Rapid Paper

Inhibition of Platelet Aggregation
by Dietary Linseed OilMasataka ISHINAGA, Masanori KAKUTA,
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Male Sprague-Dawley rats were fed diets containing linseed oil, safflower oil, or cacao butter for 27 weeks. The collagen-induced aggregation of washed platelets decreased only in rats fed linseed oil. Dietary oils did not influence the phospholipid composition of platelets, but did influence the fatty acid composition of platelet phospholipids. Compared with the other diets, the linseed oil diet markedly decreased the arachidonic acid content in platelet phosphatidylcholine and accumulated the eicosapentaenoic acid in all phospholipids. A similar change was not found in platelets from rats fed the other diets. These results indicate that the feeding of linseed oil, which has a high linolenic acid (a precursor of eicosapentaenoic acid) content, alters the fatty acid composition of platelet phospholipids similar to the feeding of fish oils, and thereby reduces the aggregation tendency of platelets.

The relationship between platelet function and dietary oils has been extensively investigated on the formation of thrombi. In general, saturated oils enhance platelet aggregation but polyunsaturated oils reduce it.¹⁻⁵) Eicosapentaenoic acid (20:5 ω 3) from certain fish oil markedly reduces the incidence of cardiovascular disease in Greenland Eskimos by reducing the platelet aggregation tendency.^{6,7}) This fatty acid decreases thromboxane A₂ production, which causes platelet aggregation, by inhibiting the cyclooxygenase reaction competitively with the natural substrate, arachidonic acid (20:4 ω 6).⁸) These fatty acids are supplied from the platelet membrane phospholipids. However, it is still uncertain how the fatty acids of platelet membrane phospholipids modified by dietary oils can affect the platelet aggregation.

From these points of view, we have investigated the effects of dietary oils on platelet

aggregation by using washed platelets to correlate this aggregation to changes in platelet phospholipids. The mechanism of arachidonic acid release from the platelet membrane is discussed on the basis of precise analysis of the fatty acid composition of platelet phospholipids modified by dietary oils.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats (5 weeks old) were used. The basal diet was prepared with the following weight percentage composition: vitamin-free casein 25, corn starch 38, sucrose 21, salt mixture 6, vitamin mixture 2, and cellulose powder 8. Linseed oil (LSO), safflower oil (SO), or cacao butter (CB) was supplemented to account for 20% (w/w). The fatty acid composition of these oils was determined (Table I). To 1 kg of the LSO- or SO-diet, 250 mg of tocopherol and 500 mg of butylated hydroxytoluene (BHT) were added. The low fat diet (LF) contained 10% of sucrose and 10% of corn starch in place of 20% of oil. The diets were fed *ad libitum* to 4 groups of 15 rats each for 27 weeks.

Preparation of washed platelets. The animals were anesthetized with ether, and blood was collected from the abdominal aorta in the presence of acid citrate dextrose 10% (v/v). Blood (11~13 ml) from one or more rats was centrifuged at 800 $\times g$ for 10 min, after which the supernatant was recentrifuged at 100 $\times g$ for 10 min. The resulting supernatant, platelet-rich plasma (PRP), was centrifuged at 1000 $\times g$ for 10 min and the platelets derived were washed with Ca²⁺-free Tyrode solution containing 0.25% of gelatin, pH 6.6, and resuspended in the Tyrode solution, pH 7.4.

TABLE I. FATTY ACID COMPOSITION
OF DIETARY OILS

Fatty acid	Linseed oil	Safflower oil % (w/w)	Cacao butter
16:0 ^a	6.3	6.8	26.4
18:0	3.5	2.3	31.0
18:1 (ω 9)	17.2	12.8	37.7
18:2 (ω 6)	15.7	77.9	3.8
18:3 (ω 3)	57.3	T	T

T, trace amounts (less than 1%).

^a The first number gives the number of carbon atoms in the fatty acid molecule. The second indicates the number of double bonds. ω indicates the position of the first double bond in relation to the methyl end of the fatty acid molecule.

Lipid analysis. The platelet suspension from 3 or 4 rats was added to cold chloroform/methanol (1:2, v/v) containing 0.001% of BHT and 0.1 mM tocopherol, and lipids from the platelets were extracted by the method of Arthur and Sheltawy,⁹⁾ in which the water was replaced with 2 M KCl and 0.1 M EDTA. The separation and determination of the phospholipids have been described previously.¹⁰⁾ Phosphatidylethanolamine (PE) was methylated with boron trifluoride/methanol and the other phospholipids with 0.5 N sodium methoxide/methanol (Applied Science Lab. Inc., Penn., USA). The analysis of the fatty acid methyl esters has been described previously.¹⁰⁾ The identification was accomplished by GLC-mass spectrometry. All developing solvents contained 0.01% of BHT.

Other analysis. Statistical analysis was performed with Student's *t* test. Phosphorus was determined according to the method of Bartlett¹¹⁾ and Keenan *et al.*¹²⁾

RESULTS AND DISCUSSION

Aggregation of washed platelets

Many factors such as collagen, ADP, thrombin, and platelet activating factor, are concerned in platelet aggregation during hemostasis. Collagen was used as an aggregating reagent, since it is thought to be the initial, and therefore most important, stimulus. The tendency for aggregation of PRP of rats fed oils was significantly lower than that of rats fed the LF-diet ($p < 0.01$, data not shown). The aggregation tendency of washed platelets was similar among rats fed the LF-, SO-, and CB-diets, whereas it was significantly reduced in rats fed the LSO-diet (about 38%, $p < 0.01$) (Fig. 1). This indicates that only in the case of the LSO-diet is alteration in the platelet aggregation ability attributable to the modification of platelet phospholipids. Hornstra and Haddeman have shown that PRP aggregation of rats fed hydrogenated coconut oil is lower than that of rats fed linseed or sunflower oils.¹³⁾ However, experiments using washed platelets were not carried out in this case.

Effects of dietary oils on platelet phospholipids

The phospholipid composition of the platelets did not change after feeding of various oils for 27 weeks (Table II). The fatty acid composition of the various platelet phospholipid classes, however, was differentially affected by

FIG. 1. Collagen-induced Aggregation of Washed Platelets.

Platelet number was adjusted to $2 \times 10^8/0.2$ ml. One μ g of collagen (Horn-Chemie, Munchen, West Germany), was used as an aggregating reagent. The extent of aggregation was measured as the maximum transmission of light (in percent) by using Tyrode solution as the control in a platelet aggregation tracer (Model PAT-2M, Niko Bioscience Co. Ltd., Japan). The different asterisks are significantly different ($p < 0.01$).

dietary oils (Table III). The major changes in fatty acid composition by LSO-diet are important to explain its inhibitory effect on platelet aggregation. The LSO-diet accumulated 18:2 ω 6 but reduced 20:4 ω 6 levels in all phospholipids except phosphatidylinositol (PI). The reduction of 20:4 ω 6 in PC was notable. In addition 20:5 ω 3 was significantly accumulated in all phospholipids. Sanders *et al.* have also observed the accumulation of 20:5 ω 3 in total phospholipids of human platelets by a dietary supplement of LSO.¹⁴⁾ This suggests that the conversion of 18:3 ω 3 to 20:5 ω 3 precedes that of 18:2 ω 6 to 20:4 ω 6 according to a higher affinity of the ω 3 family to desaturation and elongation enzymes.

In a cyclooxygenase reaction, the substrate 20:4 ω 6 is supplied from phospholipid in the platelet membranes. Two alternative pathways are proposed in this mechanism: 1) phospholipase A₂ directly releases 20:4 ω 6 from PC and/or PE^{15,16)}; 2) phospholipase C specific for PI initially makes diglyceride from PI and 20:4 ω 6 is then released from diglyceride^{17,18)} or phosphatidic acid.¹⁹⁾ Inhibition of the cyclooxygenase reaction by 20:5 ω 3 is competitive with 20:4 ω 6, and its affinity to the en-

zyme seems to be equal to that of 20:4 ω 6.⁸⁾ In addition, the product thromboxane A₂ has little effect on platelet aggregation.²⁰⁾ Therefore, the inhibition of platelet aggregation by 20:5 ω 3 may be attributed to dilution of the effective free 20:4 ω 6 by 20:5 ω 3. We can calculate the dilution effect by 20:5 ω 3 (20:5 ω 3/20:4 ω 6 + 20:5 ω 3) in individual phospholipids from Table III; 0.38, 0.28, and

0.12 for PC, PE, and PI, respectively. The inhibition of platelet aggregation (about 38%) observed in a LSO-diet suggests that PC and/or PE are the principal donors of 20:4 ω 6, since the 20:5 ω 3 content in PI is too small to explain the degree of inhibition. Recently, Hornstra *et al.* or Siess *et al.* have indicated that 20:5 ω 3 inhibited the formation of thromboxan A₂ from 20:4 ω 6 in the platelets of rats

TABLE II. PHOSPHOLIPID COMPOSITION OF PLATELETS

Values are means $\pm$ SE of 4 samples composed of 3 or 4 rats.

Phospholipid	Dietary oils			
	Low fat	Linseed oil	Safflower oil % (mol)	Cacao butter
Lysophosphatidylcholine	2.8 $\pm$ 0.6	3.7 $\pm$ 0.5	2.8 $\pm$ 0.2	2.7 $\pm$ 0.4
Sphingomyelin	9.6 $\pm$ 1.0	11.1 $\pm$ 0.3	10.6 $\pm$ 0.6	11.7 $\pm$ 0.7
Phosphatidylcholine	43.1 $\pm$ 1.9	39.2 $\pm$ 0.6	39.2 $\pm$ 1.2	39.8 $\pm$ 0.9
Phosphatidylserine	11.4 $\pm$ 0.8	12.5 $\pm$ 0.5	12.4 $\pm$ 1.1	11.5 $\pm$ 0.9
Phosphatidylinositol	2.3 $\pm$ 0.4	2.1 $\pm$ 0.2	2.5 $\pm$ 0.4	2.4 $\pm$ 0.3
Phosphatidylethanolamine	29.6 $\pm$ 1.7	30.9 $\pm$ 0.4	31.7 $\pm$ 0.6	31.4 $\pm$ 1.2

TABLE III-1. FATTY ACID COMPOSITION OF PLATELET PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

Values are means $\pm$ SE of 4 samples composed of 3 or 4 rats. Values with a different superscript letter are significantly different ($p < 0.05$).

Fatty acid	Low fat		Linseed oil		Safflower oil		Cacao butter	
	PC % (w/w)	PE % (w/w)	PC % (w/w)	PE % (w/w)	PC % (w/w)	PE % (w/w)	PC % (w/w)	PE % (w/w)
16:0*	—	6.9 $\pm$ 0.1	—	6.6 $\pm$ 0.2	—	5.7 $\pm$ 0.2	—	6.0 $\pm$ 0.1
16:0	57.8 $\pm$ 0.6	5.3 $\pm$ 0.1	56.1 $\pm$ 1.1	5.1 $\pm$ 0.1	56.1 $\pm$ 2.1	4.5 $\pm$ 0.1	53.2 $\pm$ 0.2	3.6 $\pm$ 0.1
16:1	4.4 $\pm$ 0.1	—	1.4 $\pm$ 0.3	—	T	—	T	—
18:0*	—	7.5 $\pm$ 0.1	—	9.1 $\pm$ 0.4	—	7.6 $\pm$ 0.3	—	11.4 $\pm$ 0.0
18:0	6.5 $\pm$ 0.3	13.8 $\pm$ 0.7	8.9 $\pm$ 0.5	14.5 $\pm$ 0.7	9.0 $\pm$ 0.3	12.4 $\pm$ 0.3	10.7 $\pm$ 0.2	12.7 $\pm$ 0.3
18:1*	—	7.5 $\pm$ 0.1	—	4.3 $\pm$ 0.1	—	3.0 $\pm$ 0.1	—	3.5 $\pm$ 0.1
18:1 (ω 9)	15.8 $\pm$ 0.2	7.2 $\pm$ 0.2	7.6 $\pm$ 0.1	5.3 $\pm$ 0.1	5.8 $\pm$ 0.3	5.6 $\pm$ 0.1	12.3 $\pm$ 0.4	4.2 $\pm$ 0.3
18:2 (ω 6)	2.0 $\pm$ 0.1	—	14.6 $\pm$ 0.3	5.7 $\pm$ 0.1	13.1 $\pm$ 0.4	3.9 $\pm$ 0.1	3.2 $\pm$ 0.1	T
18:3 (ω 3)	—	—	2.3 $\pm$ 0.1	1.3 $\pm$ 0.1	—	—	—	—
20:2	—	—	—	—	2.3 $\pm$ 0.2	—	—	—
20:3 (ω 9)	3.6 $\pm$ 0.1	11.4 $\pm$ 0.2	—	—	—	—	1.1 $\pm$ 0.0	2.9 $\pm$ 0.3
20:3 (ω 6)	—	—	—	—	T	2.5 $\pm$ 1.4	T	3.4 $\pm$ 1.3
20:4 (ω 6)	8.6 $\pm$ 0.4 ^a	28.6 $\pm$ 0.5 ^b	4.8 $\pm$ 0.4 ^c	25.7 $\pm$ 0.7 ^b	11.9 $\pm$ 0.8 ^d	34.8 $\pm$ 0.8 ^c	15.9 $\pm$ 0.2 ^d	37.2 $\pm$ 0.0 ^e
20:5 (ω 3)	—	—	2.9 $\pm$ 0.3	10.1 $\pm$ 0.8	—	—	—	—
22:3	—	6.1 $\pm$ 0.3	—	T	—	1.2 $\pm$ 0.4	—	3.0 $\pm$ 0.1
22:4	—	3.5 $\pm$ 0.1	—	2.2 $\pm$ 0.2	—	15.8 $\pm$ 0.1	—	10.4 $\pm$ 0.3
22:5	T	T	1.3 $\pm$ 0.1	8.8 $\pm$ 0.6	—	T	—	1.7 $\pm$ 0.3
22:6	—	T	—	1.2 $\pm$ 0.1	—	1.0 $\pm$ 0.0	—	T

* Dimethylacetal derivatives of aldehydes.

T, trace amounts (less than 1%); PC, phosphatidylcholine; PE, phosphatidylethanolamine.

TABLE III-2. FATTY ACID COMPOSITION OF PLATELET PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLSELINE

Superscript letters: see footnote to Table III-1.

Fatty acid	Low fat		Linseed oil		Safflower oil		Cacao butter	
	PI % (w/w)	PS % (w/w)	PI % (w/w)	PS % (w/w)	PI % (w/w)	PS % (w/w)	PI % (w/w)	PS % (w/w)
16:0	4.0±0.7	1.3±0.1	3.4±0.4	1.0±0.1	3.1±0.5	1.0±0.2	3.0±0.1	1.0±0.0
16:1	1.0±0.1	1.0±0.2	T	T	1.4±0.0	T	2.3±0.2	1.0±0.0
18:0	28.8±1.1	33.4±1.3	33.8±1.0	35.6±0.8	33.0±0.6	35.3±0.6	36.5±0.6	35.0±0.5
18:1 (ω 9)	6.9±0.7	13.0±0.2	5.6±0.2	7.7±0.2	4.2±0.2	5.0±0.2	7.2±0.2	9.1±0.6
18:2 (ω 6)	1.1±0.0	—	1.7±0.2	11.0±0.8	3.3±0.3	5.8±0.4	1.0±0.1	3.1±0.2
18:3 (ω 3)	—	—	—	1.1±0.2	—	1.0±0.0	—	—
20:0	—	2.2±0.1	—	—	—	—	—	—
20:1	1.6±0.6	1.9±0.4	—	—	—	—	—	1.4±0.0
20:2	—	—	—	—	1.0±0.1	3.6±0.0	—	—
20:3 (ω 9)	15.0±0.2	11.9±0.9	—	—	—	—	—	3.4±0.4
20:3 (ω 6)	—	—	2.1±0.4	3.0±0.1	1.5±0.3	2.0±0.1	3.2±0.4	2.0±0.1
20:4 (ω 6)	34.3±1.3 ^a	26.4±0.2 ^b	42.2±1.3 ^c	26.7±1.0 ^b	51.0±1.5 ^d	40.1±0.3 ^e	42.7±1.5 ^e	35.9±0.7 ^a
20:5 (ω 3)	—	—	5.9±0.6	3.4±0.3	—	—	—	—
22:0	—	—	—	2.1±0.1	—	—	—	—
22:3	7.1±0.1	7.4±1.0	T	T	2.0±0.3	T	2.8±0.4	3.8±0.6
22:4	1.9±0.4	1.5±0.3	3.0±0.6	—	3.0±0.2	5.5±0.6	1.8±0.1	2.9±0.1
22:5	—	—	—	6.2±0.3	—	T	—	1.0±0.1
24:0	—	—	—	1.6±0.1	—	—	—	—

PI, phosphatidylinositol; PS, phosphatidylserine.

fed cod liver oil, or in the platelets of men taking mackerel diet.^{21,22} However, the fatty acid composition of individual phospholipids remains unknown.

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