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Stabilization of egg phosphatidylcholine liposomes by the insertion of sulfatide

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The characteristic effects of sulfatide and its derivatives on the stability of small unilamellar vesicles of egg phosphatidylcholine liposomes in phosphate-buffered saline were investigated by measuring the leakage of carboxyfluorescein that had been entrapped in these vesicles. We found that both the sulfate group and a long acyl chain, such as lignoceric acid, of the sulfatide are essential for the stabilization. The sulfatide derivatives that contain a somewhat shorter acyl chain such as stearic acid had no effect to suppress the leakage of carboxyfluorescein. The galactose residue of sulfatide is not essential to suppress the leakage. ^{1}H-NMR study using a paramagnetic shift reagent demonstrated that the distribution of phosphatidylcholine in the vesicles containing sulfatide is homogeneous, which seems to contribute to the stability of the membrane.

Introduction

Sulfatide (CSE) is an acidic glycolipid consisting of a hydrophobic ceramide and a hydrophilic sulfogalactose residue. Our previous EPR study on liposomes composed of egg phosphatidylcholine (PC) showed that CSE inserted in the liposomes reduces the membrane fluidity in a manner different from either cerebroside (CMH) or cholesterol, and has a strong rigidifying effect on the membrane [1]. Cestaro et al. [2] reported that CSE has a condensing effect above the phase-transition temperature.

From these results, insertion of CSE into the liposomal membrane was expected to increase the stability of liposomes and to prolong their circulation time in the blood. In fact, notable effects of CSE on stablization of small unilamellar vesicles (SUV) composed of PC (PC-SUV) in the presence of human plasma in vitro [3] and on prolongation of their circulation time in vivo [4,5] were reported.

For better understanding of the part(s) of the CSE molecule responsible for these advantages, we synthesized its derivatives having partial structures of CSE (Fig. 1) and examined the effect of CSE structure on the stability of PC-SUV in phosphate-buffered saline (PBS). The stability of liposomes was estimated in terms of leakage of carboxyfluorescein (CF) [6], the fluorescence of which is quenched when fluorochrome is entrapped in liposomes [7]. In relation to the stability, the effect of the insertion of CSE, phosphatidylserine (PS), or cholesterol on the outside-inside distribution of PC in SUV was studied with ^{1}H-NMR spectroscopy by the addition of praseodymium chloride, $PrCl_3$, as a paramagnetic shift reagent.

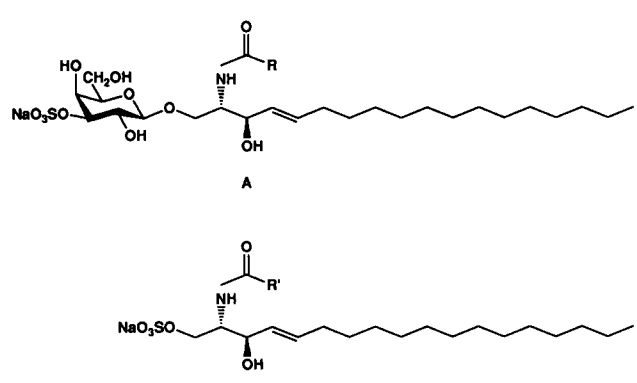

Fig. 1. Chemical structures of CSE (A) and *N*-acylsphingosine sulfate ester (B). For *N*-stearoylsphingosine-1-*O*-sulfate sodium salt (C18CS), $R' = -(CH_2)_{16}CH_3$; for *N*-lignoceroylsphingosine-1-*O*-sulfate sodium salt (C24CS), $R' = -(CH_2)_{22}CH_3$.

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Abbreviations: CSE, sulfatide; PC, egg phosphatidylcholine; CMH, cerebroside; SUV, small unilamellar vesicles; CF, carboxyfluorescein; PS, phosphatidylserine; DLCPC, dilignoceroylphosphatidylcholine; C18ES, sodium 1,2-bis(octadecyloxycarbonyl)ethane-1-sulfonate; C24CS, *N*-lignoceroylsphingosine-1-*O*-sulfate ester sodium salt; C24SP, *N*-lignoceroylsphingosine; C18CS, *N*-stearoylsphingosine-1-*O*-sulfate ester sodium salt; PBS, phosphate-buffered saline.

Materials and Methods

Materials

CSE sodium salt and CMH were prepared from bovine brain according to the method of Svennerholm and Thorin [8]. In brief, the extract made with chloroform-methanol from acetone powder of bovine brain tissue was saponified. Then crude glycolipids were extracted with chloroform-methanol and separated on a silica gel column to obtain CSE and CMH. Other materials used and their source were as follows: PC, from Nippon Fine Chemical Co., Tokyo; cholesterol, PS and sphingosine, from Sigma, St. Louis, MO; lignoceric acid (C24:0) and sulfur trioxide-pyridine complex, from Aldrich, Milwaukee, WI; stearic acid (C18:0) and $PrCl_3$, from Nacalai Tesque, Inc., Kyoto,; dilignoceroylphosphatidylcholine (DLCPC), from Avanti Polar Lipids, Pelham, AL; sodium 1,2-bis(octadecyloxycarbonyl)ethane-1-sulfonate (C18ES), from Sogo Pharmaceutical Co., Tokyo; CF, from Eastman-Kodak, Rochester, NY, and purified as described by Ralston et al. [9]; and Iatrobeads, from Iatron Laboratories, Tokyo.

Synthesis of N-lignoceroylsphingosine-1-O-sulfate ester sodium salt (C24CS)

N-Lignoceroylsphingosine (C24SP) and *N*-stearoylsphingosine were synthesized according to the procedure reported by Ong and Brandy [10], by coupling fatty acid esters of *N*-hydroxysuccinimide directly to sphingosine. To synthesize C24CS, sulfur trioxide-pyridine complex (294 mg) was added to a solution of C24SP (1.00 g) in anhydrous benzene (40 ml) under an atmosphere of argon gas, and the mixture was stirred for 2.5 h at 52°C. Then the solvent was evaporated under vacuum and the residue was suspended in distilled water (60 ml). Aqueous 0.1 N NaOH solution (14.6 ml) was added dropwise to the above suspension to raise the pH from 1.7 to 7.3, and the suspension was then stirred for 1 h at room temperature. The mixture was concentrated to dryness, and C24CS (257 mg) was obtained by chromatography on an Iatrobeads column by use of chloroform/methanol (15:1, v/v) as developer. In this preparation, the contamination of 3-*O*-sulfate derivatives was not found by NMR analysis.

IR (ν^{KBr} max) cm^{-1}: 3388, 2902, 2842, 1617, 1533, 1209, 972; ^{1}H-NMR (90 MHz, $CDCl_3:CD_3OD = 2:1$): δ 0.88 (t, 6H, $-CH_3$), 1.27 (s, 64H, CH_2), 2.05 (m, 2H, CH_2), 2.20 (m, 2H, CH_2), 3.99–4.33 (complex, 4H), 5.47 (d.d, $J = 15$, 6 Hz, 1H, -CH =), 5.76 (d.t, 15, 6 Hz, 1H, = CH-); FAB-MS (negative FAB; matrix, glycerol): m/z 728 $(M - Na)^-$.

N-Stearoylsphingosine-1-*O*-sulfate ester sodium salt (C18CS) was also synthesized as above by use of *N*-stearoylsphingosine as a starting material.

Preparation of liposomes

PC and other lipids in the desired molar ratio were dissolved in chloroform-methanol (2:1, v/v), evaporated to dryness in a rotary evaporator, and stored overnight under high vacuum in the dark. The dried lipids were suspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.2 M CF. The suspension was sonicated for 40 min at 25°C with a probe-type sonicator (Model W-375, Heat Systems Ultrasonics, Plainview, NY) under argon gas, and then the dispersion was centrifuged to remove multilamellar vesicles at $100\,000 \times g$ and at 20°C for 40 min. SUV that had entrapped CF were separated from free CF by passage through a column of Sepharose CL-6B (Pharmacia, Uppsala) equilibrated with PBS. The size of these liposomes was estimated to be 25–50 nm in diameter by electron microscopy.

Assay for stability of liposomes

Stability of liposomal membranes was estimated according to the procedure of Senior and Gregoriadis [11]. Briefly, 1 ml of the suspension of SUV consisting of 1 μmol of total lipids containing CF was added to 4 ml of PBS and incubated at 37°C. The total amount of CF in SUV was determined fluorophotometrically (emission, 516 nm; excitation, 492 nm; fluorospectrophotometer, Model 650–10S, Hitachi, Tokyo) with purified CF as standard. The amounts of CF leaked from liposomes were determined at various times and extent of leakage was expressed as percent of the total amount before incubation. The latency of various liposomes was represented by rate constant calculated from the slope of each linear plot of CF leakage by the least-squares method.

Nuclear magnetic resonance study

A film consisting of the lipids in the desired molar ratio was suspended in D_2O and sonicated, and SUV were obtained by means of ultra-centrifugation as described above. Then 5 μmol of $PrCl_3$ was added to the SUV containing 10 μmol of total lipids. Since low p*D* interferes with the shifting effect of Pr^{3+} on the NMR signals of phospholipids, as pointed out by Fernández [12], all ^{1}H-NMR experiments were carried out within 15 min after the p*D* had been adjusted to 6–8 with dilute NaOD or DCl solution. The p*D* of SUV solution was estimated by adding 0.4 to the measured pH values. ^{1}H-NMR was done at 90 MHz by use of a JEOL FX-90Q spectrometer (JEOL, Tokyo) operating in the Fourier transform mode at 22°C, and homogated decoupling was employed as the irradiation mode in order to suppress the solvent peak. For the determination of outside-inside ratio ($R_{o/i}$) of PC across the bilayer, the integral intensity of *N*-methyl protons of both external and internal monolayers was measured.

Other analyses

The fatty acid composition of CSE and of CMH was determined by the GC-MS analysis of fatty acid methyl esters prepared as described by Ramsey et al. [13]. Each sample (*ca.* 10 mg) was treated with 1 ml of 14% methanolic boron trifluoride in sealed tubes at 85°C for 18 h to form the methyl esters. In order to separate non-hydroxy fatty acid methyl esters from hydroxy fatty acid methyl esters, the reactants were chromatographed on Kieselgel 60 plates by use of *n*-hexane-ethyl ether (5 : 1, v/v) as developer. Hydroxy fatty acid methyl esters were treated with 1 ml of bis(trimethylsilyl)trifluoroacetamide in sealed tubes at 92°C for 23 h to prepare trimethylsilyl derivatives. GC-MS analysis of each fatty acid methyl ester was performed on a JEOL JMS-DX300 gas chromatograph-mass spectrometer (JEOL, Tokyo). GC was carried out on a column of 5% Advance-DS on 80–100 mesh Chromosorb W at 200°C with N_2 at a flow rate of 50 ml/min.

PC concentration in SUV was determined with an assay kit for PC (Phospholipid B-Test Wako, Wako, Osaka).

Results

Fatty acid composition of CSE and CMH

To clarify what part of the structure of CSE, viz., galactose, sulfate ester group, long acyl chain, or hydroxyl group of the sphingosine base, contributes to the stabilizing effect on the liposomal membrane, we examined the CF latency of liposomes containing CSE or its derivatives.

First, the fatty acid composition of CSE and of CMH was determined (Table I). As can be seen from the table, the fatty acids in CSE are mainly lignoceric acid (24:0) and nervonic acid (24:1). In this respect, CMH is very similar to CSE.

TABLE I

Fatty acid composition of CSE and CMH from bovine brain

Values are the means of duplicate analyses, and the amount of each lipid is expressed as the percentage of the total amount of fatty acids.

Fatty acid	Non-hydroxy (%)		Hydroxy (%)	
	CSE	CMH	CSE	CMH
16:0	0.9	6.1		
18:0	4.2	17.7	5.7	15.8
18:1		0.8		
20:0		0.6	1.1	1.6
22:0	5.1	4.1	4.5	4.7
22:1	0.6		0.9	0.7
23:0	5.6	5.0	6.2	6.3
23:1	1.1	0.9	0.5	0.6
24:0	22.7	18.4	35.4	25.7
24:1	39.3	30.5	14.6	19.2
25:0	5.1	4.0	11.4	8.5
25:1	5.8	4.6	3.2	3.9
26:0	1.8	1.3	7.1	4.4
26:1	7.5	5.5	8.9	8.4

TABLE II

Effect of CSE or its derivatives on CF latency of PC-SUV in PBS

Each liposome containing 0.2 M CF was incubated in PBS at 37°C. At definite times, samples (30 μl) were taken and assayed for CF latency which is expressed in rate constant of CF leakage (see text).

Liposome	Molar ratio	CF latency rate
PC		−0.50
PC:CSE	9:1	−0.21
PC:DLCPC	9:1	−0.58
PC:CMH	9:1	−0.36
PC:C24CS	9:1	−0.25
PC:C24SP	9:1	−0.32
PC:C18CS	9:1	−0.37
PC:C18ES	9:1	−0.39

Effect of CSE or its derivatives on the stability of PC liposomes

CF latency of PC-SUV with either 10 mol% CSE or its derivatives inserted in the bilayer was determined at 37°C in PBS (Table II). SUV composed of only PC released over 50% of the entrapped CF within a 120-min incubation, and DLCPC-containing PC-SUV showed an even greater degree of leakage.

In contrast, PC-SUV containing CSE showed the most suppressed leakage of CF. Incorporation of CMH, which is a neutral glycolipid lacking a sulfate ester group and having a similar fatty acid composition as CSE, decreased CF latency to 80% of that of CSE-containing PC-SUV at 120 min of incubation.

PC-SUV containing C24CS, which has sulfate group in place of the sulfogalactose moiety of CSE, showed the same degree of latency as CSE-containing PC-SUV. The removal of the negatively charged sulfate ester from C24CS promoted CF leakage from PC-SUV, for C24SP-containing PC-SUV showed lower latency than C24CS-containing PC-SUV. CF leakage from PC-SUV containing C18CS having rather shorter acyl chain was greater than that from the vesicles containing C24CS. CF latency of PC-SUV containing either C18CS or C18ES was almost the same.

The effect of insertion of 10 mol% CSE or its derivatives into PC-SUV made with 40 mol% cholesterol, which stabilizes the liposomal membrane, was examined as above. After incubation in PBS, all of SUV composed of PC, cholesterol and CSE or its derivatives retained about 95% of the entrapped CF (data not shown).

Effect of the concentration of CSE, cholesterol and PS on the stability of PC liposomes

Leakage of entrapped CF from PC-SUV containing various amounts of CSE was determined at 37°C in

PBS. For comparison, the effects of variation of the molar ratio of cholesterol [11,14] and PS [3], both of which exert the stabilizing effect, were also examined. In PBS, the effect of the insertion of 10 mol% cholesterol or PS into PC-SUV was small, whereas 10 mol% CSE exhibited an appreciable stabilizing effect (Table III). Upon raising the concentration to 50 mol%, CSE or cholesterol strongly suppressed leakage of entrapped CF. While, PS was less effective.

The stabilizing effect of CSE and cholesterol on the liposomes was dependent on the increase in their concentrations.

Effect of the incorporation of CSE on the outside-inside distribution of PC in vesicles

Relating to the phenomenon described above, we considered that the outside-inside distribution of PC in various vesicles contributes to the membrane stability, and we examined it by use of ^{1}H-NMR.

The insertion of 5 mol% CSE into PC-SUV did not have any effect on the outside-inside distribution of PC (Table IV). By raising the concentration of CSE to 20 mol% the value of $R_{o/i}$ increased, but the extent was slight, suggesting that the outside-inside distribution of PC was rather homogeneous regardless of the amount of CSE in the vesicles. Since PC-SUV containing 30 mol% CSE gave very low resolution in the ^{1}H-NMR spectrum just before the addition of $PrCl_3$ to the external environment of the vesicles, the distribution of PC in this liposome could not be measured. $R_{o/i}$ of PC in PC-SUV was appreciably decreased by the incorporation of 5 mol% cholesterol and further decreased by raising the concentration of cholesterol up to 20 mol%, but increased slightly at 30 mol% cholesterol. This indicates that cholesterol in cholesterol-containing PC-SUV localizes excessively in the outside monolayer, and PC in the inside monolayer. In the case of PS, the outside-inside ratio of PC increased markedly by the incorporation of PS up to 30 mol%. Upon, the incorporation of 30 mol% PS, it was found that the number of PC molecule on the outside surface was approx. 80% of the total amount of PC of the vesicles. This indicates that PS preferentially distributed to a high degree in the inside monolayer.

TABLE III

Effect of CSE, cholesterol and PS contents in PC liposomes on the liposomal stability upon incubation in PBS

Each liposome containing 0.2 M CF was incubated at 37°C in PBS. CF latency is expressed in rate constant of CF leakage (see text).

Liposome	Molar ratio	CF latency rate
PC		−0.50
PC:CSE	9:1	−0.21
PC:CSE	7:3	−0.19
PC:CSE	5:5	−0.06
PC:cholesterol	9:1	−0.37
PC:cholesterol	7:3	−0.12
PC:cholesterol	5:5	−0.002
PC:PS	9:1	−0.37
PC:PS	7:3	−0.22
PC:PS	5:5	−0.24

TABLE IV

Outside-inside ratio ($R_{o/i}$) of PC in PC-SUV containing CSE, cholesterol, or PS

Values are the means of duplicate measurements. The outside-inside distribution of PC in each type of liposome was measured at 22°C in the presence of $PrCl_3$, and the $R_{o/i}$ was determined by the measurement of integral intensities of each split signal.

Liposome	Molar ratio	$R_{o/i}$
PC		1.84
PC:CSE	95:5	1.90
PC:CSE	90:10	2.17
PC:CSE	80:20	2.25
PC:cholesterol	95:5	1.53
PC:cholesterol	90:10	1.43
PC:cholesterol	80:20	1.40
PC:cholesterol	70:30	1.48
PC:PS	95:5	2.16
PC:PS	90:10	2.60
PC:PS	80:20	3.63
PC:PS	70:30	3.83

Discussion

In our previous paper [1], EPR study showed that CSE reduced the membrane fluidity of PC liposomes, compared with CMH. The present study showed that CF latency of CSE-containing PC-SUV was greater than that of CMH-containing PC-SUV, reflecting the strong correlation between the membrane fluidity and liposomal stability.

In the present study, we found that the galactose residue is not essential for the stabilizing effect. Since the stabilizing effect of both CSE and C24CS exceeds that of C24SP, the negatively charged sulfate group is considered to be essential for the stabilization. As for the effect of the length of a long acyl chain of CSE, Viani and his co-workers [15] presented evidence that at temperatures below the gel-to-liquid crystalline phase transition the insertion of stearoyl-CSE into distearoylphosphatidylcholine-containing liposomes did not change the steady-state anisotropy, whereas that of lignoceroyl-CSE decreased it. In our experiments, CF leakage from C24CS-containing PC-SUV in PBS was lower than that from C18CS-containing ones at 37°C. It seems that the long acyl chain of CSE contributes to the condensing effect, while the insertion of DLCPC,

having the same C24 acyl chain, showed higher CF leakage. We suggest that the incorporated DLCPC might exhibit either lateral phase separation [16] or lipid immiscibility [17] in the bilayer, resulting in the increment of permeability that promotes CF leakage from the vesicle. The difference in the effect on leakage between CSE and DLCPC should be ascribed to the existence of sulfate group in the former.

In comparison of CF leakage from CSE-containing PC-SUV with that from cholesterol- and PS-containing PC-SUV, it was noted that the incorporation of CSE at the level of only 10 mol% reduced CF leakage. Above 30 mol%, the insertion of cholesterol showed high CF latency as CSE. Although the condensing effect of cholesterol is brought about by hydrogen bonding between hydroxyl groups of cholesterol and ester carbonyl groups of phospholipids [18], cholesterol at 10 mol% had not such an effect. In the case of CSE, either the amide or hydroxyl group of the sphingosine base and the carbonyl groups of phospholipids can participate in intra- and intermolecular hydrogen bonding, and the potential hydrogen acceptor sulfate can strengthen the hydrogen bonding network in the liposomal membrane. The stabilization effect of PS inserted in PC-SUV was small compared with CSE- or cholesterol-inserted PC-SUV.

When PC distributes at a higher concentration in the outside monolayer than in the inside one, the head group interaction on the external surface of the membrane through hydrogen bonding and electrostatic interaction will certainly decrease, since PC has only the hydrogen-accepting groups and no hydrogen-donating ones. In view of the above considerations, the outside-inside distribution of PC across the bilayer of SUV was measured by ^{1}H-NMR in the presence of $PrCl_3$. The incorporation of CSE into PC-SUV did not appreciably affect the value of $R_{o/i}$, showing the homogeneous distribution of CSE across the bilayer. The incorporation of cholesterol in PC-SUV decreased the ratio, indicating the excessive localization of PC in the inside monolayer of PC-SUV. The tendency of the distribution of liposomal PC was similar to that reported by De Krujiff *et al.* [19]. On the contrary, the incorporation of PS up to 30 mol% increased $R_{o/i}$ remarkably in agreement with the report of Barsukov *et al.* [20]. From these results on the relationship between the $R_{o/i}$ of liposomal PC and the leakage of entrapped CF, we consider that the insertion of either CSE or cholesterol might rigidify the liposomal membrane due to the hydrogen bonding network on the external surface of the membrane. In contrast, the stabilization effect of PS is smaller than CSE probably due to the excessive localization of the charged group in the internal surface and due to an insufficient hydrogen bonding network on the external surface of the membrane.

The present results indicate that CSE has a potent stabilizing effect on liposomal membrane and that for this stabilization, both the sulfate group and long acyl chain such as lignoceric acid of CSE are essential. In addition, the homogeneous distribution of PC outside and inside the membrane seems to contribute to the membrane stability.

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