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The inverted hexagonal phase is more sensitive to hydroperoxidation than the multilamellar phase in phosphatidylcholine and phosphatidylethanolamine aqueous dispersions

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The effect of phase behaviour (hexagonal II phase and lamellar phase) on the peroxidation of membrane phospholipids has been investigated in dilinoleoyl phosphatidylcholine (DLPC)/dilinoleoyl phosphatidylethanolamine (DLPE) aqueous dispersions. Peroxidation was initiated with a water-soluble radical inducer 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPN). The phospholipid morphology was monitored by ^{31}P-nuclear magnetic resonance (NMR). Phospholipid hydroperoxides (PCOOH and PEOOH) were determined by chemiluminescence high-performance liquid chromatography (CL–HPLC). In pH-induced phase transition systems, DLPE in the bilayer state was much less oxidized than in the hexagonal II state. In composition-induced phase transition systems, the formation of total hydroperoxides and the consumption of α-tocopherol in the hexagonal II phase were greater than in the bilayer phase. These data suggest that the hexagonal II phase is more sensitive to hydroperoxidation than the bilayer phase in phospholipid aqueous dispersions.

Lamellar; Hexagonal; Hydroperoxide; Phosphatidylcholine; Phosphatidylethanolamine; Peroxidation

1. INTRODUCTION

In recent years there has been much interest in the bilayer-to-inverted-hexagonal (Lα–HII) transition zone, because it is thought that the presence of the non-lamellar structures involves many important physiological phenomena of the membrane including membrane fusion, protein insertion, and increased membrane activity [1–3]. It has been demonstrated that under physiological conditions many membrane lipids can adopt non-lamellar lipid structures such as the inverted hexagonal (HII) phase. PE is a good example of such a lipid; it can undergo a reversible transition from the lamellar to the hexagonal II phase depending on pH and temperature [4].

Lipid peroxidation in biological membranes has been focused on, because its participation in the development of a great variety of human diseases has been suggested [5–8]. The lipid peroxidation cannot only cause chemical modification of membrane components such as membrane proteins [9,10], but also affect the molecular dynamics of phospholipids in membranes, such as enhancement of the transbilayer movements of phospholipids in mammalian cell membranes [11], increase in the rigidity of the outer layer of neuronal membranes [12], increase in the Lα–HII phase transition temperature of egg-PE in aqueous dispersions [13], and induction of lipid vesicle fusion [14]. Gel-to-liquid crystalline phase transition, on the other hand, which involves an order-to-disorder transition of lipid fatty acyl chains in the lamellar form, was found to affect the peroxidation rate of arachidonic acid in small unilamellar vesicles of dipalmitoylphosphatidylcholine [15]. Actually, the geometrical packing of lipids in the non-lamellar phase transition changes more drastically, but its effect on phospholipid peroxidation of the membrane has been little studied. Many studies on the antioxidative mechanism of biomembranes have been devoted to a major lipid soluble antioxidant, α-tocopherol, although it is a minor constituent in biomembranes. α-tocopherol has also a stabilizing effect on membranes either by forming

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Abbreviations: Lα, lamellar; HII, inverted hexagonal; PC, phosphatidylcholine; PE, phosphatidylethanolamine; AAPN, 2,2′-azobis (2-amidino-propane) dihydrochloride; NMR, nuclear magnetic resonance; DLPC, L-α-dilinoleoyl phosphatidylcholine; DLPE, L-α-dilinoleoyl phosphatidylethanolamine; α-Toc, D-α-tocopherol; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; BHT, butylated hydroxytoluene; MLV, multilamellar vesicles; CL-HPLC, chemiluminescence high-performance liquid chromatography; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.

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complexes with potentially toxic unsaturated fatty acids or by restricting the molecular mobility of the membrane components [16,17].

In the present study, DLPC and DLPE were used to prepare various compositions of phospholipid aqueous dispersions. The hexagonal II structure was obtained either by acidification of the DLPE dispersing matrix or by enhancement of the DLPE ratio in DLPC/DLPE mixtures. PC hydroperoxide (PCOOH) and PE hydroperoxide (PEOOH) were determined individually by the CL-HPLC method. ^{31}P-NMR, which has been very extensively and successfully used to study the HII phase in phospholipid systems, was used to monitor the structural organization of the phospholipid dispersions. The effect of the phase state on α-tocopherol consumption during peroxidation was also examined.

2. MATERIALS AND METHODS

2.1. *Materials*

DLPC and DLPE (>99.9%) were purchased from Avanti Polar Lipids (Birmingham, AL). Horse heart cytochrome *c* (type VI) was from Sigma (St. Louis, MO, USA). Luminol (3-aminophthaloylhydrazine) and AAPN [2,2'-azobis (2-amidino-propane) dihydroxychloride] were from Wako Pure Chemical Industries (Osaka, Japan). D-α-tocopherol (α-Toc, 98.5% purity) and 2,2,5,7,8-pentamethyl-6-hydrochroman (PMC) were generous gifts of Eisai Co., Ltd. (Tokyo, Japan). D_2O (99.8%) was from CEA (France). Phospholipids were stored at −20°C until use.

2.2. *Preparation of multilamellar lipid dispersions*

The phospholipid mixture in chloroform was dried by an evaporator to form a thin film on the glass vial wall. Residual trace chloroform was removed under a stream of nitrogen. Multilamellar vesicles (MLV) were prepared by vortexing the dry lipids with buffers vigorously for 30 s. The suspensions were further incubated overnight at 4°C in the dark to ensure proper hydration of the samples. For pH-sensitive dispersions, 5 mM borate buffer (150 mM NaCl, 0.1 mM EDTA, pH 9.5) and its acetic acid-adjusted buffer (pH 5.0) were used to suspend DLPE and DLPC/DLPE (1:1 in molar ratio) [18]. The final phospholipid concentrations were about 0.5 mM, which were determined by phosphorus analysis [19]. For α-Toc-containing dispersions, chloroform solutions of DLPC and DLPE in molar ratios of 1:0, 1:1, 1:4 and 0:1 were mixed with α-Toc (PMC as an internal standard, 0.5 mol% of α-Toc and 0.3 mol% of PMC in total phospholipids), and suspended in 10 mM Tris-HCl buffer (154 mM NaCl, pH 7.4). The final phospholipid concentrations were adjusted to 10 μmol/ml.

2.3. *Peroxidation measurements*

AAPN (10 mM at final concentration) was added to phospholipid dispersions. After various intervals of incubation at 37°C, the phospholipids were extracted with chloroform/methanol (2:1, v/v) containing 0.002% BHT. The chloroform layer was injected directly into a CL-HPLC unit for phospholipid hydroperoxide analysis [20–22]. The CL-HPLC system included a JASCO Finepak SIL-NH_2 column (5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Ltd., Tokyo, Japan) which was placed in a JASCO 860-CO oven at 40°C, with a mobile phase of hexane/isopropanol/methanol/water (5:7:2:1, v/v/v/v) at a flow rate of 1.1 ml/min by a JASCO 880-PU pump. In the post-column detection system, phospholipid absorption at 210 nm was monitored with a JASCO 875-UV detector and hydroperoxide-specific chemiluminescence was measured with a JASCO 825-CL detector. The CL reagent was a mixture of 10 mg/l of cytochrome *c* and 2 mg/l of luminol in 50 mM borate buffer at pH 10.0. As calibration curves of PCOOH and PEOOH almost overlap each other, the PCOOH of egg yolk PC was used as a standard for preparing a calibration curve for both PCOOH and PEOOH [22]. The concentration of authentic PCOOH was determined iodometrically according to the official method of the Japan Oil Chemists' Society [23]. The content of α-Toc was determined by HPLC with a fluorescent detector [24].

2.4. *^{31}P-NMR of hydrated phospholipids*

Lipid dispersions prepared by the method described above were centrifuged, and the pellets were transferred into 5 mm NMR tubes using 0.5 ml of various buffers which contained 10% of D_2O. About 20 mg phospholipids were used for each determination. ^{31}P-NMR measurements were performed at 37°C with a Bruker MSL-400 NMR spectrometer. Accumulated free induction decays were obtained from up to 10,000 transients employing a 15 μs 90° r.f. pulse, a 30 kHz sweep width and gated proton decoupling with an interpulse time of 0.1–0.5 s. Chemical shifts were measured from 85% phosphoric acid in D_2O as an external reference.

3. RESULTS

3.1. *^{31}P-NMR spectra in pH-induced phase transition systems*

Pure DLPE aqueous dispersions at pH 5.0 gave a spectrum with the characteristic asymmetry (a high-field shoulder and a low-field peak) and a chemical shift anisotropy of about 25 ppm. This shape is typical for the HII phase. When the pH of the dispersing matrix was increased to 9.5, the spectrum shifted to the low-field shoulder and high-field peak, and was separated by approximately 40 ppm. Although a small isotropic signal appeared in the spectrum, the lineshape is typical of the bilayer (Fig. 1). The result is fully consistent with that observed for egg PE by the fluorometric method [18,25].

We further investigated by ^{31}P-NMR the phase behaviour of the mixture of DLPC/DLPE in a molar ratio of 1:1 at both pHs. Both of them show a spectrum of the bilayer type, which indicates that the morphology

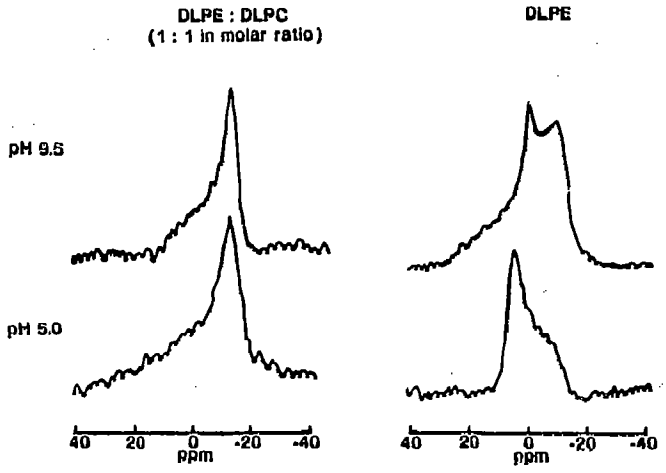

Fig. 1. ^{31}P-NMR spectra of phospholipid aqueous dispersions composed of DLPC and DLPE at pH 9.5 and pH 5.0.

of the DLPC/DLPE aqueous dispersions was not affected by pH (Fig. 1).

3.2. *Peroxidation of phospholipids in pH-dependent phase transition systems*

The effect of pH on lipid peroxidation of DLPC and DLPE in aqueous dispersions was assessed by measurements of hydroperoxide formation using the CL-HPLC method. In pure DLPE systems, PEOOH formation at pH 5.0 was greater than that at pH 9.5 (Fig. 2c). In the DLPC/DLPE (1:1 in molar ratio) system, however, both PCOOH and PEOOH were formed more significantly at high pH (Fig. 2a,b). As shown in Fig. 1, the pH did not affect the ^{31}P-NMR characteristics of the lamellar DLPC/DLPE system, but a bilayer-type spectrum was obtained in the pure DLPE system when the pH was enhanced to 9.5. These data indicate that DLPE was more peroxidized at the hexagonal II phase than at the bilayer phase.

3.3. *^{31}P-NMR spectra of phospholipid dispersions with various ratios of DLPC and DLPE*

Pure DLPC and the mixture of DLPC/DLPE in a molar ratio of 1:1 show spectra with the asymmetrical lineshape characteristic of bilayer structures. High content of DLPE (above 80 mol% of total phospholipids) induced a shift to lineshapes with a reversed asymmetry and a reduced line width, typical of phospholipid molecules organized in a hexagonal II phase (Fig. 3). These data are in agreement with the egg PC/egg PE systems reported by Hui et al. [26].

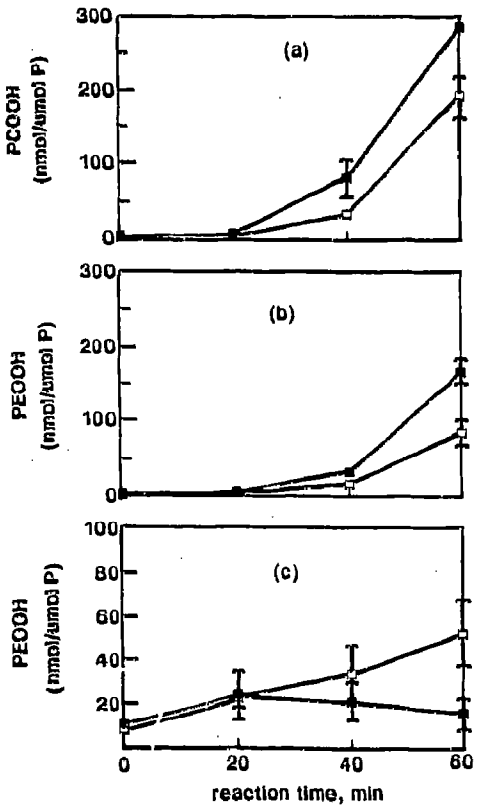

Fig. 2. Effect of pH on peroxidation of (a) DLPC and (b) DLPE in DLPC/DLPE (1:1 in molar ratio) dispersion and (c) pure DLPE dispersion. The extent of phospholipid peroxidation is expressed as levels of PCOOH and PEOOH, which were determined by the CL-HPLC method. □, pH 5.0; ■, pH 9.5. Bars represent mean ± S.D., $n = 4$.

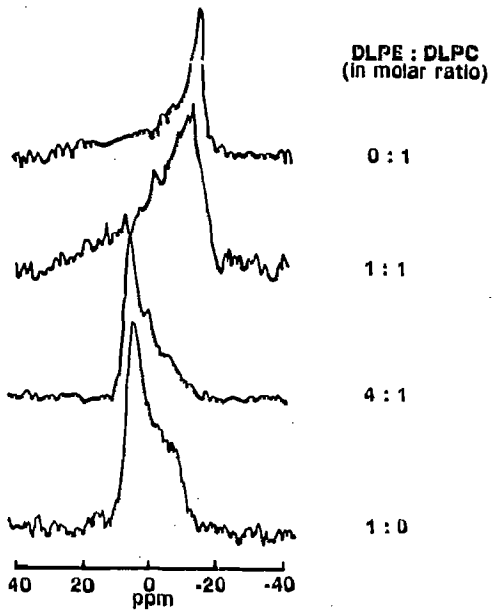

Fig. 3. ^{31}P-NMR spectra of phospholipid aqueous dispersions composed of various ratios of DLPC and DLPE (pH 7.4).

3.4. *Hydroperoxide formations and α-tocopherol consumption in phospholipid composition-dependent phase transition systems*

Equal concentrations of phospholipid aqueous dispersions composed of various ratios of DLPC and DLPE and 0.5 mol% α-Toc and 0.3 mol% PMC were oxidized by AAPN. Formations of total hydroperoxide in DLPC/DLPE (1:4 in molar ratio) and pure DLPE aqueous dispersions were much higher than those in DLPC/DLPE (1:1 in molar ratio) and pure DLPC aqueous dispersions (Table I). α-Toc was also consumed more quickly in DLPC/DLPE (1:4 in molar ratio) and in pure DLPE aqueous dispersions than in DLPC/DLPE (1:1 in molar ratio) and pure DLPC aqueous dispersions (Table I). These data suggested strongly that not only are the phospholipids in HII phase more sensitive to peroxidation, but also that α-Toc is more easily consumed in the hexagonal II phase than in the lamellar phase.

4. DISCUSSION

The aim of the present study was to establish whether the phase behaviour (Lα and HII) of DLPC and DLPE aqueous dispersions affects the protective ability of the membrane against peroxidation.

Table I

Comparison of the formation of PCOOH, PEOOH and α-tocopherol consumption between the Lα phase and HII phase in DLPC/DLPE aqueous dispersions, which contained equal concentrations of total phospholipids (pH 7.4). Samples were subjected to peroxidation with AAPN for 4 h at 37°C. Values for hydroperoxide levels are expressed as pmol OOH per μmol P; values for α-tocopherol consumption are expressed as the peak height of PMC to the peak height of α-tocopherol in HPLC charts.

		DLPC/DLPE (μmol/μmol)			
		1/0	1/1	1/4	0/1
0 h	PCOOH	145.6 ± 9.8	104.4 ± 40.6	112.4 ± 51.3*	
	PEOOH		77.8 ± 13.7	163.2 ± 0.4	130.7 ± 63.3
	Total OOH	145.6 ± 9.8	182.2 ± 40.6	275.6 ± 51.3	130.7 ± 63.3
	α-toc	0.80 ± 0.03	0.75 ± 0.07	0.74 ± 0.07	0.82 ± 0.06
4 h	PCOOH	229.0 ± 24.2	142.3 ± 34.8	257.5 ± 28.9	
	PEOOH		100.2 ± 36.8	430.8 ± 93.6	676.9 ± 251.3
	Total OOH	229.0 ± 24.2[a]	242.5 ± 36.8[a]	688.3 ± 93.6[b]	676.9 ± 251.3[b]
	α-toc	0.99 ± 0.03[a]	1.03 ± 0.14[a]	2.15 ± 0.24[b]	4.11 ± 0.84[b]

Values are mean ± S.D., $n = 3$ (*$n = 2$). Values with different superscripts in the same line are significantly different ($P < 0.05$, Student's t-test).

In a wide concentration range, unsaturated PE does not form closed vesicles at neutral pH. It can form the lamellar structure, however, at high pH or in combination with another component [27,28]. According to the method of Nieva et al., PE dispersions at pH 5.0 and pH 9.5 were prepared. The latter has been reported to form a bilayer structure [18]. ^{31}P NMR spectra of DLPE at pH 5.0 (Fig. 1) exhibited a typical hexagonal II phase. At pH 9.5, the characteristic spectrum of the bilayer structure appeared. DLPC/DLPE (1:1 in molar ratio) aqueous dispersion was used as a control which took a bilayer structure at both the pH values (Fig. 1). It should be noted that the phospholipid concentration (approx. 50 mM) used for ^{31}P-NMR determination was about 5–100 times higher than that used in the peroxidation experiments. Hong et al. have reported that the bilayer-to-hexagonal transition of egg yolk PE dispersions measured by the fluorometric method is consistent with result obtained by differential scanning calorimetry, although the phospholipid concentrations used in the two methods differ greatly [25]. Therefore, the NMR spectra observed here can provide reliable information about the polymorphism in phospholipid-water systems.

Quantitative measurements of PCOOH and PEOOH in phospholipid dispersions of DLPC/DLPE (1:1 in molar ratio) revealed that both DLPC and DLPE were oxidized more easily at higher than at lower pH. The phase states of phospholipid were not affected by the pHs. In contrast, pH-dependent phase transition from the hexagonal II phase to the bilayer phase in pure DLPE dispersions showed the tendency that little PEOOH was formed at higher pH. It has been reported that the oxidation rate of DLPE in an emulsion state is in the order of pH 8.0>7.0>5.8 without addition of ferrous ion [29]. The mechanism for the acceleration of PE oxidation at pH 8.0 is deduced as follows. At alkaline pH, the $-NH_2$ form is predominant over the $-NH_3^+$ form in the amino group of PE. The former seems to be responsible for homolysis of hydroperoxides resulting in significant autoxidation of PE at pH 8.0 [30]. We consider that this deduction is also applicable to explain the result of DLPE peroxidation in DLPC/DLPE systems. For pure DLPE dispersions, the explanation may be reliable when the pH is below 8.0, but when the pH is above 8.0, phase transition derived difference in the peroxidation is dominant. The pH-dependent phase transition of unsaturated PE is said to occur only with increase of the pH above 8–9 [31,32].

Although pH can modify the molecular packing of PC in the bilayer, for example, acidification induces reduction to the degree of disorder and motion in the liquid-crystalline state of dipalmitoyl phosphatidylcholine, the major changes in the structure and function of membranes with pH involve PE or negative phospholipids [33]. Our data on phospholipid peroxidation in model membranes also indicate that the difference of the hydroperoxide formation between pH 5.0 and 9.5 in both DLPC/DLPE (1:1 in molar ratio) and pure DLPE systems is derived from the PE component which is pH-sensitive. There was no difference in PCOOH levels between pH 5.0 and 9.5 in pure DLPC MLV dispersions (data not shown).

Recently, a stress model on nonbilayer structure has been proposed [34]. Within the context of this model, the curvature-related lateral stress is a major physiochemical factor which dictates the bilayer-to-nonbilayer phase transition of the lipids. It is further argued that the stress is also present and already developed within the bilayer of cell membranes, which contain the nonbi-

layer preferring lipids. The Lα to HII phase transition involves a change in the lipid/water surface curvature and a topologically discontinuous arrangement of the lipids, which might be contributed to the sensitive peroxidability of the HII phase.

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