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Primary Aminophospholipids in the External Layer of Liposomes Protect Their Component Polyunsaturated Fatty Acids from 2,2′-Azobis(2-amidinopropane)-dihydrochloride-Mediated Lipid Peroxidation

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We showed in our previous study that docosahexaenoic acid-rich phosphatidylethanolamine in the external layer of small-size liposomes, as a model for biomembranes, protected its docosahexaenoic acid from 2,2′-azobis(2-amidinopropane)dihydrochloride- (AAPH-) mediated lipid peroxidation in vitro. Besides phosphatidylethanolamine, both phosphatidylserine and an alkenyl-acyl analogue of phosphatidylethanolamine, phosphatidylethanolamine plasmalogen, are reported to possess characteristic antioxidant activities. However, there are few reports about the relationship between the protective activity of phosphatidylethanolamine plasmalogen and/or phosphatidylserine against lipid peroxidation and their distribution in a phospholipid bilayer. Furthermore, it is unclear whether phosphatidylethanolamine plasmalogen and/or phosphatidylserine protect their component polyunsaturated fatty acids (PUFAs) from lipid peroxidation. In the present study, we examined the relationship between the transbilayer distribution of aminophospholipids, such as phosphatidylethanolamine rich in arachidonic acid, phosphatidylethanolamine plasmalogen, and phosphatidylserine, and the oxidative stability of their component PUFAs. The transbilayer distribution of these aminophospholipids in liposomes was modulated by coexisting phosphatidylcholine bearing two types of acyl chain: dipalmitoyl or dioleoyl. The amounts of these primary aminophospholipids in the external layer became significantly higher in liposomes containing dioleoylphosphatidylcholine than in those containing dipalmitoylphosphatidylcholine. Phosphatidylethanolamine rich in arachidonic acid, phosphatidylethanolamine plasmalogen or phosphatidylserine in the external layer of liposomes, as well as external docosahexaenoic acid-rich phosphatidylethanolamine, were able to protect their component PUFAs from AAPH-mediated lipid peroxidation.

KEYWORDS: Acyl chain region; aminophospholipid; docosahexaenoic acid; lipid peroxidation; liposome; plasmalogen

INTRODUCTION

Since liposomes have a characteristic structure and can encapsulate various compounds, they have been used as carriers of pharmaceutical ingredients and for some foods. In Japan, a large-scale project for development of manufacturing technology using liposomes for health food production is currently in progress. In this project, research efforts are being focused on the establishment of liposome technology and production of functional liposomes, which should ultimately lead to a dramatic improvement of physiological effectiveness in comparison with conventional health foods. It is postulated that the intake of liposomes, encapsulating high-concentration active ingredients, would facilitate absorption of the ingredients directly from mucous membranes into blood vessels, and ultimately into cells within the body. Under the present circumstances in the food industry, where studies aimed at the establishment of liposome technology and commercialization of such functional liposomes are being intensively conducted, liposomes containing polyunsaturated fatty acids (PUFAs) with useful physiological functions play a very important role in the development of microcapsulated forms of functional foods and/or dietary supplements. At this time, PUFAs such as docosahexaenoic acid are highly susceptible to lipid peroxidation, leading to the generation of an unpleasant odor after lipid peroxidation, and therefore stabilization of the quality, for example by preventing the production of such odor, is crucial.

In model membranes, increasing the molar ratio of phosphatidylethanolamine in phosphatidylethanolamine/phosphatidylcholine liposomes decreases the amount of external phosphatidylethanolamine in a liposomal phospholipid bilayer (*1*−*4*) and promotes lipid peroxidation in the metal ion-induced (*5*)

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and 2,2′-azobis(2-amidinopropane)dihydrochloride- (AAPH-) mediated (*6*) systems, respectively. A higher content of phosphatidylethanolamine induces an inverted hexagonal phase that is more sensitive to lipid peroxidation than a lamellar phase in phosphatidylcholine/phosphatidylethanolamine aqueous dispersions (*6*). In most studies, however, the transbilayer distribution of phosphatidylethanolamine has not been considered. The arrangement of phospholipid molecules in bilayer membranes is a significant factor determining resistance to free radical attack during lipid peroxidation. We have previously shown that a difference in the acyl chain region of phosphatidylcholine molecules modulates the transbilayer distribution of docosahexaenoic acid-rich phosphatidylethanolamine in liposomes and that phosphatidylethanolamine in the external layer of liposomes protects its docosahexaenoic acid from AAPH-mediated lipid peroxidation (*7*). This observation may be applicable to the development of oxidatively more stable docosahexaenoic acid-containing functional food materials with microcapsulated forms and to transport of docosahexaenoic acid-containing foods at room temperature.

If the primary amino group of phosphatidylethanolamine in the external layer possesses antioxidant activity, other primary aminophospholipids may also possess similar activity. An alkenyl-acyl analogue of phosphatidylethanolamine, phosphatidylethanolamine plasmalogen (*8*−*12*), and also phosphatidylserine (*13*, *14*) are each reported to possess characteristic antioxidant activities. However, there are few reports about the relationship between the antioxidant activity of phosphatidylethanolamine plasmalogen and/or phosphatidylserine against lipid peroxidation and their transbilayer distribution in a phospholipid bilayer. Furthermore, it is unclear whether phosphatidylethanolamine plasmalogen and/or phosphatidylserine protect their component polyunsaturated fatty acids (PUFAs) from lipid peroxidation.

Our objectives in this study were, first, to investigate whether the relative area or volume of the acyl chain region of phosphatidylcholine modulates not only the transbilayer distribution of docosahexaenoic acid-rich phosphatidylethanolamine but also that of other aminophospholipids containing various acyl chains and a different polar headgroup, and second, to investigate the relationship between the transbilayer distribution of these primary aminophospholipids in bilayer model membranes and the susceptibility of their component PUFAs to AAPH-mediated lipid peroxidation.

MATERIALS AND METHODS

Materials. Phosphatidylethanolamine originated from porcine liver (arachidonic acid-rich phosphatidylethanolamine, PE-AA) and 1-radyl-2-acyl-*sn*-glycero-3-phosphatidylethanolamine from bovine brain (phosphatidylethanolamine plasmalogen, PLE) were purchased from Doosan Serdary Research Laboratories (Toronto, Canada). L-α-Phosphatidylserine (PS) originated from soybean and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (dipalmitoylphosphatidylcholine, PC16:0) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Docosahexaenoic acid-rich phosphatidylethanolamine (PE-DHA) and docosahexaenoic acid-rich phosphatidylcholine (PC-DHA) were obtained from the liver of rats fed a diet containing docosahexaenoic acid ethyl esters. Docosahexaenoic acid ethyl esters (78% pure) prepared from orbital fat of tuna were donated by Maruha Corporation (Tsukuba, Japan). *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) and Triton X-100 were purchased from Sigma (St. Louis, MO). 2,6-Di-*tert*-butyl-4-methylphenol (BHT), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (dioleoylphosphatidylcholine, PC18:1), 2,4,6-trinitrobenzenesulfonic acid, AAPH, boron trifluoride/methanol solution, DL-methionine, glucose, and choline bitartrate were purchased from Wako Pure Chemical Co. (Osaka, Japan). Hydrogen chloride−methanol solution, *n*-hexane, methanol, and sodium hydroxide were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). For identification of peaks in gas−liquid chromatography (GLC), standard mixtures (GLC-85 and GLC-421) of fatty acid methyl esters of 46 species were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

Preparation of Docosahexaenoic Acid-Rich Phosphatidylethanolamine and Docosahexaenoic Acid-Rich Phosphatidylcholine. The animal breeding procedures used in this study met the guidelines of the Animal Committee of Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan). Male Sprague-Dawley rats (Murai Jikken Dobutsu, Saitama, Japan), 1 year of age, were fed an experimental diet containing docosahexaenoic acid and linoleic acid in the dietary lipids (10 wt %) based on the AIN-76 (*15*, *16*) for 30 days. The proportions of docosahexaenoic acid and linoleic acid in the fatty acid composition of dietary lipid were 38.6 and 9.5 wt %, respectively. Total lipids were extracted from the liver according to the method of Folch et al. (*17*). Docosahexaenoic acid-rich phosphatidylethanolamine (PE-DHA) and docosahexaenoic acid-rich phosphatidylcholine (PC-DHA) were separated from the total lipids by using a silica Sep-Pak column (35 cm^3, 10 g) from Waters Associates (Milford, MA) (*18*). Phosphatidylethanolamine was eluted with chloroform/methanol (1:2), and then docosahexaenoic acid-rich phosphatidylcholine was eluted with methanol. The identification of each phospholipid species was confirmed by thin-layer chromatography on Merck type 60 silica gel plates (Merck A.G., Darmstadt, Germany). α-Tocopherol in each phospholipid preparation was analyzed by HPLC as described (*19*), but it was not detected.

Preparation of Liposomes. Phospholipid concentration was determined as phosphorus by a modification of the microprocedure (*20*) of Bartlett (*21*), and the liposomal PE-DHA/phosphatidylcholine, PE-AA/phosphatidylcholine, PLE/phosphatidylcholine, and PC-DHA/phosphatidylcholine molar ratios were adjusted to 1:2. Phospholipid mixtures were dried quickly by purging with nitrogen gas to form a thin film on the glass vial wall. The thin film was suspended in 0.1 M K^+/Hepes buffer, pH 7.6, and the suspension was frozen in liquid nitrogen and thawed in a water bath-type sonicator to tear the thin film off from the glass vial wall completely. This freeze−thaw cycle was repeated more than 10 times under nitrogen. A polycarbonate filter (Nuclepore, Pleasanton, CA) was mounted in a miniextruder (Avanti Polar Lipids, Inc., Alabaster, AL) fitted with two 1.0-mL microsyringes (Ito Co., Shizuoka, Japan), which had been modified by Ito Co. The suspension was passed through a polycarbonate filter (pore size 80 nm) 41 times to give an optically clear suspension and to form unilamellar vesicles. The liposomes were diluted in 0.1 M K^+/Hepes buffer, pH 7.6, to the concentrations shown in each figure.

Determination of Aminophospholipid Distribution in Liposomes. Aminophospholipid distribution in the liposomes was determined by a modified spectroscopic method for determination of the transbilayer distribution of phosphatidylethanolamine (*22*). The total concentration of the amino groups of each aminophospholipid was determined after solubilization of the liposomes with Triton X-100 (final concentration of 2.7%). A solution of 2,4,6-trinitrobenzenesulfonic acid (final concentration 2 mM), as a probe for aminophospholipid, was freshly prepared in the same buffer used to prepare the liposomes. Formation of the resulting trinitrophenyl derivatives was detected spectrophotometrically at 420 nm with a DU650 spectrophotometer (Beckman, Fullerton, CA). The distribution of aminophospholipid in the external layer of liposomes (aminophospholipid$_{external}$ %) was calculated from the ratio of external absorbance ($A_{external}$), which measures the reaction between aminophospholipid in the external layer of liposomes and 2,4,6-trinitrobenzenesulfonic acid, to total absorbance (A_{total}), which measures the reaction between total aminophospholipid in liposomes and 2,4,6-trinitrobenzenesulfonic acid when A_{total} reached a plateau.

Oxidation of Liposomes. AAPH was added to the solution containing liposomes at a final concentration (outside of the liposomes) of 1 mM, but 2 mM in the case of the phosphatidylserine/phosphatidylcholine liposome solution. The liposomes were incubated for 20, 44, 68, and 92 h at 25 °C under darkness.

Analysis of Fatty Acid Composition. Fatty acid compositions of the dietary lipids, aminophospholipids, docosahexaenoic acid-rich phosphatidylcholine, and liposomal lipids were analyzed by GLC (*7*).

Table 1. Fatty Acid Composition of Docosahexaenoic Acid-Rich Phosphatidylethanolamine, Arachidonic Acid-Rich Phosphatidylethanolamine, Phosphatidylethanolamine Plasmalogen, Phosphatidylserine, and Docosahexaenoic Acid-Rich Phosphatidylcholine

fatty acid (g/100 g)	PE-DHA	PE-AA	PLE	PS	PC-DHA
C16:0 dimethyl acetal	0.0	0.0	9.6	0.0	0.0
C16:0	14.7	7.6	2.6	11.4	21.3
C16:1 (*n*-9)	1.3	0.0	0.0	0.2	1.0
C18:0 dimethyl acetal	0.0	0.0	6.0	0.0	0.0
C18:1 dimethyl acetal	0.0	0.0	6.4	0.0	0.0
C18:0	22.9	35.2	6.8	3.3	18.8
C18:1 (*n*-9)	7.6	6.4	23.8	9.8	6.9
C18:2 (*n*-6) linoleic acid	9.5	10.8	0.0	60.4	10.9
C18:3 (*n*-3)	0.0	0.0	0.0	4.0	0.0
C20:1 (*n*-9)	0.0	0.0	5.6	0.0	0.0
C20:3 (*n*-6)	1.1	0.6	0.9	0.0	0.8
C20:4 (*n*-6) arachidonic acid	13.0	29.0	5.1	0.0	13.6
C20:5 (*n*-3) eicosapentaenoic acid	4.0	0.5	0.0	0.0	5.3
C22:1 (*n*-9)	0.0	0.0	0.6	0.0	0.0
C22:5 (*n*-6)	1.1	0.0	0.2	0.0	0.0
C22:5 (*n*-3)	1.4	3.1	1.5	0.0	0.6
C22:6 (*n*-3) docosahexaenoic acid	23.1	3.5	6.5	0.0	13.7
C24:0	0.0	0.0	2.0	0.0	0.0
others	0.3	3.5	22.5	11.0	7.1
<chgrow;lp;4q>saturated fatty acid (%)	37.5	42.7	27.0	14.6	40.1
monounsaturated fatty acid (%)	8.9	6.4	36.3	10.0	7.9
polyunsaturated fatty acid (%)	53.3	47.4	14.2	64.4	44.9

Fatty acid methyl esters (FAME) of the dietary lipid were prepared by saponification with 0.5 mol/L sodium hydroxide/methanol solution and the resultant free fatty acids were converted into methyl esters by addition of boron trifluoride/methanol solution (140 g/L). Liposomal phospholipids used for the oxidation reaction were extracted by the Bligh and Dyer method (*23*) in the presence of 1.4 μmol of BHT. FAME of aminophospholipids, docosahexaenoic acid-rich phosphatidylcholine, and liposomal phospholipids used for the oxidation reaction were prepared with hydrogen chloride/methanol solution as described by Kates (*24*). The internal standard used was 17:0 methyl ester. The methyl esters were extracted with *n*-hexane and analyzed by GLC on a GC-18A chromatograph (Shimadzu, Kyoto, Japan) using a 25 m $\times$ 0.25 mm i.d. HR-SS-10 glass capillary column coated by nitrilesilicon (Shinwa Chemical Industries, Ltd., Kyoto, Japan) (*7*). The column temperature was programmed from 160 to 210 °C. The injector and detector temperatures were 250 °C. Helium was used as the carrier gas. Standard mixtures of FAME were used for identification of the peaks.

Statistical Analysis. Values are expressed as means $\pm$ standard deviation of the means (SD). Data were first analyzed by the *F*-test for homogeneity of variance with significant levels of 5%. If the test revealed homogeneity of variance, difference in percent unoxidized fatty acids in two phosphatidylcholine species of liposomes was assessed by Student's *t*-test (*25*) by comparing each value at each time point. In the case of heterogeneity of variance, difference in percent unoxidized fatty acids in two phosphatidylcholine species of liposomes was assessed by Aspin-Welch's *t*-test (*26*) by comparing each value at each time point. Differences were considered significant at $^{**}p < 0.01$ or $^{*}p < 0.05$. All calculations were performed with StatLight software (Yukms Co., Ltd, Tokyo, Japan).

RESULTS

Fatty Acid Composition of Aminophospholipids and Docosahexaenoic Acid-Rich Phosphatidylcholine Used for Preparation of Liposomes. As shown in **Table 1**, the proportions of major PUFA of docosahexaenoic acid-rich phosphatidylethanolamine (PE-DHA) were docosahexaenoic acid (23.1%) and arachidonic acid (13.0%), those of arachidonic acid-rich phosphatidylethanolamine (PE-AA) were arachidonic acid (29.0%) and linoleic acid (10.8%), those of docosahexaenoic acid-rich phosphatidylcholine (PC-DHA) were docosahexaenoic acid (13.7%) and arachidonic acid (13.6%), those of phosphatidylethanolamine plasmalogen (PLE) were docosahexaenoic acid (6.5%) and arachidonic acid (5.1%), and that of phosphatidylserine (PS) was linoleic acid at approximately 60%, respectively.

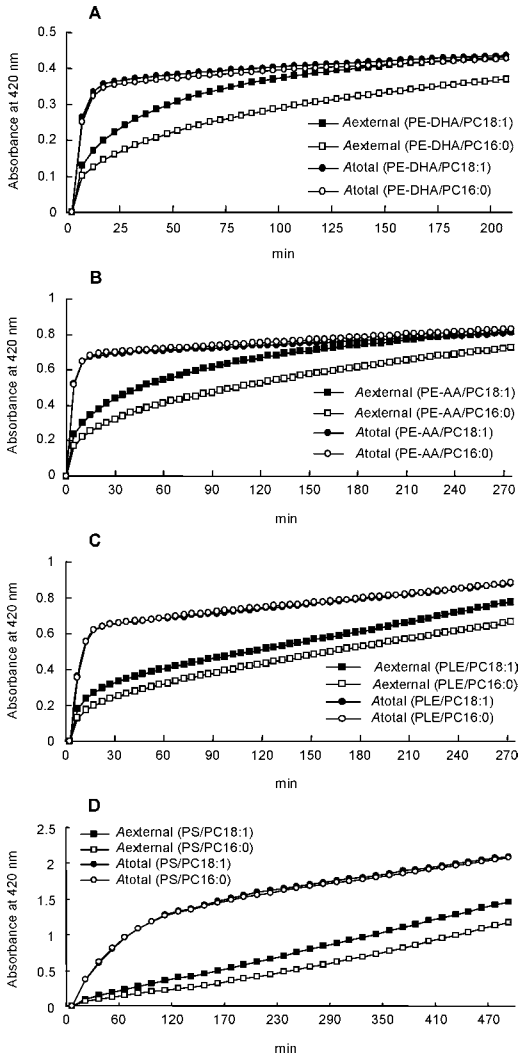

Figure 1. Distribution of aminophospholipid in the external layer of aminophospholipid/phosphatidylcholine (1:2) liposomes (Hepes buffer, pH 7.6): time course of a representative case of aminophospholipid labeling by 2,4,6-trinitrobenzenesulfonic acid in different species of liposomes. (**A**) PE-DHA/PC18:1 (1.7 mM) vs PE-DHA/PC16:0 (1.7 mM); (**B**) PE-AA/PC18:1 (1.9 mM) vs PE-AA/PC16:0 (1.9 mM); (**C**) PLE/PC18:1 (1.8 mM) vs PLE/PC16:0 (1.9 mM); (**D**) PS/PC18:1 (3.6 mM) vs PS/PC16:0 (3.6 mM).

Distribution of Aminophospholipid in the External Layer of Liposomes. The time course of aminophospholipids labeling by 2,4,6-trinitrobenzenesulfonic acid in different species of liposomes is shown in **Figure 1**. On mixing liposomes with excess 2,4,6-trinitrobenzenesulfonic acid, a biphasic time course is observed as shown elsewhere (*22*) and in **Figure 1**. A first rapid phase is followed by a slower phase. The slower phase observed with liposomes corresponds therefore to the formation of trinitrophenyl derivatives with the aminophospholipid molecules assembled in the internal layer of the liposomal membrane. A_{total} of liposomal species containing PE-DHA, PE-AA, or PLE reached a plateau after 30 min (**Figure 1A**−**C**) but after 150 min in the case of phosphatidylserine/phosphatidylcholine liposomes (**Figure 1D**). Therefore, PE-DHA$_{external}$ %, PE-

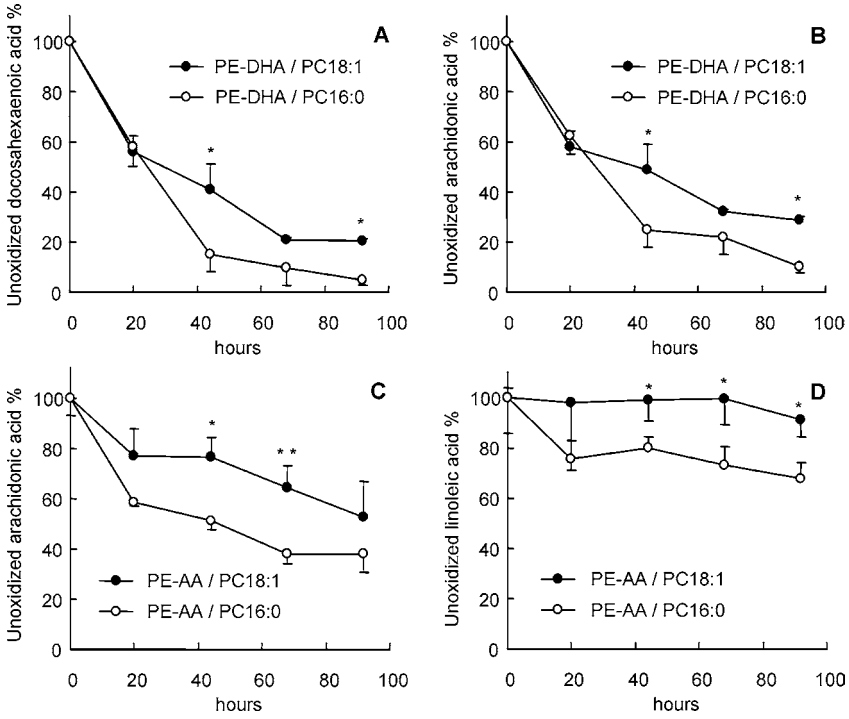

Figure 2. Unoxidized docosahexaenoic acid (**A**), arachidonic acid (**B**, **C**), and linoleic acid (**D**) in phosphatidylethanolamine/phosphatidylcholine (1:2) liposomes at various times of lipid peroxidation induced by 1 mM AAPH (Hepes buffer, pH 7.6). (**A**, **B**) (●) PE-DHA/PC18:1 (1.7 mM) vs (○) PE-DHA/PC16:0 (1.7 mM); (**C**, **D**) (●) PE-AA/PC18:1 (1.9 mM) vs (○) PE-AA/PC16:0 (1.9 mM). Values are the mean ± SD, $n = 3$. $^{**}p < 0.01$; $^{*}p < 0.05$.

$AA_{external}$ %, and $PLE_{external}$ % were calculated from A_{total} and $A_{external}$ at 30 min, respectively, and at 150 min in the case of $PS_{external}$ %. $PE\text{-}DHA_{external}$ % (66.8% ± 0.73%) of PE-DHA/PC18:1 liposomes was significantly higher than that (54.0% ± 1.70%) of PE-DHA/PC16:0 liposomes ($p < 0.01$). $PE\text{-}AA_{external}$ % (63.0% ± 1.32%) of PE-AA/PC18:1 liposomes was significantly higher than that (46.4% ± 0.57%) of PE-AA/PC16:0 liposomes ($p < 0.01$). $PLE_{external}$ % (51.1% ± 0.49%) of PLE/PC18:1 liposomes was significantly higher than that (38.4% ± 0.61%) of PLE/PC16:0 liposomes ($p < 0.01$). $PS_{external}$ % (33.9% ± 1.32%) of PS/PC18:1 liposomes was significantly higher than that (21.3% ± 0.14%) of PS/PC16:0 liposomes ($p < 0.01$). This tendency was retained at 4 °C under darkness for 4 weeks (data not shown).

Unoxidized PUFA Remaining in Liposomes after Various Periods of AAPH-Mediated Lipid Peroxidation. Figures 2 and **3** show the proportions of residual PUFA (unoxidized PUFA %) in liposomes after various periods of lipid peroxidation mediated by AAPH. As shown in **Figure 2**, the residual unoxidized docosahexaenoic acid (**Figure 2A**) and arachidonic acid (**Figure 2B**) of PE-DHA/PC18:1 were significantly higher than those of PE-DHA/PC16:0 at 44 h ($p < 0.05$) and 92 h ($p < 0.05$), respectively. The differences of unoxidized docosahexaenoic acid were 26% (at 44 h) and 15% (at 92 h), respectively (**Figure 2A**), and those of arachidonic acid were 24% (at 44 h) and 19% (at 92 h), respectively (**Figure 2B**). The residual unoxidized arachidonic acid of PE-AA/PC18:1 was significantly higher than that of PE-AA/PC16:0 at 44 h ($p < 0.05$) and 68 h ($p < 0.01$), where the differences were 25% (at 44 h) and 26% (at 68 h), respectively (**Figure 2C**). The residual unoxidized linoleic acid of PE-AA/PC18:1 was significantly higher than that of PE-AA/PC16:0 at 44, 68, and 92 h ($p < 0.05$, respectively), where the differences were 19%, 26%, and 23%, respectively (**Figure 2D**). The residual unoxidized docosahexaenoic acid of PLE/PC18:1 was significantly higher than that of PLE/PC16:0 at 20 h ($p < 0.01$) and 92 h ($p < 0.05$), where the differences were 26% (at 20 h) and 20% (at 92 h) (**Figure 3A**). The residual unoxidized arachidonic acid of PLE/PC18:1 was significantly higher than that of PLE/PC16:0 at 20 h ($p < 0.05$) and 92 h ($p < 0.05$), where the differences were 21% (at 20 h) and 7.5% (at 92 h), respectively (**Figure 3B**). The residual unoxidized linoleic acid of PS/PC18:1 was significantly higher than that of PS/PC16:0 at 20 h ($p < 0.05$), where the difference was 52%, but no significant differences between the two phosphatidylcholine species were observed at 44, 68, and 92 h (**Figure 3C**). The residual unoxidized docosahexaenoic acid and arachidonic acid of both PC-DHA/PC18:1 and PC-DHA/PC16:0 decreased with incubation time, and no significant differences were observed between the two phosphatidylcholine species (**Figure 3D**,**E**).

DISCUSSION

Aminophospholipids in the brain and nervous tissues are characterized by a high content of docosahexaenoic acid (*27*−*30*). When fed to rats, docosahexaenoic acid is utilized preferentially for phosphatidylethanolamine synthesis in the liver (*28*) and is also incorporated into hepatic phosphatidylcholine, but less is incorporated into phosphatidylcholine than into phosphatidylethanolamine (*28*). In fact, the proportion of docosahexaenoic acid in PE-DHA was considerably higher than that in other phospholipids (**Table 1**). Although the proportion of docosahexaenoic acid in phosphatidylserine, as well as that in phosphatidylethanolamine or phosphatidylethanolamine plasmalogen, is generally high in the brain and nervous tissues (*29*, *30*), phosphatidylserine used in this study was characterized by a high content of linoleic acid, since phosphatidylserine originating from soybean was used. We used these phospholipids, which differ characteristically in their PUFA or acyl chain compositions.

The charge of polar headgroups (*31*−*33*), curvature of vesicles (*34*, *35*), and molar ratios of phospholipid species (*1*−

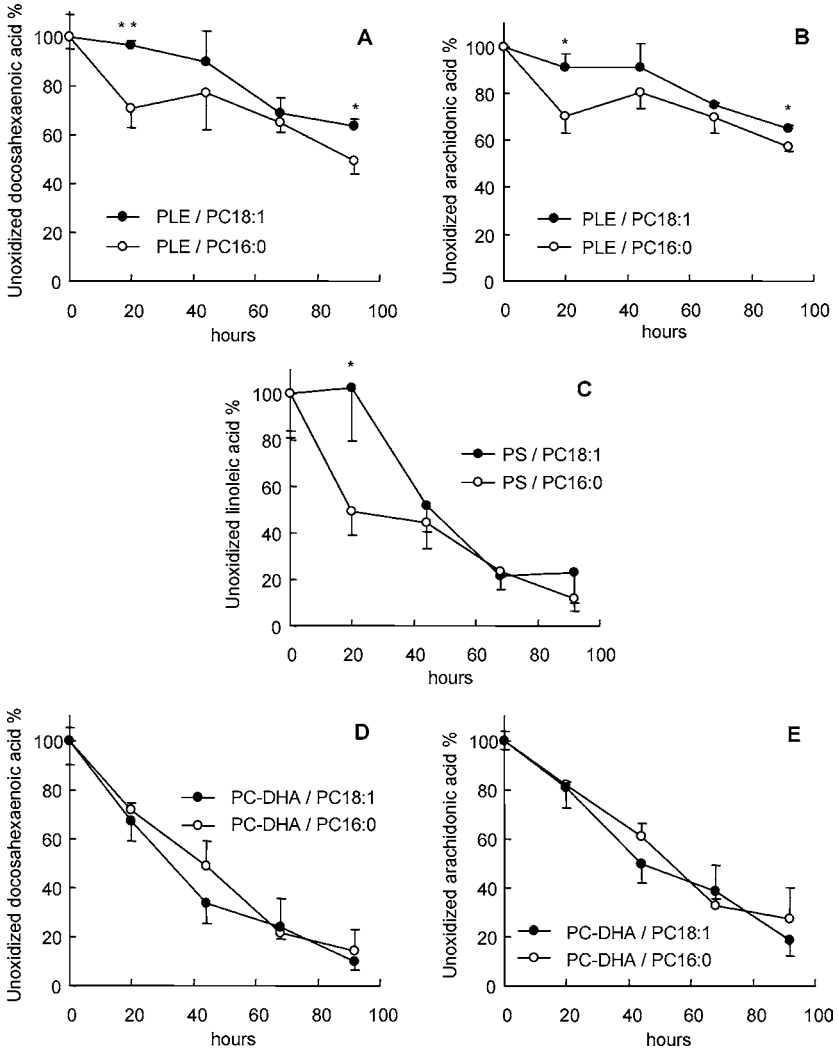

Figure 3. Unoxidized docosahexaenoic acid (**A**, **D**), arachidonic acid (**B**, **E**), and linoleic acid (**C**) in phosphatidylethanolamine plasmalogen/phosphatidylcholine (**A**, **B**), phosphatidylserine/phosphatidylcholine (**C**), and docosahexaenoic acid-rich phosphatidylcholine/phosphatidylcholine (**D**, **E**) (1:2) liposomes at various times of lipid peroxidation induced by 1 mM AAPH (Hepes buffer, pH 7.6). (**A**, **B**) (●) PLE/PC18:1 (1.8 mM) vs (○) PLE/PC16:0 (1.9 mM). (C) (●) PS/PC18:1 (3.6 mM) vs (○) PS/PC16:0 (3.6 mM). (**D**, **E**) (●) PC-DHA/PC18:1) (1.9 mM) vs (○) PC-DHA/PC16:0 (1.6 mM). Values are the mean ± SD, $n = 4$–5. $^{**}p < 0.01$; $^{*}p < 0.05$.

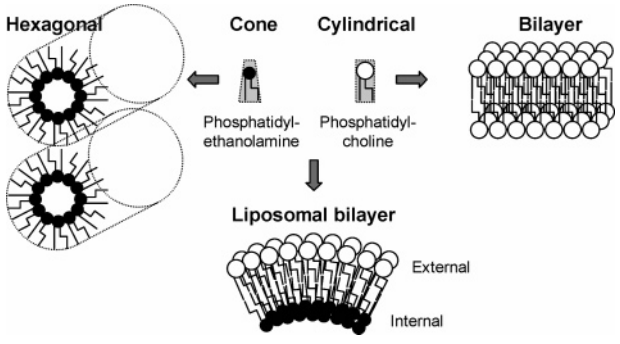

Figure 4. Molecular shape and phase of phospholipids.

4, *36*) have been shown to be factors influencing the asymmetric distribution of phospholipids in bilayer membranes. On the other hand, as shown in **Figure 4**, a cone-shaped molecule such as phosphatidylethanolamine tends to be distributed in the internal layer, since the relative area or volume of the acyl chain region in comparison to the headgroup of a phosphatidylethanolamine molecule is greater than that of a phosphatidylcholine molecule, as was suggested first by Cullis and de Kruijff (*37*, *38*). When the acyl chain region of phosphatidylcholine molecules is considered, therefore, a molecule containing unsaturated chains, such as dioleoylphosphatidylcholine (PC18:1), is expected to adopt a more pronounced cone shape than a molecule containing saturated chains, such as dipalmitoylphosphatidylcholine (PC16:0). In liposomes, dioleoylphosphatidylcholine tends to be distributed more in the internal layer than is the case for dipalmitoylphosphatidylcholine, and thus the coexisting phospholipid may be pushed out to the external layer. Indeed, in our previous study, when the curvature of vesicles was uniformly high, the proportion of PE-DHA in the external layer was significantly higher in liposomes containing dioleoylphosphatidylcholine than in those containing dipalmitoylphosphatidylcholine, and this tendency for a transbilayer distribution became more pronounced in smaller-sized liposomes with a pore size of less than 100 nm (*7*). In the present study, therefore, we used a filter with a pore size of 80 nm and confirmed that the transbilayer distribution of PE-AA, PLE, or PS in liposomes was also modulated by the difference in the acyl chain region of the phosphatidylcholine molecule. The relative area or volume of the acyl chain region of a phosphatidylcholine molecule affected not only the transbilayer distribution of PE-DHA but also that of other aminophospholipids with various acyl chains and different polar headgroups (**Figure 5A**).

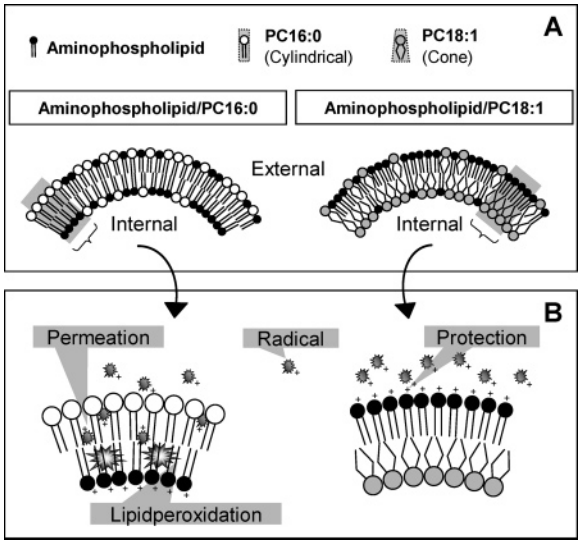

Figure 5. (**A**) Difference in the acyl chain region of the phosphatidylcholine molecule modulates the transbilayer distribution of coexisting aminophospholipids in liposomes. (**B**) Hypothetical illustration that aminophospholipids in the external layer of liposomes protect their component PUFA from AAPH-mediated lipid peroxidation.

In liposomes with diameters of 60−80 nm, phosphatidylethanolamine is known to be preferentially localized in the external layer at low phosphatidylethanolamine concentration (10 mol % of total phospholipids) (*39*). In egg yolk phosphatidylcholine liposomes containing dipalmitoylphosphatidylethanolamine or dipalmitoylphosphatidylserine at 10 mol % of total phospholipids, formation of phosphatidylcholine hydroperoxide and accumulation of thiobarbituric acid-reactive substances were lower than in the control (egg yolk phosphatidylcholine liposomes containing dipalmitoylphosphatidylcholine at 10 mol % of total phospholipids) in iron-induced lipid peroxidation (*14*). However, increasing the molar ratio of phosphatidylethanolamine in phosphatidylethanolamine/phosphatidylcholine liposomes decreased the amounts of external phosphatidylethanolamine in liposomes (*1*−*4*) and increased the oxygen consumption rate in the iron-induced system (*5*), and also increased the phosphatidylethanolamine hydroperoxide in the AAPH-mediated system (*6*). A higher content of phosphatidylethanolamine (above 80 mol % of total phospholipids) induces an inverted hexagonal phase that is more sensitive to AAPH-mediated lipid peroxidation than the multilamellar phase in phosphatidylcholine/phosphatidylethanolamine aqueous dispersions (*6*).

Phosphatidylethanolamine has been suggested to accelerate lipid peroxidation (*40*, *41*). In most studies, however, the transbilayer distribution of phosphatidylethanolamine was not considered. In the case of the inverted hexagonal phase, the phosphatidylethanolamine molecule may have higher susceptibility to lipid peroxidation since the acyl chain region of phosphatidylethanolamine is oriented in the direction of free radical attack (aqueous phase). Hence, it is reasonable to assume that the phosphatidylethanolamine molecule itself can weaken its susceptibility to lipid peroxidation when its polar headgroup is oriented in the direction of free radical attack. If this unique protective activity, as shown in both the present study and our previous study (*7*), is conferred by a function of the polar headgroup of PE-DHA, it is likely that docosahexaenoic acid, which lies behind the polar headgroup, would be protected from lipid peroxidation. This idea, as shown by the external phosphatidylethanolamine in liposomes, may be similarly applicable to arachidonic acid- and linoleic acid-rich phosphatidylethanolamine (**Figure 2**). Furthermore, since the percentage of unoxidized PUFA remaining after AAPH-mediated lipid peroxidation was higher in liposomes with a higher distribution of PLE or PS in the external layer (**Figure 3**), the radical initiators may penetrate into the hydrophobic inner region in liposomes with a lower distribution of aminophospholipids in the external layer (**Figure 5**) and thus promote the propagation reaction of lipid peroxidation of aminophospholipids.

Several researchers have observed that phosphatidylethanolamine undergoes oxidation more rapidly than phosphatidylcholine during lipid peroxidation mediated by metal ion species such as iron(Fe^{2+}) (*5*, *41*), hemoglobin (*42*) or copper (*43*). The phosphatidylethanolamine hydroperoxide formed is decomposed more rapidly than phosphatidylcholine hydroperoxide (*44*), and thus phosphatidylethanolamine hydroperoxide tends to generate active secondary degradation products in the presence of iron (Fe^{2+}) (*5*, *45*, *46*). However, it is reported that secondary degradation products are not generated when AAPH is used as the radical initiator (*5*). If this is so, phosphatidylethanolamine hydroperoxide is produced in the AAPH-mediated lipid peroxidation system but phosphatidylethanolamine hydroperoxide is not decomposed, and thus the propagation of peroxidation of PUFA combined with phosphatidylethanolamine is suppressed under current experimental conditions.

In contrast, it has been reported that phosphatidylethanolamine plasmalogen (PLE) inhibits iron- and copper-dependent lipid hydroperoxide formation, whereas it is ineffective for AAPH-mediated lipid hydroperoxide formation (*47*). PLE is a glycerophospholipid with a vinyl ether bond ($-CH_2-O-CH=CH-$) at the *sn*-1 position of the glycerol backbone, and the action of scavenging radicals at this bond is well recognized to be responsible for the antioxidant property of PLE (*8*−*10*). It has been proved that the inhibitory mechanism of PLE against lipid peroxidation is based not merely on iron chelation (*48*). Overall, the results obtained so far are consistent with the suggestion that PLE interferes with the propagation, rather than the initiation, of lipid peroxidation (*11*).

The PLE used in the present study originates from bovine brain tissue, and thus represents a unique class of glycerophospholipids rich in PUFA, such as docosahexaenoic acid and arachidonic acid. The action of scavenging radicals at the vinyl ether bond involves specific destruction of the PLE molecule, and this is proposed to involve characteristic reactivity imparted by the polyunsaturated fatty acyl group esterified at the sn-2 position (*49*). PLE is suggested to undergo oxidative destruction more easily than the diacyl analogue, phosphatidylethanolamine. If the protective activity of PLE in the external layer is dependent on this vulnerability to oxidative destruction, then the proportion of unoxidized PUFA remaining after AAPH-mediated lipid peroxidation cannot be higher in liposomes containing dioleoylphosphatidylcholine than in those containing dipalmitoylphosphatidylcholine (**Figure 3A,B**). Hence, the protective activity of the external PLE may be related to some other undetermined factor, as observed in liposomes rich in external phosphatidylethanolamine, which has no vinyl ether bond.

On the other hand, it has been shown that phosphatidylserine in phospholipid bilayers also possesses antioxidant activity during iron-induced lipid peroxidation (*13*, *14*). This antioxidant activity of phosphatidylserine was suggested to be due to not only its iron-binding ability but also its effective prevention of preformed hydroperoxide decomposition (*14*). Since the amidinium groups of AAPH typically exhibit a $pK_a > 12$ (*50*), AAPH-derived radicals ought to be charged positively, as shown

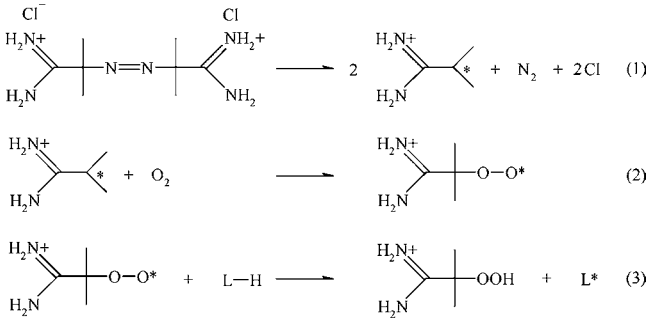

Figure 6. Commonly accepted mechanism to explain the initiation of lipid peroxidation with AAPH. PC16:0, dipalmitoylphosphatidylcholine; PC18:1, dioleoylphosphatidylcholine.

in **Figure 6**. The pK_a of the carboxyl group of phosphatidylserine in liposomes with different phosphatidylserine/phosphatidylcholine lipid ratios is 3.6 ± 0.1 (*51*). Therefore, a negatively charged carboxyl group of external phosphatidylserine in liposomes may be able to trap the AAPH-derived radicals more effectively than that of internal phosphatidylserine, until the negative charges of the external layer become saturated by trapping the positively charged amidinium groups. Indeed, as shown in **Figure 3C**, the percentage of residual unoxidized linoleic acid of PS/PC18:1 did not change at 20 h and was significantly higher than that of PS/PC16:0, but no significant differences between the two phosphatidylcholine species were observed after incubation times of 44 h and beyond. Furthermore, if decomposition of generated hydroperoxide is effectively prevented by another type of antioxidant activity of phosphatidylserine, as pointed out by Dacaranhe and Terao (*14*), the propagation of lipid peroxidation in phosphatidylserine might be suppressed, although the mechanism responsible for prevention of hydroperoxide decomposition remains to be clarified.

Although phosphatidylethanolamine plasmalogen and phosphatidylserine show characteristically different inhibitory actions against lipid peroxidation, as mentioned above, the important point to note is that the unique protective activity of external primary aminophospholipids in liposomes is not the self-sacrificing form of antioxidation that is observed for antioxidants in general. This raises the question of the unique protective activity of the external aminophospholipids in liposomes observed in the present study.

The rate of peroxidation of methyl linoleic acid with AAPH as an initiator in Hepes buffer was markedly increased as the pH rose from 5 to 7 and reached a plateau at about pH 8 (*52*). Since Hepes buffer at pH 7.6 was used in the present study, the rate of lipid peroxidation would have been almost maximal. The commonly accepted mechanism for explaining the initiation of lipid peroxidation by AAPH is given in **Figure 6**. The pK_a of the amino group of phosphatidylethanolamine in phosphatidylethanolamine/phosphatidylcholine liposomes is 9.6 ± 0.1 and that of phosphatidylserine in liposomes with different phosphatidylserine/phosphatidylcholine ratios is 9.8 ± 0.1 (*51*). These pK_a values are independent of both the aqueous-phase ionic strength and the liposomal surface potential due to the presence of these partially charged lipids (*51*). So, under these experimental conditions, the primary amino group of phosphatidylethanolamine, phosphatidylethanolamine plasmalogen, or phosphatidylserine and the amidinium groups of AAPH-derived radicals ought to exist in the protonated ionic form, and thus there must be high repulsion between the primary amino groups in the external layer and AAPH-derived radicals (**Figure 5B**). Specifically, a stronger barrier would be formed at the interface between the solution and the liposomal external layer, leading to enhancement of the oxidative stability of liposomes rich in external primary aminophospholipids as illustrated in **Figure 5B**.

On the other hand, the results shown in **Figure 3D,E** suggest that the antioxidant ability of external phosphatidylcholine is very feeble compared with that of the primary aminophospholipids in the external layer. The choline base of the phosphatidylcholine molecule is a quaternary ammonium salt. One positive charge of the choline base is surrounded by three methyl groups and a methylene group and is thus separated from the aqueous phase. Consequently, the external phosphatidylcholine might not be able to protect its component PUFA from AAPH-derived radicals (**Figure 5**). An electrostatic surface such as that afforded by the primary amino group at the interface of the membrane may alter the intensity of interaction between the AAPH-derived radicals and the lipid core.

At present, since research aimed at the establishment of liposome technology and commercialization of functional liposomes is being carried out intensively in the food industry, our present observations are expected to be applicable to the development of functional foods and/or dietary supplements containing oxidatively more stable PUFAs as liposomes, which might allow, for example, their shelf life to be extended soon. In conclusion, we have shown in this study that a difference in the acyl chain region of the phosphatidylcholine molecule modulates the transbilayer distribution of the coexisting phospholipid in liposomes, and that phosphatidylethanolamine plasmalogen and/or phosphatidylserine in the external layer of liposomes, as well as external phosphatidylethanolamine, protect their component PUFA from AAPH-mediated lipid peroxidation. These observations may be applicable to the development of oxidatively stable docosahexaenoic acid-containing functional food materials and/or dietary supplements.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; FAME, fatty acid methyl esters; GLC, gas−liquid chromatography; PC16:0, dipalmitoylphosphatidylcholine; PC18:1, dioleoylphosphatidylcholine; PC-DHA, docosahexaenoic acid-rich phosphatidylcholine; PE-AA, arachidonic acid-rich phosphatidylethanolamine; PE-DHA, docosahexaenoic acid-rich phosphatidylethanolamine; PLE, phosphatidylethanolamine plasmalogen; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid.

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LITERATURE CITED

(1) Litman, B. J. Lipid model membranes. Characterization of mixed phospholipid vesicles. *Biochemistry* **1973**, *12*, 2545−2554.
(2) Litman, B. J. Determination of molecular asymmetry in the phosphatidylethanolamine surface distribution in mixed phospholipid vesicles. *Biochemistry* **1974**, *13*, 2844−2848.
(3) Nordlund, J. R.; Schmidt, C. F.; Thompson, T. E. Transbilayer distribution in small unilamellar phosphatidylglycerol−phosphatidylcholine vesicles. *Biochemistry* **1981**, *20*, 6415−6420.
(4) Kumar A.; Gupta, C. M. Effect of altered polar headgroup of phosphatidylethanolamines on transbilayer aminophospholipid distribution in sonicated vesicles. *Biochim. Biophys. Acta* **1983**, *730*, 1−9.

(5) Wang, J. Y.; Wang, Z. Y.; Kouyama, T.; Shibata T.; Ueki T. Significance of amino groups of phosphatidylethanolamine in phospholipid peroxidation of mixed liposomes. *Chem. Phys. Lipids* **1994**, *71*, 197−203.
(6) Wang, J. Y.; Miyazawa, T.; Fujimoto, K.; Wang Z. Y.; Nozawa, T. The inverted hexagonal phase is more sensitive to hydroperoxidation than the multilamellar phase in phosphatidylcholine and phosphatidylethanolamine aqueous dispersions. *FEBS Lett.* **1992**, *310*, 106−110.
(7) Kubo, K.; Sekine, S.; Saito, M. Docosahexaenoic acid-containing phosphatidylethanolamine in the external layer of liposomes protects docosahexaenoic acid from 2,2′-azobis(2-aminopropane)dihydrochloride-mediated lipid peroxidation. *Arch. Biochem. Biophys.* **2003**, *410*, 141−148.
(8) Zoeller, R. A.; Lake, A. C.; Nagan, N.; Gaposchkin, D. P.; Legner, M. A.; Lieberthal, W. Plasmalogens as endogenous antioxidants: Somatic cell mutants reveal the importance of the vinyl ether. *Biochem. J.* **1999**, *338*, 769−776.
(9) Reiss, D.; Beyer, K.; Engelmann, B. Delayed oxidative degradation of polyunsaturated diacyl phospholipids in the presence of plasmalogen phospholipids in vitro. *Biochem. J.* **1997**, *323*, 807−814.
(10) Khaselev, N.; Murphy, R. C. Structural characterization of oxidized phospholipid products derived from arachidonate-containing plasmenyl glycerophosphocholine. *J. Lipid Res.* **2000**, *41*, 564−572.
(11) Murphy, R. C. Free-radical-induced oxidation of arachidonoyl plasmalogen phospholipids: antioxidant mechanism and precursor pathway for bioactive eicosanoids. *Chem. Res. Toxicol.* **2001**, *14*, 463−472.
(12) Khaselev, N.; Murphy, R. C. Susceptibility of plasmenyl glycerophosphoethanolamine lipids containing arachidonate to oxidative degradation. *Free Radical Biol. Med.* **1999**, *26*, 275−284.
(13) Yoshida, K.; Terao, J.; Suzuki, T.; Takama, K. Inhibitory effect of phosphatidylserine on iron-dependent lipid peroxidation. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 1077−1081.
(14) Dacaranhe, C. D.; Terao, J. A unique antioxidant activity of phosphatidylserine on iron-induced lipid peroxidation of phospholipid bilayers. *Lipids* **2001** *36*, 1105−1110.
(15) American Institute of Nutrition. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. *J. Nutr.* **1977**, *107*, 1340−1348.
(16) American Institute of Nutrition. Second report of the Ad Hoc Committee on Standards for Nutritional Studies. *J. Nutr.* **1980**, *110*, 1726.
(17) Folch, J.; Lees, M.; Sloan-Stanley, G. A. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−507.
(18) Juaneda, P.; Rocquelin, G. Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. *Lipids* **1985**, *20*, 40−41.
(19) Saito, M.; Nakatsugawa, K.; Oh-hashi, A.; Nishimuta, M.; Kodama, N. Comparison of vitamin E levels in human plasma, red blood cells, and platelets following varying intakes of vitamin E. *J. Clin. Biochem. Nutr.* **1992**, *12*, 59−68.
(20) Marinetti, G. V. Chlomatographic separation, identification, and analysis of phosphatides. *J. Lipid Res.* **1962**, *3*, 1−20.
(21) Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1959**, *234*, 466−468.
(22) Sarti, P.; Molinari, A.; Arancia, G.; Meloni, A.; Citro, G. A modified spectroscopic method for the determination of the transbilayer distribution of phosphatidylethanolamine in soyabean asolectin small unilamellar vesicles. *Biochem. J.* **1995**, *312*, 643−648.
(23) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.
(24) Kates, M. *Techniques of Lipidology*; Elsevier Science: Amsterdam, 1986; pp 113−115 and 248−250.
(25) Dixon, W. J.; Massey, F. J., Jr. Inference: Two populations. *Introduction to statistical analysis*; McGraw-Hill Publishing Co.: New York, 1983; pp 116−131.
(26) McArdle, B. H. The significance of differences between means: A simulation study. *Comp. Biochem. Physiol. A* **1987**, *87*, 979−982.
(27) Kubo, K.; Saito, M.; Tadokoro, T.; Maekawa, A. Dietary docosahexaenoic acid dose not promote lipid peroxidation in rat tissue to the extent expected from peroxidizability index of the lipids. *Biosci. Biotechnol. Biochem.* 1998, *62*, 1698−1706.
(28) Kubo, K.; Saito, M.; Tadokoro, T.; Maekawa, A. Preferential incorporation of docosahexaenoic acid into nonphosphorus lipids and phosphatidylethanolamine protects rats from dietary DHA-stimulated lipid peroxidation. *J. Nutr.* 2000, *130*, 1749−1759.
(29) Kitajka, K.; Puskas, L. G.; Zvara, A.; Hackler, L. Jr; Barcelo-Coblijn, G.; Yeo, Y. K.; Farkas, T. The role of n-3 polyunsaturated fatty acids in brain: modulation of rat brain gene expression by dietary n-3 fatty acids. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2619−2624.
(30) Svennerholm, L. Distribution and fatty acid composition of phosphoglycerides in normal human brain. *J. Lipid Res.* **1968**, *9*, 570−579.
(31) Berden, J. A.; Barker, R. W.; Radda, G. K. NMR studies on phospholipid bilayers. Some factors affecting lipid distribution. *Biochim. Biophys. Acta* **1975**, *375*, 186−208.
(32) Massari, S.; Pascolini, D.; Gradenigo, G. Distribution of negative phospholipids in mixed vesicles. *Biochemistry* **1978**, *17*, 4465−4469.
(33) Papahadjopoulos, D.; Miller, N. Phospholipid model membranes. I. Structural characteristics of hydrated liquid crystals. *Biochim. Biophys. Acta* **1967**, *135*, 624−638.
(34) Eigenberg, K. E.; Chan, S. I. The effect of surface curvature on the headgroup structure and phase transition properties of phospholipid bilayer vesicles. *Biochim. Biophys. Acta* **1980**, *599*, 330−335.
(35) Thomas, P. D.; Poznansky, M. J. Curvature and composition-dependent lipid asymmetry in phosphatidylcholine vesicles containing phosphatidylethanolamine and gangliosides. *Biochim. Biophys. Acta* **1989**, *978*, 85−90.
(36) Koynova, R. D.; Tenchov, B. G. Effect of ion concentration on phosphatidylethanolamine distribution in mixed vesicles. *Biochim. Biophys. Acta* **1983**, *727*, 351−356.
(37) Cullis, P. R.; de Kruijff, B. The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A ^{31}P NMR study. *Biochim. Biophys. Acta* **1978**, *513*, 31−42.
(38) Cullis, P. R.; de Kruijff, B. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* **1979**, *559*, 399−420.
(39) Nordlund, J. R.; Schmidt, C. F.; Dicken, S. N.; Thompson, T. E. Transbilayer distribution of phosphatidylethanolamine in large and small unilamellar vesicles. *Biochemistry* **1981**, *20*, 3237−3241.
(40) Yazu, K.; Ukegawa, K.; Yamamoto, Y.; Niki, E. Effects of the nitrogen moieties of phosphatidylethanolamine and phosphatidylcholine on the autoxidation of these phospholipids in aqueous dispersions. *J. Jpn. Oil Chem. Soc.* **1994**, *43*, 350−356.
(41) Kawakatsu, M.; Terao, J.; Matsushita, S. Phospholipid oxidation catalyzed by ferrous ion and ascorbic acid. *Agric. Biol. Chem.* **1984**, *48*, 1275−1279.
(42) Szebeni, J.; Toth, K. Lipid peroxidation in hemoglobin-containing liposomes. Effects of membrane phospholipid composition and cholesterol content. *Biochim. Biophys. Acta* **1986**, *857*, 139−145.
(43) Morita, M.; Fujimaki, M. Characterization of the copper-binding of phosphatidylethanolamine emulsion in its rapid autoxidation. *Agric. Biol. Chem.* **1972**, *36*, 1751−1754.
(44) Terao, J. Stabilization of phosphatidylethanolamine hydroperoxides in human plasma. *Biochim. Biophys. Acta* **1989**, *1003*, 221−224.
(45) Tappel, A. L. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. 4, pp 2−47.

(46) Shimasaki, H.; Ueta, N.; Mowri, H. O.; Inoue, K. Formation of age pigment-like fluorescent substances during peroxidation of lipids in model membranes. *Biochim. Biophys. Acta* **1984**, *792*, 123−129.

(47) Zommara, M.; Tachibana, N.; Mitsui, K.; Nakatani, N.; Sakono, M.; Ikeda, I.; Imaizumi, K. Inhibitory effect of ethanolamine plasmalogen on iron- and copper-dependent lipid peroxidation. *Free Radical Biol. Med.* **1995**, *18*, 599−602.

(48) Sindelar, P. J.; Guan, Z.; Dallner, G.; Ernster, L. The protective role of plasmalogens in iron-induced lipid peroxidation. *Free Radical Biol. Med.* **1999**, *26*, 318−324.

(49) Khaselev, N.; Murphy, R. C. Susceptibility of plasmenyl glycerophosphoethanolamine lipids containing arachidonate to oxidative degradation. *Free Radical Biol. Med.* **1999**, *26*, 275−284.

(50) Sykes, P. A. *Guidebook to mechanism in organic chemistry*, 6th ed.; Longman: New York, 1986; pp 66−69.

(51) Tsui, F. C.; Ojcius, D. M.; Hubbell, W. L. The intrinsic pK_a values for phosphatidylserine and phosphatidylethanolamine in phosphatidylcholine host bilayers. *Biophys. J.* **1986**, *49*, 459−468.

(52) Hanlon, M. C.; Seybert, D. W. The pH dependence of lipid peroxidation using water-soluble azo initiators. *Free Radical Biol. Med.* **1997**, *23*, 712−719.

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