As dietary cholesterol interferes with Δ^6 -desaturation,^{6,25,29)} the complex interplay of these dietary components should also be taken into account. In fact, the fatty acid composition data shows no preferable desaturation of linoleic acid in the SBO group (Table IV) as has been observed elsewhere.^{30,31)} On the while, the fatty acid profile of liver phosphatidylcholine suggested a greater availability of the eicosanoid substrates in rats fed EPO than in those fed SFO or SBO.

In conclusion, GLA as evening primrose oil was effective in lowering serum cholesterol of rats. It seems that GLA in relation to linoleic acid predictably serves as a source of the preferential substrate for eicosanoids even when the desaturation of polyunsaturated fatty acids is depressed by dietary cholesterol.

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