Ethanolamine plasmalogens prevent the oxidation of cholesterol by reducing the oxidizability of cholesterol in phospholipid bilayers

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Abstract The aims of the present study are to establish an appropriate system for assessing the oxidizability of cholesterol (CH) in phospholipid (PL) bilayers, and to explore the effect of ethanolamine plasmalogens on the oxidizability of CH with the system, through comparing with those of choline plasmalogens, phosphatidylethanolamine, and antioxidant α-tocopherol (Toc). Investigation of the effects of oxidants, vesicle lamellar forms, saturation level, and constituent ratio of PLs in vesicles on CH oxidation revealed the suitability of a system comprising unilamellar vesicles and the water-soluble radical initiator 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH). As CH oxidation in the system was found to follow the rate law for autoxidation without significant interference from oxidizable PLs, the oxidizability of CH in PL bilayers could be experimentally determined from the equation: $k_p/(2k_t)^{1/2}=R_p/[LH]R_i^{1/2}$ by measuring the rate of CH oxidation. It was found with this system that bovine brain ethanolamine plasmalogen (BBEP), bovine heart choline plasmalogen, and egg yolk phosphatidylethanolamine lower the oxidizability of CH in bilayers. Comparison of the dose-dependent effects of each PL demonstrated the greatest ability of BBEP to reduce the oxidizability. A time course study of CH oxidation suggested a novel mechanism of BBEP for lowering the oxidizability of CH besides the action of scavenging radicals.—Maeba, R., and N. Ueta. **Ethanolamine plasmalogens prevent the oxidation of cholesterol by reducing the oxidizability of cholesterol in phospholipid bilayers. *J. Lipid Res.* 2003.** 44: **164–171.**

Supplementary key words free radicals • unilamellar vesicles • radical initiator • antioxident • phosphatidylethanolamine

Cholesterol (CH) oxidation caused by free radicals in vivo is of considerable interest, as is intake of CH oxidation products in food, owing to potential pathological application as in the case of atherosclerosis (1, 2). CH oxidation is characterized by the following features: CH has a very low ability to propagate radical chain reactions (3), and its oxidation products, i.e., oxysterols, have various biological activities such as cytotoxicity, atherogenicity, mutagenicity, and carcinogenicity (4), in addition to their effects on CH metabolism in cells (5). Considering the characteristics of CH oxidation, in vivo defensive mechanisms appear to differ from those for other oxidizable lipids, such as polyunsaturated fatty acids, possessing a high ability to propagate radical chain reactions. One of the mechanisms of these oxidizable lipids is to suppress the expansion of oxidative injury via active radical species by blocking chain reactions by chain-breaking antioxidants such as α-tocopherol (Toc) (6). On the other hand, in CH oxidation, the most effective way to avoid the damage seems to be to inhibit the formation of oxysterols as far as possible by reducing the susceptibility of CH to radical attack.

Protecting CH in biomembranes from oxidation is especially important, because CH in the phospholipid (PL) bilayer is the form most susceptible to attack by free radicals generated in the water phase (7). CH content in biomembranes differs noticeably among various species of cells or intracellular organelles, and is abundant in nervous-system myelin and red blood cells at almost the equivalent molar ratio of CH to PLs (8, 9). Nervous-system myelin and red blood cells may readily incur oxidative injury, being the sites of oxygen consumption and exposure to oxygen, but the vivo life spans of these tissues are comparatively long. These biomembranes would appear to possess structures capable of resisting oxidative stress, especially CH oxidation.

These biomembranes contain major distribution of 1-*O*-alk-1 eny-2-acyl-*sn*-glycero-3-phosphoethanolamine (i.e., ethanolamine plasmalogen) in glycerophosphoethanolamines (10). Plasmalogens are glycerophospholipids

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Abbreviations: AAPH, 2,2'-azobis (2-amidino-propane) dihydrochloride; BBEP, bovine brain ethanolamine plasmalogen (1-*O*-alk-1 enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine); BHCP, bovine heart choline plasmalogen (1-*O*-alk-1 eny-2-acyl-*sn*-glycero-3-phosphocholine); CH, cholesterol; EYPE, egg yolk phosphatidylethanolamine; LUV, large unilammelar vesicle; PL, phospholipid; Toc, α-tocopherol.

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with vinyl ether double bonds (-CH2-O-CH=CH-) at the *sn*-1 position of the glycerol backbone, and are widely distributed in most mammalian cells and tissues (11). The physiological role of plasmalogens is not fully understood, but recent studies on plasmalogen-deficient mutant cells lead to the proposal that these ether lipids serve to protect cells from oxidative stress as endogenous antioxidants by scavenging radicals at the vinyl ether linkage (12–14). However, their ability to scavenge radicals is far less than that of Toc (15). On the other hand, it is known that ethanolamine plasmalogens have a stronger propensity for hexagonal phase formation than diacyl analog (16), which contributes to membranes fusion (17). Such a modification of the physical features of membranes may serve to reduce the oxidizability of membranes.

Ethanolamine plasmalogens are considered by the authors to prevent the oxidation of CH by lowering susceptibility to attack by free radicals. To confirm this possibility, in the present study an appropriate system for assessing the oxidizability of CH in PL bilayers has been established, and the effect of bovine brain ethanolamine plasmalogen (BBEP) on the oxidizability of CH in bilayers has been explored with this system by comparing them with those of bovine heart choline plasmalogen (BHCP), egg yolk phosphatidylethanolamine (EYPE), and an antioxidant (Toc).

MATERIALS AND METHODS

Materials

CH, 7-ketocholesterol (7K), 7β-hydroxycholesterol (7βOH), CH 5α,6α-epoxide (α-EPOX), cholestane-3β,5α,6β-triol (α-TRIOL), dioleoyl phosphatidylethanolamine (DOPE), bovine brain phosphatidylserine (BBPS), Toc, and L-ascorbic acid (AsA) were purchased from Sigma Chemicals (St. Louis, MO). 1-Radyl-2-acyl-*sn*-glycero-3-phosphoethanolamine from bovine brain (BBPE), BBEP, 1-radyl-2-acyl-*sn*-glycero-3-phosphocholine from bovine heart (BHPC), 1-*O*-alk-1 eny-2-lyso-*sn*-glycero-3-phosphoethanolamine from bovine brain (LyEP), and 1-acyl-2-lyso-*sn*-glycero-3-phosphoethanolamine from porcine liver (LyPE) were obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). EYPE and dimyristoyl phosphatidylcholine (DMPC) were purchased from Nichiyu Liposome Co., Inc. (Tokyo, Japan). Soybean phosphatidylcholine (SPC) was kindly provided form Tsuji Seiyu Co., Inc. (Mie, Japan) and purified by chromatography prior to use. 2,2′-Azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Pure Chemical Industries (Osaka, Japan). 7K was purified by preparative thin layer chromatography prior for use as a standard for analysis. All other chemicals were of the highest purity available from commercial sources.

Purification of BBEP and bovine heart choline plasmalogen

Ethanolamine- and choline-plasmalogens were respectively purified from commercial 1-radyl-2-acyl-*sn*-glycero-3-phosphoethanolamine from bovine brain (BBPE) and 1-radyl-2-acyl-*sn*-glycero-3-phosphocholine from bovine heart (BHPC) with *Rhizopus arrhizus* lipase (18). The purity was estimated as 82–91% for BBEP and 86–94% for BHCP by acid-catalyzed hydrolytic procedure with HCL fumes followed by thin layer chromatography (19) and phosphorus analysis (20). The purified plasmalogens were stored in chloroform at −80°C under N^2 gas atmosphere until use.

Preparation and oxidation of unilamellar and multilamellar vesicles

CH was mixed with various glycerophospholipids and, in some cases, supplemental Toc in chloroform at a specified molar ratio. The solvent was evaporated completely in a stream of nitrogen (N^2) gas. The dried lipids were dispersed in phosphate buffered saline (PBS; 10 mM, pH 7.4) kept at 42°C, above the phase transition temperature for all lipids used, with or without 0.1 mM ethylenediamine tetraacetic acid (EDTA) in a vortex mixer for preparation of multilamellar (ML) vesicles. Large unilamellar vesicles (LUV) were prepared by passing ML vesicles through polycarbonate filters (Corning Glass Works, Corning, NY) of pore sizes 3.0 μm, 1.0 μm, 0.4 μm, and 0.1 μm in that order by N^2 gas pressure with an extruder (Lipex Biomembranes Inc., Vancouver, Canada) (21). The final extrusion was recycled five times with a dual 0.1 μm filter. Vesicle lamellar structure was visually confirmed by negative dyeing with uranyl acetate (Merck, Darmstadt, Germany) under a transmission electron microscope (JEM-2000FX; JEOL, Tokyo, Japan) (22). Particle sizes of vesicles were measured with a light scattering instrument (NICOMP 370; Particle Sizing Systems Inc.) (23). The constituent molar ratio of CH and PLs in vesicles have been checked by means of enzymatic method with a commercial kit for CH (CH C-test; Wako), TLC separation, and phosphorus analysis for PLs after preparation of vesicles with extrusion. The content of Toc in vesicles was checked by high performance liquid chromatography (880-PU; JASCO, Tokyo, Japan) using a Senshupak NH2-1251-N column (4.6 mm × 25 cm; SSC Corporation, Tokyo, Japan) (24). Freshly prepared vesicles were used for oxidation experiments.

The oxidation of vesicles was carried out in an open glass tube, and incubation with 0.4 mM FeSO4 and 4 mM ascorbic acid (Fe/AsA) or 5–100 mM water-soluble azo radical initiator (AAPH) for the designated periods at 37°C with shaking at 150 oscillations per min.

TABLE 1. PLs composition

Component	SPC	BBPE	BBEP	BHPC	BHCP	EYPE
			mol %			
16:0	15.9	6.1	2.4	25.6	0.7	19.5
18:0	4.0	16.1	8.0	5.1		30.7
18:1	11.4	25.4	24.9	20.9	5.7	15.8
18:2	64.0			28.8	26.3	8.9
18:3	5.7			1.3		
20:1		5.3	6.5			
20:3				1.6	3.4	
20:4		6.0	5.0	6.1	8.1	15.4
22:4		6.5	5.5			
22:5						4.1
22:6		7.8	6.3			5.6
16:0 ald		8.3	12.0	10.5	43.7	
18:0 ald		8.2	13.6			
18:1 ald		10.3	15.8			

BBEP, bovine brain ethanolamine plasmalogen; BBPE, bovine brain glycero- phosphoethanolamine; BHCP, bovine heart choline plasmalogen; BHPC, bovine heart glycerophosphocholine; EYPE, egg yolk phosphatidylethanolamine; SPC, soybean phosphotidylcholine. Fatty acid methyl esters and dimethylacetal derivatives from fatty aldehydes analyzed by GC/MS, and shown as means of duplicate determinations.

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Samples preparation for lipid analysis by GC and GC/MS

For CH and oxysterols analyses, lipids were extracted from incubation mixtures by the method of Bligh and Dyer (25) with chloroform-methanol (1:2, v/v) containing butylated hydroxytoluene (50 μg/ml; Sigma) as an antioxidant and 5α-cholestane (100 μg/ml; Sigma) as internal standard. The lipids were subjected to mild saponification with alcoholic KOH (26), and then derived to CH and oxysterols trimethylsilyl ethers (TMSi) with *N,O*-Bis-TMS-trifluoroacetamide-trimethylchlorosilane (5:1, v/v; Tokyo Kasei Kogyo) as described detail in the literature (27).

For quantification of PLs component, fatty acids and fatty aldehydes derived from alkenyl chains of plasmalogens were transesterified with anhydrous HCl-methanol (5% HCl, w/w; Muto Pure Chemicals, Tokyo, Japan) to yield fatty acid methyl esters and dimethylacetal derivatives from fatty aldehydes. The PLs composition used in the present experiments are shown in **Table 1**, and almost agree with those previously reported in the literature (28–30).

Reactivities of plasmalogens and Toc toward galvinoxyl radical

The reactivities of BBEP, lyso ethanolamine plasmalogen (LyEP), and Toc toward galvinoxyl radical were measured with a spectrophotometer equipped with a rapid-mixing stopped-flow apparatus (RX-1000, Applied Photophysics) by following the decrease in maximum absorption of galvinoxyl at 429 nm (31).

RESULTS AND DISCUSSION

Oxidation system for assessing the oxidizability of CH in PL bilayers

To establish an appropriate system for assessing the oxidizability of CH in PL bilayers, the effects of oxidant (Fe/AsA vs. AAPH), vesicle lamellar form [unilamellar (UL) vs. ML], saturation level of phosphatidylcholine [unoxidizable saturated (S) vs. oxidizable unsaturated (U)], and constituent molar ratio of CH to PL in vesicles (CH/PL= 0.2 vs. 1.0) on the percentage of CH oxidized (**Fig. 1**) and oxysterols formed (**Table 2**) were investigated. In oxidation with Fe/AsA, the percentage of CH oxidized was noticeably promoted by the unsaturation and increase in the constituent ratio of phosphatidylcholine in either UL or ML vesicles, which indicates that CH oxidation by Fe/AsA is largely dependent on the amount of unsaturated PLs in vesicles. On the other hand, in oxidation with AAPH, the percentage of CH oxidized was only slightly enhanced by the unsaturation and increase in the constituent ratio of phosphatidylcholine in UL vesicles, although it was doubled by the unsaturation of PL in ML vesicles (Fig. 1). Major oxysterols in the CH oxidation products formed in vesicles were classified into C-7 (isomeric 7α- and 7βOH and 7K) and C-5,6 (isomeric α- and β-EPOX and the reduced form, TRIOL) oxidation products (Table 2). It is known that the latter products are enhanced by the oxidation of unsaturated PLs present with CH (32). Therefore, the degree of involvement of PL oxidation in CH oxidation was estimated by the product ratios of C-5,6 to C-7 oxidation

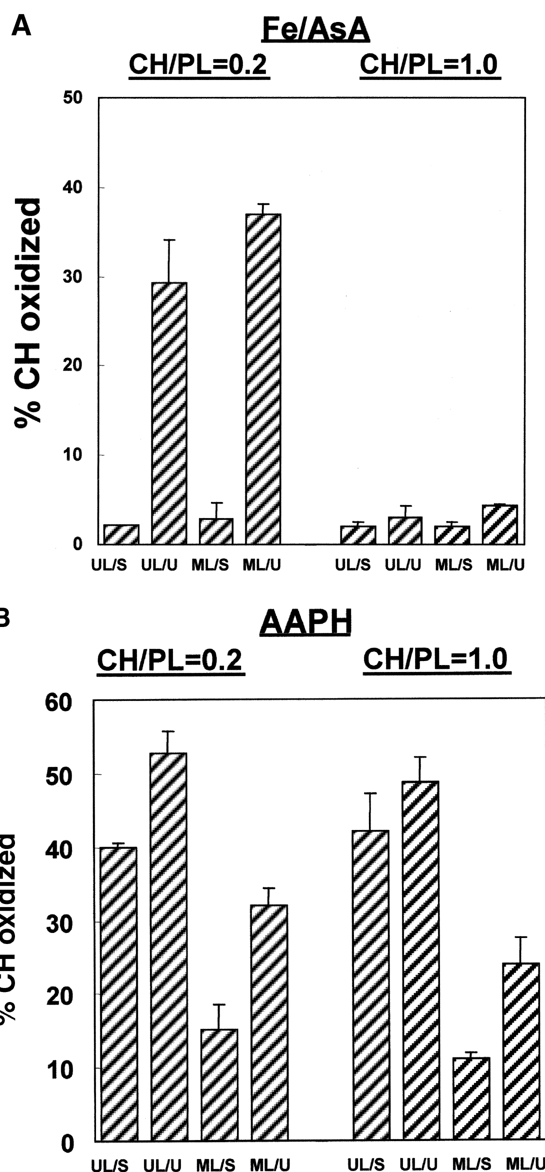

Fig. 1. Comparison of the percentage of cholesterol (CH) oxidized in unilamellar (UL) and multilamellar (ML) vesicles comprising saturated or unsaturated phosphatidylcholine in two different oxidation systems. Vesicles containing CH (∼3 mM) were incubated in PBS with (A) Fe/AsA (0.4 mM/4 mM) in the absence of EDTA, or with (B) 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (10 mM) in the presence of EDTA, at 37°C for 17 h. Vesicles are indicated as UL or ML vesicles composed of saturated dipalmitoyl phosphatidylcholine (UL/S and ML/S) or unsaturated soybean phosphatidylcholine (UL/U and ML/U). CH/phospholipid (PL) (=0.2 or 1.0) is the molar ratio of CH to PL in the vesicles. The % CH oxidized was obtained from triplicate determinations. Columns and bars represent means and standard deviations, respectively.

products. A maximum product ratio, 0.27, was obtained for the oxidation of ML/U vesicles (CH/PL=0.2) with Fe/AsA, indicating the large participation of PL oxidation in CH oxidation. A minimum ratio, 0.08, obtained for UL/S vesicles (CH/PL=1.0) with AAPH represents the formation ratio in the absence of participation of unsaturated PLs. The ratio for UL/U (CH/PL=1.0) with AAPH, 0.14, was significantly smaller than that for ML/U (CH/PL=0.2) with AAPH, 0.22,

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TABLE 2. Major oxysterols formed in vesicles

Vesicle	CH/PL Molar Ratio	Oxidant	CH Oxidized	C-7 Oxidation Products				C-5,6 Oxidation Products				C-5,6/C-7 Products Ratio
				7αOH	7βOH	7K	Total	α-EPOX	β-EPOX	TRIOL	Total	
			%	%[a]				%[a]				
UL/S	1.0	AAPH	42.7 ± 4.9	3.0 ± 0.5	2.2 ± 0.4	32.1 ± 2.3	37.3 ± 2.8	1.1 ± 0.3	0.6 ± 0.1	1.1 ± 0.2	2.8 ± 0.6	0.08
UL/U	1.0	AAPH	49.0 ± 3.4	2.0 ± 0.2	4.0 ± 0.2	19.4 ± 1.2	25.3 ± 1.2	1.4 ± 0	1.9 ± 0	0.2 ± 0.2	3.5 ± 0.3	0.14
ML/U	0.2	AAPH	32.9 ± 2.2	4.7 ± 0.2	7.6 ± 1.2	20.9 ± 0.7	33.2 ± 0.6	3.1 ± 0.2	2.4 ± 0.5	1.9 ± 0.5	7.3 ± 1.2	0.22
ML/U	0.2	Fe/AsA	38.9 ± 1.4	3.9 ± 0.3	5.4 ± 0.2	18.6 ± 0.2	28.0 ± 0.7	3.1 ± 0.2	1.5 ± 0.2	2.9 ± 0.3	7.5 ± 0.4	0.27

AAPH, 2,2'-azobis (2-amidino-propane) dihydrochloride; 7α or 7βOH, 7α- or 7β-hydroxycholesterol; α- or β-EPOX, cholesterol 5α,6α- or 5β,6β-epoxide; 7K, 7-ketocholesterol; TRIOL, cholestane-3β,5α,6β-triol. Experimental conditions the same as in the legend to Fig. 1. CH and oxysterols trimethylsilyl ether derivatives were quantified by GC with 5α-cholestane as the internal standard. Value determination based on triplicate results, expressed as average ± SD.

[a] Percentage is the value for amount of CH oxidized.

indicating the reduced effect of unsaturated PLs in UL vesicles compared with ML vesicles (Table 2). These results suggest that a system comprising UL vesicles and the water-soluble radical initiator AAPH would be most appropriate for assessing the oxidizability of CH in bilayers without significant interference from oxidizable PLs.

To elucidate the characteristics of CH oxidation in the system, the effects of the concentrations of CH and AAPH

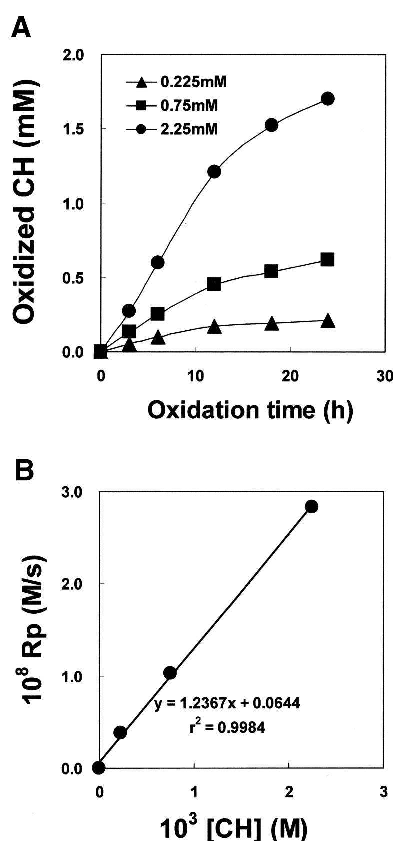

Fig. 2. Effect of the concentration of CH on the rate of CH oxidation in large unilamellar vesicle (LUV) by AAPH. A: LUV composed of CH and dimyristoyl phosphatidylcholine at the equivalent molar ratio (CH/DMPC=1/1) were incubated at indicated concentrations (0.225 mM, 0.75 mM, and 2.25 mM CH) in PBS with 25 mM AAPH in the presence of EDTA at 37°C until 24 h. The amounts of oxidized CH (mM) in each LUV were determined by quantifying unoxidized CH with GC. B: The rates of CH oxidation, R_p (M/s), were estimated from the straight lines obtained within the time holding a linear correlation, and are plotted against the concentration of CH in the reaction mixtures, [CH] (M).

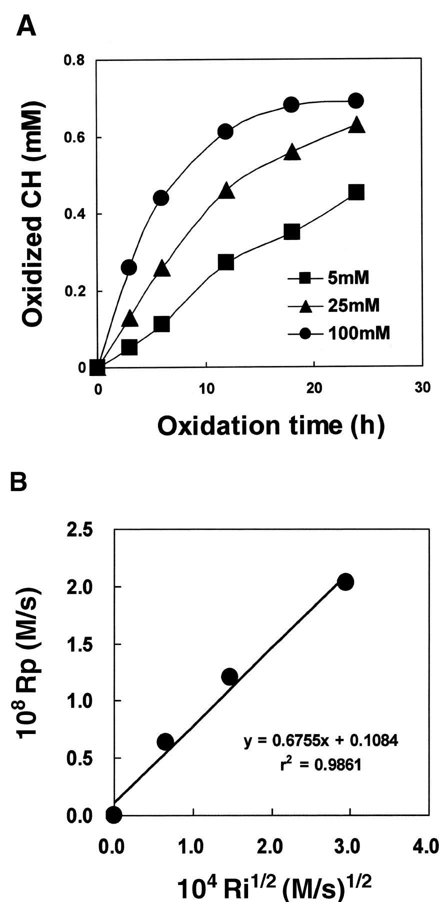

Fig. 3. Effect of the concentration of AAPH on the rate of CH oxidation in LUV. A: LUV (CH/DMPC=1/1, molar ratio, 0.75 mM CH) were incubated in PBS with indicated concentrations of AAPH (5 mM, 25 mM, and 100 mM) in the presence of EDTA at 37°C until 24 h. B: The rates of CH oxidation, R_p (M/s), were estimated from the straight lines obtained within the time holding a linear correlation, and are plotted against the square root of the rate of radical initiation, R_i, which was calculated from the following equation: $R_i = 2ki[\text{AAPH}]e$, as described in detail on the text.

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TABLE 3. Effects of the saturation level and constituent ratio of PLs on the oxidizability of CH in bilayers

LUV	CH/DMPC			CH/SPC		
Constituent molar ratio	1/1	1/3	1/10	1/1	1/3	1/10
Oxidizability $(Ms)^{-1/2} \times 10^{-2}$	9.2	8.7	8.2	9.4	9.4	11.1

The oxidizability of CH was determined by measuring the rate of CH oxidation in each LUV with 25 mM AAPH.

on the rate of CH oxidation were examined with LUV composed of CH and dimyristoyl phosphatidylcholine (DMPC) at the equivalent molar ratio (CH/DMPC=1/1) (**Figs. 2**, **3**). CH was linearly oxidized with time until 6–12 h (Figs. 2A, 3A), and the rate of CH oxidation, R_p, estimated from the straight line obtained within the time holding a linear correlation, was exactly dependent on the concentration of CH in the reaction mixtures (Fig. 2B) and on the square root of the rate of radical initiation, R_i, (Fig. 3B), which was calculated from:

$$R_i = 2ki[\mathrm{AAPH}]e \qquad (Eq.\ 1)$$

where [AAPH] is the concentration of initiator in the reaction mixtures, $2ki$ for AAPH is taken as $1.36 \times 10^{-6}\ s^{-1}$ at 37°C (33), and the efficiency of free radical production, e, is taken as 0.64 from the average value measured 15 times by the induction period method (34) with a water-soluble antioxidant, Trolox (35).

These results indicate that the CH oxidation in the system follows the classical rate law for autoxidation given by (36):

$$R_p = k_p/(2k_t)^{1/2}[\mathrm{LH}]R_i^{1/2} \qquad (Eq.\ 2)$$

where R_p is the rate of oxidation; R_i, the rate of radical initiation; [LH], the concentration of substrate; and k_p and $2k_t$ are the rate constants for chain propagation and termination, respectively. The ratio of these rate constants, $k_p/(2k_t)^{1/2}$, is referred to as the susceptibility of a substrate to oxidation; that is, oxidizability. Accordingly, the oxidizability of CH in bilayers can be experimentally determined from:

TABLE 4. The oxidizability values of CH in various LUVs

LUV	Constituent Molar Ratio	Particle Size[a]	10^3 [CH]	Buffer	CH/PL Molar Ratio	$10^9\ R_p$[c]	Oxidizability[d]
		diameter, nm	*M*			*M/s*	$10^2\ (Ms)^{-1/2}$
CH/SPC	1.00/0.95	99.1 ± 38.1	1.00		1.05	16.7 ± 0.5	11.3 ± 0.4
CH/SPC	1.00/1.03	100.1 ± 39.6	0.85	+EDTA	0.97	13.5 ± 0.2	10.8 ± 0.1
CH/DMPC	1.00/1.58	98.7 ± 34.7	1.08	+EDTA	0.63	12.9 ± 0.2	8.1 ± 0.1
CH/SPC/Toc.	1.00/0.95/0.01	108.6 ± 44.7	1.08		1.05	15.8 ± 0.2	9.9 ± 0.1[e,i]
CH/SPC/Toc.	1.00/1.00/0.05	ND[b]	1.10		1.00	14.2 ± 0.9	8.7 ± 0.5[e,i]
CH/SPC/BBPE	1.00/1.00/0.82	ND	0.70		0.55	5.1 ± 0.9	4.9 ± 0.9[e,i]
CH/SPC/BBPS	1.00/0.56/0.45	ND	1.12		0.99	16.3 ± 0.2	9.8 ± 0.1[e,i]
CH/SPC/DOPE	1.00/1.15/0.95	131.7 ± 55.1	0.50		0.48	7.5 ± 0.5	10.2 ± 0.7[e,h]
CH/SPC/EYPE	1.00/1.74/0.48	83.3 ± 43.3	1.05		0.45	11.8 ± 0.1	7.6 ± 0.1[e,i]
CH/SPC/EYPE	1.00/0.95/0.76	ND	1.21	+EDTA	0.58	10.6 ± 0.7	5.9 ± 0.4[f,i]
CH/SPC/BBEP	1.00/0.70/0.56	126.9 ± 50.8	0.68		0.79	4.4 ± 0.9	4.4 ± 0.9[e,i]
CH/SPC/BBEP	1.00/0.78/0.56	ND	0.45	+EDTA	0.75	3.3 ± 0.1	5.0 ± 0.2[f,i]
CH/DMPC/BBEP	1.00/1.18/0.21	ND	0.95	+EDTA	0.72	5.9 ± 0.8	4.2 ± 0.6[g,i]
CH/DMPC/BBEP	1.00/0.84/0.53	117.9 ± 27.7	0.95	+EDTA	0.73	3.2 ± 0.0	2.3 ± 0[g,i]
CH/BHPC	1.00/0.88	109.1 ± 37.4	1.00		1.14	11.3 ± 0.2	7.7 ± 0.1[e,i]
CH/BHCP	1.00/1.11	ND	0.69	+EDTA	0.90	4.3 ± 0.1	4.2 ± 0.1[f,i]
CH/SPC/LyPE	1.00/0.91/0.23	ND	1.29	+EDTA	0.88	20.5 ± 0.2	10.7 ± 0.1[f]
CH/SPC/LyEP	1.00/0.93/0.21	ND	1.21	+EDTA	0.88	16.8 ± 0.2	9.4 ± 0.1[f,i]

BBEP, bovine brain ethanolamine plasmalogen; BBPE, bovine brain glycerophosphoethanolamine; BBPS, bovine brain phosphatidylserine; BHCP, bovine heart choline plasmalogen; BHPC, bovine heart glycerophosphocholine; CH, cholesterol; DMPC, dimyristoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; EYPE, egg yolk phosphatidylethanolamine; SPC, soybean phosphatidylcholine; Toc., α-tocopherol; LyEP, 1-alkenyl-2-lyso-*sn*-glycero-3-phosphoethanolamine from bovine brain; LyPE, 1-acyl-2-lyso-*sn*-glycero-3-phosphoethanolamine from porcine liver.

[a] Expressed as average ± SD based on particle size distribution, using a light scattering instrument.

[b] Not determined.

[c] Measured by quantifying unoxidized CH with GC during oxidation of LUV with 25 mM AAPH in the presence or absence of 0.1 mM EDTA in PBS at 37°C for 12 h. Values expressed as average ± standard deviation based on triplicate results.

[d] Determined by the following equation: Oxidizability = $R_p/[\mathrm{CH}]R_i^{1/2}$, where R_i is taken as 2.18×10^{-8} *M/s* as described in the text.

[e] Statistical analysis conducted by the Student's *t*-test using the oxidizability of CH in CH/SPC LUV in the absence of EDTA as control values.

[f] Statistical analysis conducted by the Student's *t*-test using the oxidizability of CH in CH/SPC LUV in the presence of EDTA as control values.

[g] Statistical analysis conducted by the Student's *t*-test using the oxidizability of CH in CH/DMPC LUV in the presence of EDTA as control values.

[h] $P < 0.05$.

[i] $P < 0.001$.

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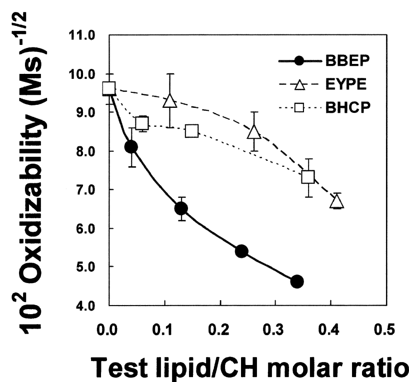

Fig. 4. Comparison of the dose-dependent effects of bovine brain ethanolamine plasmalogen (1-*O*-alk-1 enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) (BBEP), EYPE, and bovine heart choline plasmalogen (1-*O*-alk-1 eny-2-acyl-*sn*-glycero-3-phosphocholine) (BH CP) on the oxidizability of CH in bilayers. The oxidizability of CH was determined by measuring the rate of CH oxidation, R_p, in each LUV during oxidation with 10 mM AAPH in PBS containing EDTA at 37°C. Each LUV is comprised of CH, soybean phosphatidylcholine (SPC), and test lipids (BBEP, EYPE or BHCP) in variable amounts at about the equivalent molar ratio of total PLs to CH. The oxidizability values were obtained from triplicate determinations and are plotted against the molar ratio of test lipid to CH in LUV. Symbols and bars indicate means and standard deviations, respectively.

$$k_p/(2k_t)^{1/2} = R_p/[LH]R_i^{1/2} \quad (Eq.\ 3)$$

To confirm the suitability of the system, the oxidizability values were measured and compared in various LUVs composed of CH and dimyristoyl phosphatidylcholine (DMPC) or soybean phosphatidylcholine (SPC) at various constituent ratios of PLs to CH (**Table 3**). Almost the same values, 8.2×10^{-2}-11.1×10^{-2} $(Ms)^{-1/2}$, were obtained, which confirms that the system is appropriate for assessing the oxidizability of CH in PL bilayers without significant interference from oxidizable PLs.

Effect of BBEP to reduce the oxidizability of CH in bilayers

Using this system, the effects of various glyceroPLs and an antioxidant on the oxidizability of CH in bilayers were explored (**Table 4**). BBEP, BHCP, and EYPE significantly lowered the oxidizability of CH in bilayers, whereas lyso ethanolamine plasmalogen from bovine brain (LyEP) and Toc only slightly reduced the oxidizability of CH (Table 4). To compare the ability of BBEP, BHCP, and EYPE to lower the oxidizability of CH, the dose-dependent effects of each PL were examined in LUVs composed of CH (CH), soybean phospatidylcholine (SPC), and each test lipid (BBEP, EYPE, BHCP) at about the equivalent molar ratio of total PLs to CH. It was found that, among them, BBEP has the greatest ability to reduce the oxidizability of CH in bilayers (**Fig. 4**).

Mechanisms of BBEP for lowering the oxidizability of CH in bilayers

It is known that plasmalogens exert an antioxidative action via scavenging radicals at the vinyl ether linkage (12–14, 37–39). It was therefore investigated whether the effect of plasmalogens in reducing the oxidizability of CH would be due to the ability of scavenging radicals by measuring the reactivity toward galvinoxyl, which is a stable radical and has often been used to estimate the reactivity of a radical-scavenging antioxidant (31). The rate constants of reactivity of BBEP and LyEP toward galvinoxyl radical were estimated as 2.3 and 3.3×10^{-1} $(Ms)^{-1}$, respectively, whereas that for Toc was 4.0×10^3 $(Ms)^{-1}$ (**Fig. 5**). The results indicate that the radical-scavenging ability of plasmalogens is over 1,000-fold less than that of Toc in homogeneous solution, suggesting a lesser contribution of the radical-scavenging ability of plasmalogens to the reduction in the oxidizability of CH. However, since plasmalogens appear to exert an antioxidative effect comparable to that of Toc in plasma LDLs (40), plasmalogens may scavenge radicals effectively in membranes.

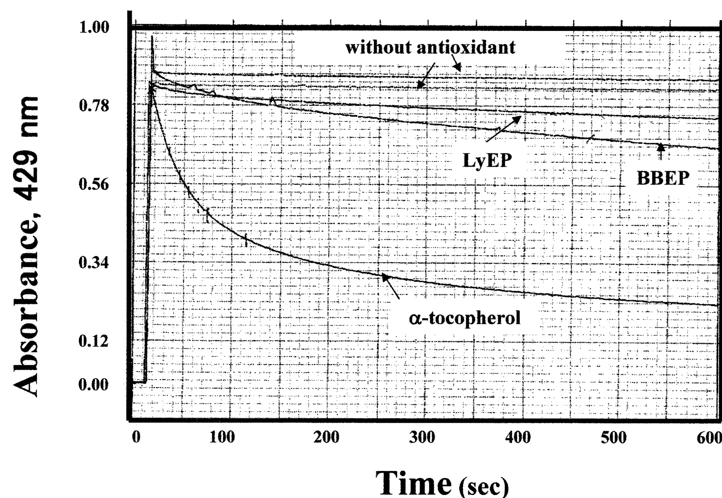

Fig. 5. Reaction of BBEP, LyEP, and α-tocopherol (Toc) with galvinoxyl radical. BBEP (1 mM), LyEP (1 mM), or Toc (5 μM) were incubated with galvinoxyl (5 μM) in methanol at 37°C in air, and the decrease in absorption at 429 nm was followed by a stopped-flow spectrophotometer as described in Materials and Methods.

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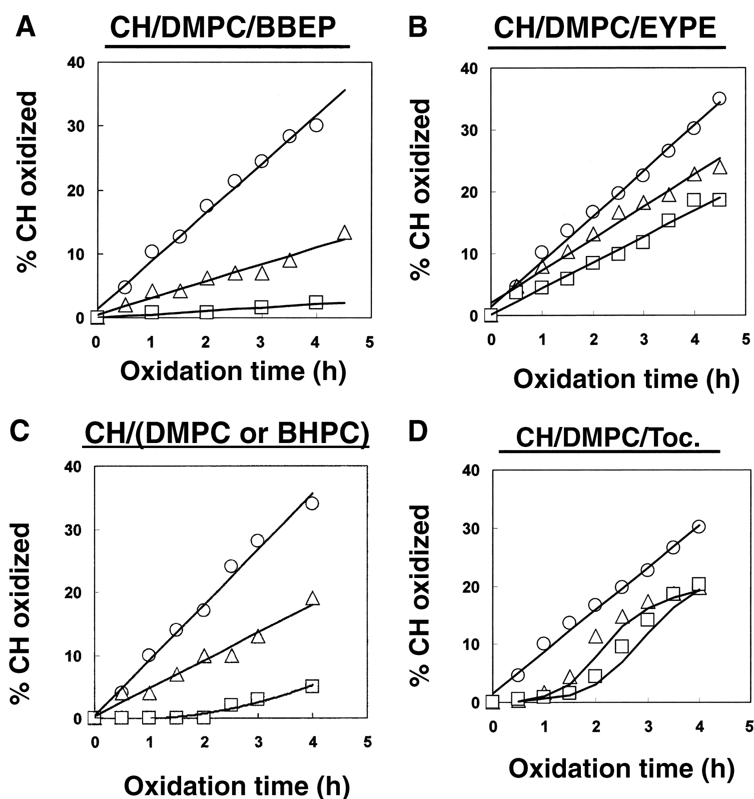

Fig. 6. Effects of BBEP, EYPE, BHPC, and Toc on time course of CH oxidation in LUV by AAPH LUV composed of CH, dimyristoyl phosphatidylcholine (DMPC), and/or test compounds (BBEP, EYPE, BHPC, or Toc) were incubated in PBS with 100 mM AAPH in the presence of EDTA at 37°C till 4.0–4.5 h. A: LUV (CH/DMPC/BBEP) contains BBEP at indicated molar ratios to CH (circle, 0; triangle, 0.12; square, 0.34). B: LUV (CH/DMPC/EYPE) contains EYPE at indicated molar ratios to CH (circle, 0; triangle, 0.17; square: 0.31). C: LUV (CH/DMPC or BHPC) contain BHPC at indicated molar ratios to CH (circle, 0; triangle, 1.0; square, 4.0). D: LUVs (CH/DMPC/Toc) contain Toc at indicated molar ratios to CH (circle, 0; triangle, 0.15; square, 0.3).

The effects of BBEP, EYPE, BHPC, and Toc on the time course of CH oxidation were examined. BBEP and EYPE decreased the rate of CH oxidation dose dependently (Fig. 6A, B), showing their effects in lowering the oxidizability of CH. Whereas the typical radical scavenger Toc induced a lag time while having no effect on the rate of oxidation (Fig. 6D), indicating no ability to reduce the oxidizability. BHPC brought about both a reduction in the rate of oxidation and induction of the lag period, probably due to the large amount of BHPC in vesicles (Fig. 6C), suggesting that choline plasmalogens also have the ability to reduce the oxidizability, though their ability is considerably less than that of ethanolamine plasmalogens. In addition to the above results, the following observations also suggest the presence of another mechanism for reducing the oxidizability of CH besides the action of scavenging radicals. *i*) BBEP has greater ability to lower the oxidizability of CH than BHCP despite equal sharing of vinyl ether double bonds in the molecule (Fig. 4). *ii*) EYPE also has a reducing ability despite lacking the action of scavenging radicals (Fig. 4, Table 4).

It is known that ethanolamine plasmalogens have a stronger propensity for hexagonal phase formation than phosphatidylethanolamine (16), which contributes to membranes fusion (17). Interestingly, it has been reported that the location of CH in PL bilayers changes from head group to fatty acyl chains during hexagonal phase formation (41). Such membrane motion may serve to relocate CH to a more hydrophobic area in the bilayers, thus making it more resistant to free radical attack in the water phase. Such a modification of the physical features of membranes, caused by ethanolamine plasmalogens, may serve to reduce the oxidizability of CH in bilayers. Further study is needed to elucidate the mechanism by which ethanolamine plasmalogens cause a lowering of the oxidizability of CH in PL bilayers.

The present study suggests that ethanolamine plasmalogens play a role in preventing CH oxidation in biomembranes by reducing the oxidizability of CH in bilayers as a physiological antioxidant for CH.

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