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Evidence of Chemical Instability of Phospholipids in Liposomes

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Chemical instability of liposomes containing dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and soybean phosphatidylcholine (4:4:2, w/w) was examined.

Various methods were employed to evaluate the phospholipids degradation: peroxidation index, malondialdehyde determination, reverse-phase high-performance liquid chromatography of DSPC and DPPC, thin layer chromatography of lysophosphatides and gas chromatography–mass spectrometry of free and total fatty acids. Formation of palmitoyllysophosphatidylcholine and stearoyllysophosphatidylcholine was detected within one month during storage.

Keywords——phospholipid; liposome; stability; lysophosphatidylcholine

Introduction

Multilamellar liposomes are spherical vesicles consisting of a number of parallel bilayers and several aqueous compartments. The bilayers consist of one or a number of phospholipids (PL). Lipophilic compounds such as cholesterol and ionic substances can be included in this structure.[1)]

The aim of this study was to evaluate the susceptibility of PL in bilayers to chemical degradation. Lipid peroxidation was assessed by measurements of peroxide index and malondialdehyde determination. Lysophosphatides formation was evaluated by their identification and phospholipids determination.

Experimental

Materials——Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were purchased from Sigma Chemicals Co. Soybean phosphatidylcholine (PC) was supplied by Lucos Meyer Co. (Epikuron 200, PC content: more than 97%). All solvents were obtained from Merck Chemicals Co., Ltd.

Liposome Preparation——Multilamellar liposomes containing DPPC, DSPC and PC (10 mg/ml) in 4:4:2 (w/w) proportions were prepared by an adaptation of Bangham *et al.*'s method.[2)] A chloroform stock solution of PL was added to a 50 ml flask and evaporated *in vacuo*. The resulting thin, dry lipid film was resuspended in 0.9% sodium chloride solution by brief bath sonication. The final PL concentration was 10 mg/ml. The suspension was placed in sterile vials, without nitrogen flushing, and the containers were sealed immediately. Liposomes were stored at +4 °C.

Peroxidation Index Measurement——The peroxidation index was calculated from the absorbance ratio at 233 nm (λ_{max} of dienes) and 215 nm (μ_{max} of phospholipids).[3)]

Malondialdehyde Determination——Malondialdehyde (MDA) was determined spectrofluorometrically in acid media with thiobarbituric acid.[4)]

Liquid Chromatography of DSPC and DPPC——DSPC and DPPC were assayed by reverse-phase liquid chromatography using modifications of a reported method.[5)] Aliquots (1 ml) of the liposome suspension were evaporated under nitrogen. The residue was dissolved in 1 ml of internal standard solution (20 mg cholesterol per ml of methanol) and 20 μl of each sample was analyzed by reverse-phase high-performance liquid chromatography.

DSPC and DPPC were eluted isocratically from a 4.6 × 250 mm Spherisorb ODS-II column (Altech, Eke, Belgium) with methanol–acetonitrile–tetrahydrofuran–water (30 : 31 : 32 : 17, v/v) at a flow rate of 1.7 ml/min. PL were detected by ultraviolet absorption measurement at 214 nm. Peak areas were recorded and compared to a calibration curve of standards.

Thin Layer Chromatography (TLC) of Lysophosphatides——Palmitoyllysophosphatidylcholine, stearoyllysophosphatidylcholine solutions (2.5 mg/ml of chloroform) and liposomes were spotted (10 μl) on a silica TLC plate (Kieselgel GF 254) and developed for 15 cm with a mixture of chloroform–methanol–acetic acid–water (100 : 60 : 25 : 10, v/v). Samples were visualized with molybdenum blue.[6]

Gas Chromatography–Mass Spectrometry (GC-MS) of Fatty Acid——Free and total fatty acids were extracted using a classical method,[7] and derivatized with trimethylammonium hydroxide (50 °C, 2 min). Fatty acid methyl esters were eluted using a gradient of temperature (120 to 290 °C—10 °C/min) from a 12 m × 0.22 mm i.d. methylsilica (OVI) column (Hewlett Packard). MS was performed by electron impact (70 eV) with a Hewlett Packard mass spectrometer (HP 5970).

Results and Discussion

Lipid Peroxidation

The results on peroxidation index (Table I) and MDA production, a useful index of lipid oxy-radical propagation reactions,[8,9] showed no clear trend. Thus, lipid peroxidation, under the conditions used in this study, could not explain the decrease of PL. The susceptibility of unsaturated fatty acid molecules to the initiation of lipid peroxidation varies according to the lability of their allylic hydrogens. The divinylmethane structures present in most polyunsaturated fatty acids are particularly susceptible to peroxidation.[10] The low concentration of such fatty acids in our liposomal preparation can explain the present results.

DPPC and DSPC Determination

The decrease of DSPC and DPPC concentrations (Table II) indicated the chemical instability of the PL. In view of the results on peroxidation, this breakdown could be due to hydrolysis of fatty acyl chains. If this is correct, lysophosphatides should be formed.

Lysophosphatides Identification

The results of TLC for lysophosphatides are shown in Table III. These results correlate well with the DSPC and DPPC concentrations found at the same times of storage.

Fatty Acids

To evaluate the ratios of saturated fatty acids (free or not) and unsaturated fatty acids (free or not), stearic acid concentration/(oleic acid + linoleic acid) concentration ratio (R) has

TABLE I. Study of Lipid Peroxidation of Fatty Acid from Phosphatidylcholine

Storage (d)	Peroxidation index	MDA (μmol/l)
0	0.2	3.0
7	0.6	3.5
28	1.0	4.4
40	1.0	3.9
70	1.0	3.0

TABLE II. DSPC and DPPC in Liposomes

Storage (d)	DPPC (mg/l)	DSPC (mg/l)
0	3.9	4.3
7	3.8	4.3
28	2.1	2.8
40	0.85	1.0
70	0.88	1.1

TABLE III. Semiquantitative Determination of Lysophosphatides in Liposomes Containing DSPC and DPPC

Storage (d)	Lysophosphatides	Storage (d)	Lysophosphatides
0	Not detected	28	< 2.5 mg/ml
8	Not detected	40	> 2.5 mg/ml

been calculated from the gas chromatographic results. The value of R increased during storage for free fatty acids (1.56 at 5 d; 2.17 at 28 d and 2.59 at 40 d) and decreased for total fatty acids (2.34 at 5 d, 1.8 at 28 d and 1.45 at 40 d). These results could be explained by stearoyllysophosphatidylcholine production and subsequent stearic acid liberation.

Conclusion

Our data suggest that lipid peroxidation is not the main deterioration mechanism.[10] DPPC and DSPC liposomes are chemically unstable, but this may be attributed to the ratio of saturated : unsaturated fatty acids. Under our conditions, lysophosphatides were the main products identified. Such modification may be correlated with the spherical vesicle disorganization observed by electron microscopy and the consequent destabilization of liposomes as colloidal particles. Lysophosphatides are not normally formed in cells or bilayers in any quantity, and they are toxic and injurious to membranes.[11] It may be possible to protect PL from hydrolysis by freezing or lyophilization of liposomes.[12,13]

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