Electron Spin Resonance Studies of Phosphatidylcholine Interacted with Cholesterol and with a Hopanoid in Liposomal Membrane

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The effects of bacteriohopane-32-ol (Monol) on liposomal membrane composed of dipalmitoylphosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (egg PC) were compared with those of cholesterol (Chol) in the change of fluidity using a spin label. The fluidity change close to the polar head groups caused by temperature increase in the DPPC membrane containing Monol was different from that of Chol. Chol had a condensing effect on DPPC membrane, whereas Monol had only a slight effect except when used at 20 mol%. Near the hydrophobic end, Chol incorporation into DPPC led to fluidization below transition temperature (T_m) and condensation above T_m . Monol incorporation into DPPC had only a fluidizing effect below T_m . On the other hand, in egg PC membrane Chol had the condensing effect at any temperature, whereas Monol had only slight effect. These results suggest that Monol may have a role in supporting constant membrane fluidity under drastic conditions.

Keywords hopanoid; cholesterol; liposome; ESR spectrum; spin label; membrane fluidity

Sterols are indispensable components of the membranes in eukaryotic cells and play functional important roles in the membranes.¹⁾ Lacking the sterols normally in prokaryotes, the existence of other molecules functional equivalent to sterols has been assumed. Hopanoids, which belong to the triterpene family, are found in strains widely scattered through numerous taxonomic groups such as cyanobacteria, methylotrophs, purple non-sulphur bacteria and gram-negative chemoheterotrophs.²⁾ According to their structural features and the characteristics of their biosynthetic pathway, hopanoids have been considered to be phylogenetic precursors of sterols and to act as membrane reinforcers on prokaryotic systems as sterols do in the membranes of eukaryotes.³⁾ Bacteriohopane-32-ol (Monol) used in this study is one of the hopanoids derived from many hopanepolyols⁴⁾ and has structural similarities to cholesterol (Chol) (Fig. 1). We recently observed that Monol can be incorporated into the lipid phase of dipalmitoylphosphatidylcholine (DPPC) liposomes and that it stabilizes the liposomal membranes.⁵⁾

In this study, we compared the reinforcing effect of Monol on the liposomal membranes composed of DPPC or egg yolk phosphatidylcholine (egg PC) using the electron spin resonance (ESR) method.

Materials and Methods

Lipids DPPC and egg PC were purchased from Nippon Oil & Fats Co., Ltd. 5-Doxyl stearic acid (5-SASL) and 16-doxy stearic acid (16-SASL) were obtained from Aldrich Chemical Co. Chol from Wako Pure Chemical Industries, Ltd. was recrystallized from $C_2H_5OH-H_2O$ (2:1, v/v). Monol was prepared as described previously.⁵⁾

Preparation of Liposomes PC (10 μ mol), various amounts of Chol or Monol (2.5, 5, 7.5 μ mol) and spin probes (PC:probe=150:1, mol/mol) were dissolved in $CHCl_3$ and the solvent was evaporated *in vacuo* to form a thin homogeneous film in a tube. Phosphate buffered saline (pH 7, 0.5 ml) was added and the mixture was vigorously vortexed for 1 min and then sonicated for 10 min at 50°C.

ESR Measurement ESR spectra were recorded with a JEOL JES-FE spectrometer (X-band, 100 kHz field modulation, 0.63 mT modulation width) equipped with a temperature controller.

Results and Discussion

Effect of Monol on DPPC Membranes Figure 2 shows typical spectra of 5-SASL (a) and 16-SASL (b) at various temperatures in liposomes composed of DPPC. A maximum splitting value, which is directly related to the viscosity of

Fig. 1. Comparison of the Dimensions of Chol (a) and Monol (b) and Structural Equivalents

Fig. 2. ESR Spectra of 5-SASL (a) and 16-SASL (b) Embedded in the Liposomes Composed of DPPC

The temperatures of ESR measurements are indicated to the left of the spectra.

the environment, has been used as a convenient parameter to monitor the rotational motional freedom of the nitroxide radical group of 5-SASL. The nitroxide radical of 5-SASL is located close to the polar groups of phospholipids in the liposomal membranes. As can be seen from Fig. 2a, the splitting value decreased with increasing temperature, indicating a decrease of alkyl chain order near the phospholipid head groups. The fluidity of bilayer near the hydrophobic end can be examined using a spin probe of 16-SASL. The ESR spectra of 16-SASL in the DPPC liposomes were characterized by three peaks as shown in Fig. 2b. In these spectra, the ratio of the low-field peak height to the central one ($h_{(+1)}/h_{(0)}$) can be used as an empirical parameter for the membrane fluidity.⁶⁻⁸ An increase of the values of this parameter reflects an increase in the mobility of the nitroxide radical near the hydrophobic end of the acyl chains.

ESR spectra of 5-SASL and 16-SASL in liposomes containing Monol of 20 mol% to DPPC concentration are presented at various temperatures in Fig. 3. On the basis of these spectra, the effects of Monol on DPPC membranes can be estimated. Figure 4 shows the effects of various amounts of Chol or Monol on the splitting value as a function of temperature. The incorporation of Chol into the DPPC liposomes decreased the membrane fluidity and affected the phase transition observed at about 40 °C in the liposomes composed of DPPC alone (control) (Fig. 4a). When Monol was incorporated instead of Chol, the change of the membrane fluidity was similar to that of the control except

for the presence of 20 mol% Monol (Fig. 4b). In the liposomes containing 20 mol% Monol, the fluidity was higher than that of the control below the transition temperature (T_m) of DPPC and the fluidity change was slight even at higher temperatures. This Monol content is close to the content of hopanoids detected in *Bacillus acidocaldarius* under cultivation at high temperatures.⁹ *B. acidocaldarius* is a unique bacterium which grows in the pH range from 2 to 6 at temperatures from 45 to 70 °C.¹⁰ Therefore, its content of about 20 mol% may have an important role in maintaining bacterial membranes that are subjected to lysis-inducing stress.

Figure 5 shows the change of the $h_{(+1)}/h_{(0)}$ value as a function of temperature in the presence of various amounts of Chol or Monol. The phase transition could be observed at about 40 °C in the control. However, the change of the membrane fluidity near the hydrophobic end of the acyl chains caused by Chol was different from that observed near the polar groups of the phospholipids (Fig. 4). At temperatures below T_m , the fluidity of the membrane was increased by the addition of Chol (fluidizing effect). On the other hand, at temperatures above T_m , the fluidity was lower than that of control (condensing effect) (Fig. 5a). Such a contradictory effect of Chol has been reported by many researchers¹¹ and seems to be due to the formation of intermediate gel state resulting from a hydrophobic interaction of Chol with the fatty acyl chains of the saturated PC.¹² When Monol was incorporated, the fluidizing effect was observed below T_m , but no condensing effect above T_m was produced (Fig. 5b). These results indicate that Monol has no effect on the membrane fluidity at liquid crystalline state but that it affects the molecular order of the liposome membranes composed of saturated PC in its own specific manner. Both Chol and Monol molecules possess a quasi-planar, rigid and amphiphilic structure (Fig. 1).³ Monol seems to be oriented parallel to the fatty acyl chains of the phospholipids in an inverted orientation compared to Chol, because its hydroxy group is located in the side chain. It can be considered that there are hydrogen bonds between the hydroxyl groups at the side chain of Monol and the ester carbonyl groups of the saturated PC. Furthermore, Monol has methyl groups on both sides of the plane of the skeleton, whereas methyl groups of sterol are directed only on one side of its ring system.¹³ The hydrogen bonds and these methyl groups may produce a specific effect on the membrane composed of saturated PC. These considerations support that the membrane fluidity

Fig. 3. Temperature Dependence of ESR Spectra of 5-SASL (a) and 16-SASL (b) Embedded in the DPPC Liposomes Containing 20 mol% of Monol

All are plotted at the same instrument sensitivity so that their spectra intensities can be directly compared. (The spectrum at 60 °C in (b) was drawn on a scale of half intensity.)

Fig. 4. Change of the Maximum Splitting of 5-SASL in the DPPC Liposomes Containing Chol (a) or Monol (b) as a Function of Temperature

Content of Monol: —●—, 0 mol%; ---○---, 20 mol%; ---△---, 33.3 mol%; ---□---, 42.9 mol%.

Fig. 5. Change of the Ratio of Peak Height of 16-SASL in the DPPC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

near the polar groups at the lower temperatures showed little change by Monol addition (Fig. 4b). As the ring system is somewhat shifted towards the ends of the acyl chains of DPPC, the lower condensation effect of Monol at the higher temperatures would be due to a perturbation of the interactions of the ring system of Monol with the acyl chains.¹³⁾

Stabilization of DPPC Membranes by Monol We recently reported that a release of entrapped 6-carboxyfluorescein (6-CF) from DPPC-DCP (dicetylphosphate) liposomes at 37 °C was increased by the addition of Monol at 20 mol%.⁵⁾ In the present study, the fluidity of DPPC liposome membranes containing 20 mol% Monol was

Fig. 6. Change of the Order Parameters in 5-SASL (a) and 16-SASL (b) Embedded in the DPPC Liposomes Containing Various Amounts of Monol

—○—, at 20 °C; —●—, at 40 °C.

Fig. 7. Change of the Maximum Splitting of 5-SASL in the Egg PC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

Fig. 8. Change of the Ratio of Peak Height of 16-SASL in the Egg PC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

higher than that of the control below T_m . The destabilization of the DPPC-DCP liposomes by Monol correlates well with this fluidizing effect of Monol on the DPPC bilayer.

To clarify the peculiar effect of 20 mol% content of Monol on the DPPC membrane, ESR spectra of 5-SASL and 16-SASL were measured under various conditions. The order parameters, the maximum splitting and h_{l+1}/h_{l0} values were plotted against Monol concentration at two temperatures (Fig. 6). As shown in Fig. 6a, a trough could be observed around 20 mol% Monol at 20 °C. At this ratio, Monol should hold most loose packing near the phospholipid head groups interacting with its side chain. At 40 °C, however, the maximum splitting values were increased by addition of Monol, suggesting the condensing effect of this material above T_m .

Changes of the h_{l+1}/h_{l0} value in the 16-SASL spectra as a function of Monol concentration are shown in Fig. 6b. A crest can be observed around 25 mol% Monol at 20 °C. The bulky ring system extending to the acyl chain ends of DPPC presumably decreases the acyl chain order around this mole ratio. At 40 °C, Monol did not affect the h_{l+1}/h_{l0} value at any concentrations.

Effect of Monol on Egg PC Membranes Next, in order to elucidate the function of Monol, the effects of Monol on the liposome membranes composed of egg PC were compared with those of Chol. Figure 7 shows the change of the maximum splitting value of 5-SASL in egg PC liposomes as a function of temperature in the presence of various amounts of Chol or Monol. The increase of Chol content lowered the fluidity independent of temperature (Fig. 7a). The influence of Monol on the membrane fluidity is less than that of Chol (Fig. 7b). The peculiar effect of Monol observed in the DPPC membranes at the concentration of 20 mol% was not obvious in the egg PC membranes. When 16-SASL was used as a spin probe, comparative results with those shown in Fig. 7 were obtained (Fig. 8). In the egg PC liposomes, the contradictory effect of Chol shown in Fig. 5a was not observed. Monol seems to have a comparatively little influence on the fluidity of the membranes composed of unsaturated PC. The difference of the fluidity between the DPPC and egg PC membranes has resulted from the difference in their molecular packings with Chol or Monol in the hydrocarbon region of the bilayer.¹¹⁾ This may be due to the fit between the ring systems of Monol and the kinks of unsaturated acyl chains in egg PC molecules. From these results, it can be said that Monol has some role in the homeostatic regulation of the membrane fluidity. This fluidity is considered to be a crucial factor in regulating certain cellular processes such as permeability of small molecules,¹³⁾ proliferation,^{14,15)} fusion¹⁶⁾ and endocytosis.¹⁷⁾ A decrease^{18,19)} or increase²⁰⁾ of the membrane fluidity or activation resulted in strong inhibition of the activities of membrane bound enzymes. The incorporation of Monol into the membrane allows the fluidity to remain constant and support its normal functions under drastic conditions such as at high temperatures.

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