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Effect of EPA and DHA Containing Glycerophospholipid Molecular Species on the Fluidity of Erythrocyte Cell Membranes

Masahiro Nojima, Masashi Hosokawa, Koretaro Takahashi, and Mutsuo Hatano

Laboratory of Food Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041, Japan

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Fluorescence depolarization of erythrocytes was measured to evaluate the increase in fluidity of cell membrane due to treatment with soy phospholipids, hydrogenated soy phospholipids, icosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) containing phospholipids. When the erythrocytes were treated with EPA or DHA containing phosphatidylethanolamine or phosphatidylserine, the least fluorescence polarization was observed, followed by EPA or DHA containing phosphatidylcholine. All of the hydrogenated phospholipids increased fluorescence depolarization. 2,4,6-Trinitrobenzenesulfonic acid fluorescence quenching proved that the phospholipids used for erythrocytes treatment are incorporated mainly into the inner layer of their cell membrane.

Key words: EPA, DHA, phospholipid, erythrocytes, molecular species, polyunsaturated fatty acids

The therapeutic benefits of highly unsaturated fatty acids (HUFA) are increased when in the form of phospholipids (PL).[1] HUFA containing PL (HUFA-PL) is also currently receiving considerable attention because of their novel physiological functions. Yazawa and his coworkers[2,3] showed that HUFA-PL decreases the amount of adipose tissue. Suzuki *et al.*[4] and Kohno *et al.*[*1] showed that HUFA-PL have certain anticancer effects with retinoid. Some of these physiological functions of HUFA-PL might be extended because HUFA-PL increases the fluidity of the cell membrane by incorporation, thus making the receptors more susceptible.

The present study evaluates the effect of HUFA-PL treatment on cell membrane fluidity. Fluorescence depolarization of erythrocytes was measured, since erythrocytes are easy to handle.

Materials and Methods

Materials

Soy phosphatidylcholine (PC) of 95% purity was obtained from Avanti Polar-Lipids Inc., (Alabaster, AL) and 5% Rh-Carbon from Kawaken Fine Chemical Co., Ltd. (Tokyo, Japan). Lysophosphatidylcholine (Lyso PC) and porcine pancreatic phospholipase A_2 (protein content 38.8%) were donated by Kyowa Hakko Kogyo Ltd. (Tokyo, Japan), and lipozyme IM-60 by Novo Nordisk Bioindustries Inc. Phospholipase D was supplied by Toyo Jozo Co., Ltd. Icosapentaenoic acid (EPA, purity 90%) and docosahexaenoic acid (DHA, purity 88%) were supplied by Nippon Chemical Feed Ltd. (Hakodate, Japan). All other chemicals and solvents used were reagent grade.

Preparation of Hydrogenated and Highly Unsaturated Fatty Acid Containing Phospholipids

The fatty acid composition of substrate lipids is shown in Table 1. Soy PC of 95% purity was used as a control for the depolarization study. This soy PC was also used as a source for transphosphatidylation and hydrogenation in order to obtain various kinds of PL molecular species. Hydrogenation was achieved by blowing hydrogen gas directly into absolute ethanol solution under the catalytic reaction of 5% Rh-Carbon. Reaction was carried out for approximately 2 h under ambient conditions. Then, chloroform was added till the saturated lipids dissolved, and the solution was filtered through filter paper, finally by 0.45 μm pore size filter in order to remove the catalyst. PC which contains HUFA at position *sn*-2 (HUFA-PC) was prepared by the method of Hosokawa *et al.*[5,6] In a typical synthesis, 1100 mg of lyso PC and 1800 mg of free HUFA were dispersed into 54 g of glycerol. The enzyme solution contained 120 mg/ml of phospholipase A_2, 6 mM $CaCl_2$ and 200 mM Tris–HCl (pH 8.0). Synthetic reactions were initiated by adding 5 ml of this enzyme-buffer solution to the substrate suspensions. The temperature of the reactions were controlled at 25°C under argon atmosphere, and stirring was provided by teflon-coated stir bars. Incubations were teriminated by the addition of chloroform–methanol–water (10:5:3, v/v) after 48 h reaction, and HUFA-PCs were recovered from the chloroform layer. HUFA-PCs were purified by a silicic acid column using chloroform–methanol (4:1, v/v) in order to remove FFA, followed by 1:1, v/v, and by 2:3, v/v as dissolvents. Substitution of the fatty acid in position *sn*-1 of PC was done as follows. One gram of soy PC and 6.0 g of EPA were dissolved in 20 ml of *n*-hexane. Reaction was initiated by adding 3.0 g of lipozyme IM-60 to this solution. Incubation was continued for 48 h at 40°C. PC was recovered and purified in the same manner as above. Transphosphatidylation was carried out by the method of Juneja *et al.*[7,8] with slight modification as follows. Fifty mg

Table 1. Fatty acid composition of substrate lipids

	LPC	90% EPA	88% DHA	Soy PC
Sat.				
16:0	24.5	1.0	—	13.3
18:0	6.5	—	0.2	4.1
Mono.				
18:1 n-9	11.1	—	—	10.7
Poly.				
18:2 n-6	52.3	1.7	0.3	65.7
18:3 n-3	5.1	1.4	—	6.2
20:5 n-3	—	90.9	1.5	—
22:5 n-3	—	—	3.5	—
22:6 n-3	—	0.4	88.2	—

Abbreviations: LPC, lysophosphatidylcholine; 90% EPA, 90% purity icosapentaenoic acid; 88% DHA, 88% purity docosahexaenoic acid; Soy PC, soy phosphatidylcholine.

*1 H. Kohno, T. Ota, M. Maeda, M. Tanino, N. Yamaguchi, and S. Odashima: Effects of MIA phosphatidyl choline on differentiation and metastasis. *Proceedings of the Japanese Cancer Association*, **51**, 398 (1992).

of soy PC or HUFA-PC was dissolved in 2.2 m*l* of ethylacetate. Then, 200 mM acetate buffer (pH 5.6) containing 87.8 mg ethanolamine salt, 11.9 mg bovine serum albumin, and 80 units of phospholipase D from *Streptomyces* sp. was added to initiate the reaction. Stirring was conducted at 1,200 rpm and 30°C. Incubation was terminated by adding chloroform–methanol–water (10 : 5 : 3, v/v) after 4 h of reaction, and the recovered phosphatidylethanolamine (PE) was purified by a preparative TLC using chloroform–methanol–water (65 : 25 : 4, v/v) as a dissolvent. To prepare phosphatidylserine (PS) by transphosphatidylation, 1.7 m*l* of ethylacetate containing 50 mg of PC, 0.8 m*l* of 200 mM acetate buffer (pH 5.6), and 250 mg of L-serine were used, and 5 mg of $CaCl_2$ was added to this buffer. Recovery and purification of PS were done in the same manner as PE. Lysophospholipids were prepared as follows. Fifty mg of phospholipid was dissolved in 50 m*l* ethyl ether–methanol (98 : 2, v/v). Then, 5 m*l* of 200 mM Tris–HCl buffer (pH 8.0) containing 140 unit/mg PC of porcine pancreatic phospholipase A_2 were added while vigorously shaking for 30 s. Incubation was carried out for 12 h at 37°C at a stroke speed of 75 min^{-1}. Reaction was terminated by rinsing the ethyl ether layer with water three times. The recovered lysophospholipid obtained by evaporation of the ethyl ether layer was purified in the same manner as PE or PS. HUFA-PC from a natural source was prepared from total lipid of chum salmon roe, which was extracted according to the method of Bligh and Dyer[9] with minor modification. The cold acetone (−20°C) insoluble fraction and cold ethanol (−20°C) soluble fraction were collected from this total lipid, and the recovered crude HUFA-PC was purified by silicic acid column chromatography using ethyl ether–ethanol (1 : 1, v/v), ethanol–methanol (9 : 1, v/v), and methanol (100%) as dissolvents.

Preparation of Erythrocytes

A blood sample was drawn from a healthy man *via* venipuncture of an antecubital vein. One tenth volume of saturated EDTA solution (pH 7.4) was added to prevent coagulation. Erythrocytes were washed three times with 10 mM Na_2HPO_3, 125 mM NaCl (pH 7.4) phosphate-buffered saline (PBS), and the hematocrit value was adjusted to 1% (10,000 rpm, 10 min).

Treatment of Erythrocytes with Fluorescent Probe and Various Phospholipids

Erythrocytes were incubated in the dark for 3 h at 37°C with individual PL and with 1,6-diphenyl-1,3,5-hexatriene (DPH) which was used as a fluorescent probe dissolved in dimethylsulfoxide. The final concentration of PL and DPH was adjusted to 10 μM. Two mM adenine, 10 mM inosine, and 10 mM glucose were added to prevent emaciation of the erythrocytes.[10]

Fluorescent Depolarization Analysis

After incubation, the erythrocytes were washed three times with PBS again to remove excess DPH and PL, then adjusted to a hematocrit value of 0.05% for the fluorescent depolarization analysis. Fluorescent intensities of DPH which was incorporated into erythrocyte cell membranes were measured by a fluorescent spectrometer, Hitachi 650-40 (Hitachi Co., Ltd., Tokyo, Japan). Fluorescence intensities were corrected for background light-scattering by subtracting a probe-free blank solution. A wavelength of 360 nm was used for excitation of erythrocyte membrane-incorporated DPH. The emission intensities from DPH were detected at 450 nm. The slit widths for both excitation and emission were 10 nm. The fluorescence polarization and intensities were obtained by measuring the fluorescence intensities polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation beam. The degree of polarization (P) is defined by the following equation.[11]

$$P=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+GI_{\perp}) \quad (1)$$

where G is the correction factor ($G=I'_{\perp}/I'_{\parallel}$).

Data were analyzed using the paired t-test.[12]

2,4,6-Trinitrobenzenesulfonic Acid Quenching Method

0.5 m*l* of DPH-PL treated erythrocytes adjusted to a hematocrit value of 0.5% by PBS was mixed with 4.5 m*l* of 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution (2 mg/m*l* PBS) and stood for 1 h at 4°C.[13] Then, the excess TNBS was removed by rinsing with PBS three times. The recovered erythrocytes were resuspended in 5 m*l* of PBS and its fluorescence intensity was measured in the same manner as described.

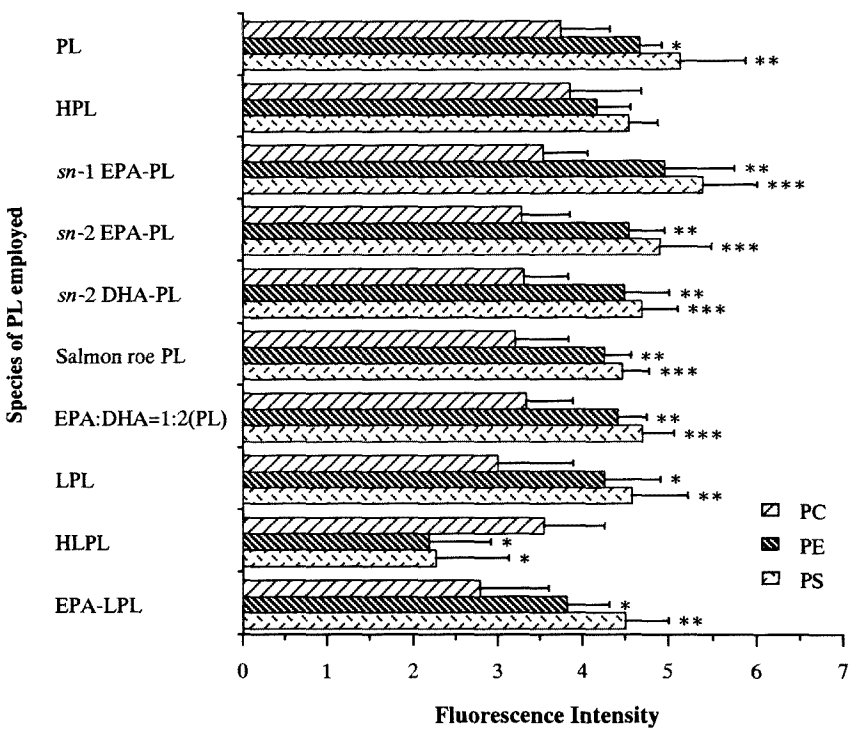

Fig. 1. Comparison of fluorescence intensity among various phospholipid treatments.

Mean ± S.D. of measurements for 5–6 times are given in each group.

(*): Significantly different ($p<0.05$) from each PC by paired t-test.

(**): Significantly different ($p<0.01$) from each PC by paired t-test.

(***): Significantly different ($p<0.001$) from each PC by paired t-test.

Abbreviations: PL, phospholipid; EPA : DHA = 1 : 2 (PL), mixed *sn*-2 EPA-PL and *sn*-2 DHA-PL with 1 : 2; same as in Tables 1–5 for other abbreviations.

Results

Fluorescence Intensity of Erythrocytes Treated with Various Phospholipids

Figure 1 compares the fluorescence intensity of various erythrocytes treated with DPH-PL. It is clear that of the PC, PE, and PS chemical forms, PS has the highest fluorescence intensity. This indicates that PS is most easily incorporated followed by PE, except the case of hydrogenated lysophospholipid (HLPL). HLPL caused cohesion during preparation, and its data is not considered to be reliable.

Table 2. Efficiency of fluorescence quenching on DPH-PL treated erythrocytes by TNBS

	Fluorescence intensity (*Ex* 360 nm; *Em* 450 nm)		Fluorescence quenching (%)
	Untreated	TNBS treated	
Control	1.90	1.39	36.4
Soy PC	2.12	1.51	37.7
Soy PE	2.14	1.41	44.5
Soy PS	2.98	1.81	47.2

Abbreviations: DPH-PL, 1,6-diphenyl-1,3,5-hexatriene and phospholipid; PC, phosphatidylcholine; Soy PE, soy phosphatidylethanolamine prepared by transphosphatidylation of Soy PC; Soy PS, soy phosphatidylserine prepared by transphosphatidylation of Soy PC; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

Table 3. Fatty acid composition of PCs prepared

	sn-2 EPA-PC	*sn*-2 DHA-PC	*sn*-1 EPA-PC	Salmon roe PC	EPA-LPC	HPC	HLPC
Sat.							
16:0	12.7	13.5	4.8	22.7	9.0	12.8	25.2
18:0	2.8	3.0	1.2	7.2	3.2	79.9	73.4
Mono.							
18:1 n-9	5.7	5.9	8.3	14.2	4.8	6.2	—
Poly.							
18:2 n-6	26.9	26.4	49.3	—	23.4	—	—
18:3 n-3	2.9	2.6	4.9	—	3.2	—	—
20:5 n-3	48.0	1.0	31.1	15.0	54.8	—	—
22:5 n-3	—	1.6	—	4.9	—	—	—
22:6 n-3	—	43.2	—	28.8	—	—	—

Abbreviations: *sn*-2 EPA-PC, EPA containing PC (at *sn*-2); *sn*-2 DHA-PC, DHA containing PC (at *sn*-2); *sn*-1 EPA-PC, EPA containing PC (at *sn*-1); Salmon roe PC, naturally occurring PC prepared from chum salmon roe; LPC, lysophosphatidylcholine; EPA-LPC, EPA containing LPC (at *sn*-1); HPC, hydrogenated PC; HLPC, hydrogenated LPC.
cf., Table 1 for detailed abbreviations.

Evaluation of Incorparation of Phospholipid by 2,4,6-Trinitrobenzenesulfonic Acid Quenching Method

Table 2 shows the results of fluorescence quenching of DPH-PL treated erythrocytes by TNBS. This experiment was carried out in order to determine the position of the erythrocyte which DPH-PL had incorporated (*cf.*, Appendix). Soy PL was used for this assay.

By TNBS treatment, all of the phospholipid classes examined showed 35–50% fluorescence quenching, suggesting that at least one-quarter of DPH-PL is incorporated adjacent to the cell membrane protein.

Fatty Acid Composition of Prepared Phospholipids

Table 3 shows the fatty acid composition of PC prepared for measuring the fluorescence depolarization of erythrocytes. The *sn*-2 EPA-PC containing EPA for 48.0% stands for the PC that binds EPA specifically to position *sn*-2, since this molecular species was prepared by the *sn*-2 positional specific phospholipase A_2 according to the method of Hosokawa *et al.*[5,6] In this case, theoretically, EPA dominates $48.0 \times 2 = 96.0\%$ at position *sn*-2. By the same reason, DHA-PC is considered to bind DHA for $43.2 \times 2 = 86.4\%$ at position *sn*-2.

Table 4 shows the fatty acid composition of PE prepared for measuring the fluorescence depolarization of erythrocytes. The *sn*-2 EPA-PE which contains EPA for 45.4% stands for the PE that binds EPA specifically to position *sn*-2, since this molecular species was prepared by transphosphatidylation of EPA-PC. In this case, theoretically, EPA dominates $45.4 \times 2 = 90.8\%$ at position *sn*-2. By the same reason, it is considered that DHA-PE binds DHA for $42.2 \times 2 = 84.4\%$ at position *sn*-2.

Table 5 shows the fatty acid composition of PS prepared for measuring the fluorescence depolarization of erythrocytes. The *sn*-2 EPA-PS containing EPA for 41.8% stands for the PS that binds EPA specifically to position *sn*-2, since this molecular species was prepared by trans-

Table 4. Fatty acid composition of PEs prepared

	Soy PE	*sn*-2 EPA-PE	*sn*-2 DHA-PE	*sn*-1 EPA-PE	Salmon roe PE	LPE	EPA-LPE	HPE	HLPE
Sat.									
16:0	12.9	12.2	12.8	3.5	17.1	44.1	6.1	21.0	19.9
18:0	3.5	3.5	3.6	—	7.9	9.0	2.3	50.1	76.6
Mono.									
18:1 n-9	10.9	5.8	5.8	7.6	12.2	11.5	3.3	25.9	—
Poly.									
18:2 n-6	66.3	26.6	25.3	47.1	—	31.5	14.7	2.1	—
18:3 n-3	6.3	3.2	2.5	4.5	—	1.7	2.1	—	—
20:5 n-3	—	45.4	1.4	35.5	14.9	—	69.3	—	—
22:5 n-3	—	—	2.1	—	6.6	—	—	—	—
22:6 n-3	—	—	42.2	—	27.8	—	—	—	—

Abbreviations: *sn*-2 EPA-PE, EPA containing PE (at *sn*-2); *sn*-2 DHA-PE, DHA containing PE (at *sn*-2); *sn*-1 EPA-PE, EPA containing PE (at *sn*-1); Salmon roe PE, naturally occurring PE prepared from chum salmon roe; LPE, lysophosphatidylethanolamine; EPA-LPE, EPA containing LPE (at *sn*-1); HPE, hydrogenated PE; HLPE, hydrogenated LPE.
cf., Tables 1 and 2 for detailed abbreviations.

Table 5. Fatty acid composition of PSs prepared

	Soy PS	*sn*-2 EPA-PS	*sn*-2 DHA-PS	*sn*-1 EPA-PS	Salmon roe PS	LPS	EPA-LPS	HPS	HLPS
Sat.									
16:0	14.1	14.3	14.9	3.3	16.6	42.1	8.6	19.1	47.0
18:0	3.3	3.8	3.9	—	7.5	8.6	4.0	58.5	52.5
Mono.									
18:1 n-9	11.2	6.3	6.3	7.7	11.9	10.9	4.4	17.8	—
Poly.									
18:2 n-6	63.7	27.7	26.7	47.0	—	34.2	16.1	2.5	—
18:3 n-3	6.3	3.2	2.3	4.5	—	4.2	1.9	—	—
20:5 n-3	—	41.8	1.1	35.2	14.6	—	60.4	—	—
22:5 n-3	—	—	2.4	—	6.2	—	—	—	—
22:6 n-3	—	—	39.6	—	30.1	—	—	—	—

Abbreviations: *sn*-2 EPA-PS, EPA containing PS (at *sn*-2); *sn*-2 DHA-PS, DHA containing PS (at *sn*-2); *sn*-1 EPA-PS, EPA containing PS (at *sn*-1); Salmon roe PS, naturally occurring PS prepared from chum salmon roe; LPS, lysophosphatidylserine; EPA-LPS, EPA containing LPS (at *sn*-1); HPS, hydrogenated PS; HLPS, hydrogenated LPS.

cf., Tables 1 and 2 for detailed abbreviations.

Table 6. Changes in fatty acid composition in erythrocytes before and after incubation with *sn*-2EPA-PC

	Untreated RBC	*sn*-2 EPA-PC treated RBC
Sat.		
16:0	30.8	28.8
18:0	9.9	9.6
Mono.		
18:1 n-9	19.3	17.6
Poly.		
18:2 n-6	13.3	13.6
18:3 n-3	—	—
20:4 n-6	13.7	13.4
20:5 n-3	0.9	2.0
22:5 n-3	1.3	1.5
22:6 n-3	4.9	5.8

Abbreviations: RBC, red blood cells (erythrocytes); *sn*-2 EPA-PC, cf. Table 3.

Table 7. Changes in fatty acid composition in erythrocytes before and after incubation with various phospholipids

	Untreated RBC	HPC treated RBC	*sn*-2 DHA-PC treated RBC	Salmon roe PC treated RBC
Sat.				
16:0	24.6	24.6	23.5	25.7
18:0	13.1	13.5	13.1	11.6
Mono.				
18:1 n-9	18.8	18.6	18.1	18.9
Poly.				
18:2 n-6	11.7	11.9	11.6	12.7
18:3 n-3	—	—	—	—
20:4 n-6	15.9	15.3	15.8	14.2
20:5 n-3	1.1	1.1	1.2	1.5
22:5 n-3	1.8	2.0	2.2	2.0
22:6 n-3	6.4	6.3	7.5	6.8

Abbreviations: RBC, red blood cell (erythrocytes); cf. Table 3 for other abbreviations.

phosphatidylation of EPA-PC. In this case, theoretically, EPA dominates $41.8 \times 2 = 83.6\%$ at position *sn*-2. By the same reason, it is considered that DHA-PS binds DHA for $39.6 \times 2 = 79.2\%$ at position *sn*-2.

Fatty acid compositions of the erythrocytes after incubation with PCs are shown in Tables 6 and 7. In hydrogenated soy PC treated erythrocytes, the difference between the treated and untreated was only after the decimal point for all fatty acids. In DHA-PC treated erythrocytes, DHA increased from 6.4% to 7.5%, while in EPA-PC treated erythrocytes, EPA increased from 0.9% to 2.0%. There was a small but significant increase in unsaturated fatty acids except arachidonic acid in salmon roe PC treated erythrocytes. The reason why all of these increases in unsaturated fatty acids were relatively small might be due to the homeostasis of cell membranes. However, these small but significant changes are considered to affect the fluidity of cell membranes.

Comparison of Degree of Polarization for Various Phospholipid Treatments

According to Eq. (1), the cell membrane fluidity and the polarization are related as follows: when the fluidity of the cell membrane increases, DPH which is the fluorescent probe and is bound on the cell membrane increases mobility. As a results, differences in $I_{\parallel}$ and $I_{\perp}$ become small, and P also becomes small as shown by Eq. (1). Therefore, the less polarization, the more fluid the cell membrane becomes. As illustrated in Fig. 2, polarizations were low in all EPA or DHA containing PLs compared to that of the control (soy PL). Of these, the PLs which contained both EPA and DHA like natural salmon roe PL or the synthetic EPA and DHA containing PL, exhibited the least polarization. On the contrary, P was higher with hydrogenated PLs. It is obvious that PE and PS increase the fluidity of erythrocytes rather than PC, since PE and PS exhibited much lower P.

It is well known that PE and PS are rich in the inner layer of the cell membrane. From the results of the TNBS quenching method (*vide supra*), we can conclude that HUFA-PE and HUFA-PS are easily incorporated in the inner layer of the cell membrane, probably by flipflop, and increase the fluidity of PE and PS in the inner layer of the cell membrane.

Discussion

Kohno *et al.**[1] and Hosokawa *et al.*[6] demonstrated

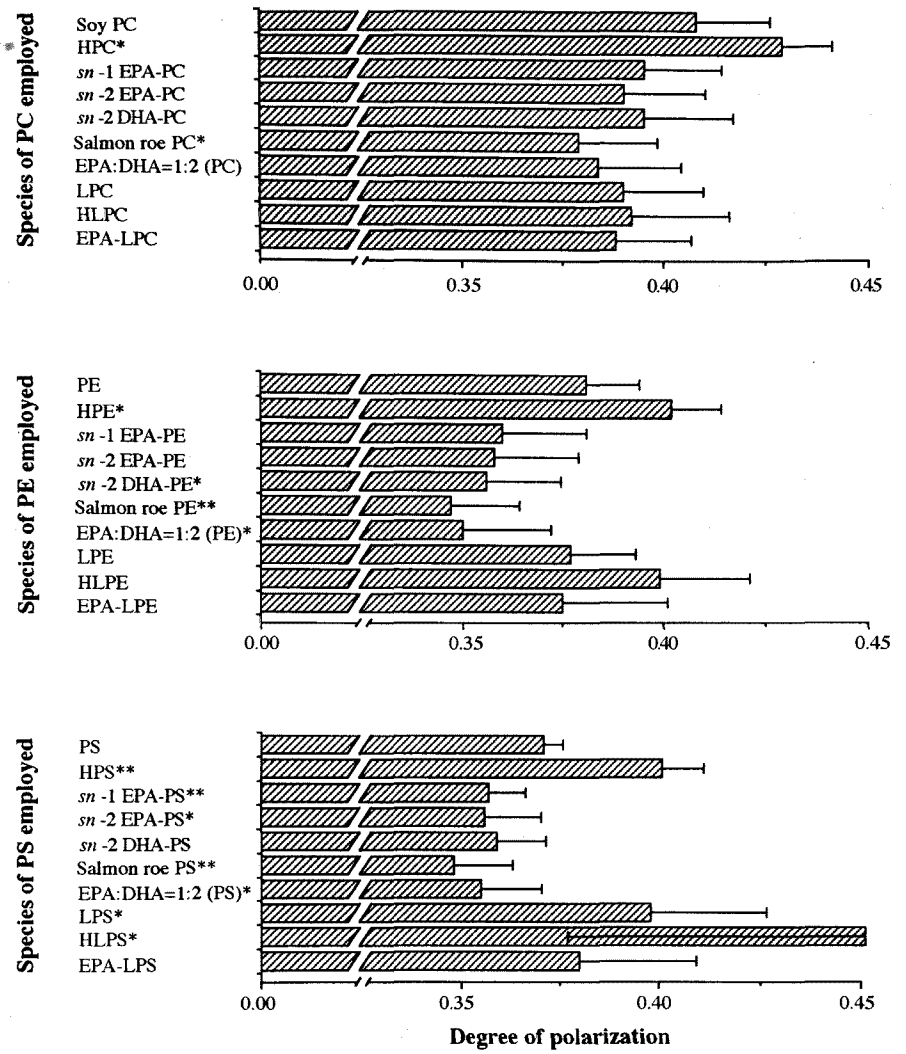

Fig. 2. Comparison of the degree of polarization among various PC, PE, PS treatments.
Mean ± S.D. of measurements for 5–6 times are given in each group.
(*): Significantly different ($p < 0.05$) from each PC by paired t-test.
(**): Significantly different ($p < 0.01$) from each PC by paired t-test.
Abbreviations: EPA : DHA = 1 : 2 (PC), mixed *sn*-2 EPA-PC and *sn*-2 DHA-PC with 1 : 2; EPA : DHA = 1 : 2 (PE), mixed *sn*-2 EPA-PE and *sn*-2 DHA-PE with 1 : 2; EPA : DHA = 1 : 2 (PS), mixed *sn*-2 EPA-PS and *sn*-2 DHA-PS with 1 : 2; same as in Tables 1–5 for other abbreviations.

that EPA or DHA containing PC has an anticancer effect on leukaemia HL-60 cell line. They postulated that EPA or DHA containing PC could easily be incorporated into cell membranes and made pliable, thus making the receptors, including the retinoic acid receptors, more susceptible physiological substances. This might have a promotional effect on retinoic acid induced cell differentiation. Cancer cells are cells which cease cell differentiation from the corresponding normal tissue. In this context, the increase in fluidity of the cell membranes of erythrocytes by EPA, DHA-PL supports the theory of Kohno *et al.*[*1] and Hosokawa *et al.*[6]

Appendix

Concept of the TNBS Quenching Method

The absorption spectrum of the yellow products trinitrophenyl-modified lysine residues obtained by reacting TNBS with proteins, and the fluorescence spectrum of DPH, have an overlapping region which can mediate the quenching of the latter.[13] The efficiency of quenching is a function of the distance between DPH and TNBS binding molecules. For this reason, if the DPH-PL has exclusively been incorporated into the cytoplasm, quenching will not be observed.

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