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Fluorescence analysis with diphenylhexatriene and its ionic derivatives of the fluidity of liposomes constituted from stratum corneum lipids: Contribution of each lipid component and effects of long-chain unsaturated fatty acids

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Summary

The fluidity of bilayer liposomes of stratum corneum lipids, consisting of ceramide, cholesterol, cholesteryl sulfate and palmitic acid, was examined by observing the fluorescence anisotropies of diphenylhexatriene (DPH) and its ionic derivatives as reporters of the fluidity of the two interfacial regions and the central region of the lipid bilayer. The effect of each lipid component was studied by comparing the fluorescence anisotropies of the probes in liposomes from which individual lipids were omitted with those in liposomes consisting of all four lipid components. The fluorescence anisotropies of DPH and its ionic derivatives at 37°C were decreased by omission of cholesterol, but not affected by omission of cholesteryl sulfate or palmitic acid. The phase transition temperature was also decreased significantly by omission of cholesterol. These results indicated the possibility that cholesterol is the main component causing solidity of the stratum corneum lipid bilayer. Addition of long-chain *cis*-unsaturated fatty acids, which are penetration enhancers, resulted in decreases in the fluorescence anisotropies of DPH and its ionic derivatives as well as decreases in the phase transition temperature. The results indicated that perturbation by these fatty acids of lamellar lipid domains in a wide range from the interfacial to deeper regions enhances penetration of drugs.

Introduction

Transdermal drug delivery systems have been investigated because of their potential therapeutic use. To be clinically effective, drugs must have sufficient transdermal permeability. However, most drugs show low permeability in the skin, presumably because of the solidity of lamellar lipid domains. Therefore, the effects of various kinds of penetration enhancers on the permeabilities of drugs have been examined (Walters, 1989). Of the compounds tested, Azone and long-chain *cis*-unsaturated fatty acids such as oleic acid have been shown to be very effective in enhancing the permeability of various drugs to the skin (Cooper, 1984; Okamoto et al., 1988; Walters, 1989). One

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of the possible mechanisms of their effect has been suggested to be via an increase in fluidity of lamellar lipids (Golden et al., 1987; Beastall et al., 1988; Francoeur et al., 1990; Ogiso et al., 1992; Sugibayashi et al., 1992), although the details of this mechanism remain unknown.

Liposomes are good models to clarify the factors governing the barrier function of stratum corneum (Abraham and Downing, 1991) and have been applied to reveal the mechanism of actions of penetration enhancers such as Azone, oleic acid and ethanol (Beastall et al., 1988; Ongpipattanakul et al., 1991; Krill et al., 1992a). Therefore, in this study we prepared liposomes consisting of stratum corneum lipid components and examined which lipid component caused solidity of the lipid bilayer. We examined fluidity in both the interfacial and central regions of the lipid bilayer using the fluorescent probes diphenylhexatriene (DPH), its cationic trimethylammonium derivative (TMA-DPH) and its anionic propionic acid derivative (DPH-PA). The latter two derivatives are probes of the fluidity of the interfacial region of the lipid bilayer, since they have ionic groups, while nonionic DPH is a reporter of the fluidity in the hydrophobic core of the lipid bilayer (Kuhry et al., 1983; Trotter and Storch, 1989; Kitagawa et al., 1991). Using these fluorescent probes, we also examined the effects of long-chain *cis*-unsaturated fatty acids on the fluidities of the interfacial and central regions of the lipid bilayer.

Materials and Methods

Materials

Ceramide (type IV), cholesterol, cholesteryl sulfate, fatty acids and DPH were purchased from Sigma Chemical Co. (St. Louis, MO). TMA-DPH and DPH-PA were obtained from Molecular Probes (Eugene, OR).

Preparation of liposomes

Ceramide, cholesterol, choresteryl sulfate and palmitic acid were dissolved in chloroform at a weight ratio of 4.0:2.5:1.0:2.5 (molar ratio, 3.2:3.2:1.0:4.4), following the procedure of Wertz et al. (1986). The solvent was evaporated, the lipids were suspended in medium A (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) and multilamellar vesicles were prepared by vortex mixing. The total lipid concentration was 1.2 mM. Then the vesicle suspension was sonicated first with a bath-type sonicator at 85°C for 15 min and then with a probe-type sonicator for 5 min at an output power of 80 W under a stream of nitrogen. Formation of liposomes of 100–300 nm diameter was confirmed by negative staining electron micrography with 2% phosphotungstic acid. Liposomes that contained ceramide and only two of the three other lipid components were prepared similarly without changing the amount of each lipid and formation of similar liposomes was confirmed as described by Wertz et al. (1986). Liposomes containing unsaturated fatty acids in addition to the four lipid components were prepared by a similar method.

Measurement of fluorescence anisotropy

The fluorescence anisotropies of DPH and its derivatives, which are markers of the fluidity of the lipid bilayer, were measured as described previously for platelet suspensions (Kitagawa et al., 1991). The liposome suspension was diluted 10-fold just before use with medium A mentioned above. A solution of TMA-DPH, DPH-PA or DPH in dimethylformamide was added to the liposome suspension at a final concentration of 0.5 μM. The final concentration of organic solvent added was limited to 0.033% to avoid perturbation of the lipid bilayer. Liposomes were incubated with TMA-DPH or DPH-PA for 2 min or with DPH for 10 min. Fluorescence in liposomes was measured in a 4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan). The excitation and emission wavelengths used for DPH, TMA-DPH and DPH-PA were 363 and 428 nm, 365 and 428 nm and 366 and 430 nm, respectively. Fluorescence anisotropy was calculated as described previously (Kubina et al., 1987). In the measurement of the temperature dependence of fluorescence anisotropy temperature was raised at the rate of about 1°C/min between two temperatures at which the fluorescence anisotropies were measured.

Results

Contribution of each lipid component to the fluidity of the stratum corneum lipid bilayer

To determine the effect of each lipid component on the fluidity of stratum corneum, we examined the fluorescence anisotropies of DPH and its analogs in liposomes consisting of the four lipid components of the stratum corneum (ceramide, cholesterol, cholesteryl sulfate and palmitic acid) and liposomes consisting of ceramide and two of the three other lipid components. Lipid mixtures from which ceramide was omitted did not form liposomes. The results at 37°C are shown in Table 1. The fluorescence anisotropies of DPH and its ionic derivatives in the liposomes were much greater than those in biological membranes such as platelet membranes (Kitagawa et al., 1991), indicating that a wide region of the lipid bilayer is solid at 37°C. DPH showed lower values than other ionic probes due to its different molecular motion in lipid bilayers (Mulders et al., 1986). However, in liposomes without cholesterol, the fluorescence anisotropies of DPH, TMA-DPH and DPH-PA were all significantly lower, although the decrease in that of DPH-PA was less than the decreases in those of the other two probes. This shows that omission of cholesterol resulted in an increase in fluidity in both the central and interfacial regions of the lipid bilayer. However, omission of either cholesteryl sulfate or palmitic acid had much less effect or none at all on the fluidity at this temperature. These results suggest the possibility that cholesterol provides the main contribution to the solidity of stratum corneum lipid bilayer.

TABLE 1

Effects of omission of cholesterol (Chol), cholesteryl sulfate (CS), or palmitic acid (PA) on fluorescence anisotropies of DPH and its derivatives in stratum corneum lipid liposomes at 37°C

Omitted component	Probe	Fluorescence anisotropy
None	DPH	0.271 ± 0.003
Chol		0.251 ± 0.005 [c]
CS		0.276 ± 0.002 [b]
PA		0.268 ± 0.005
None	TMA-DPH	0.285 ± 0.003
Chol		0.262 ± 0.005 [c]
CS		0.289 ± 0.002 [a]
PA		0.285 ± 0.002
None	DPH-PA	0.287 ± 0.002
Chol		0.278 ± 0.003 [c]
CS		0.288 ± 0.004
PA		0.288 ± 0.002

Data are means ± SD for eight experiments. Statistical significances of differences from control liposomes were determined by Student's *t*-test: [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

Studies by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy have indicated lipid phase transitions in the range between 60 and 80°C in porcine stratum corneum (Golden et al., 1987) and between 65 and 85°C in human and porcine stratum corneum (Potts et al., 1991). Studies by FTIR spectroscopy have also showed a gel to liquid-crystalline transition between 55 and 80°C in stratum corneum lipids of hairless mouse skin (Krill et al., 1992b). Similarly, in liposomes containing all four lipid components of the stratum corneum the fluorescence anisotropy of DPH decreased markedly at 70°C, indicating phase transition of the lipid bilayer from the gel to the liquid crystal state at this temperature. This finding is also consistent with the result in liposomes of the same lipid composition studied by ^{1}H-NMR, which indicated the phase transition at about 75°C (Wertz et al., 1986). These results show that the thermal behavior of lipid bilayer in the liposomes of all four components of stratum corneum is similar to that of lamellar lipids in stratum corneum, although the degrees of hydration of lipids are different.

On the other hand, in liposomes without cholesterol, the decrease started at about 60°C and decreased until 70°C; i.e., the transition temperature decreased about 10°C (Fig. 1). As revealed at 37°C, below 60°C where lipids were in the gel state, the fluorescence anisotropy of DPH was always lower than that in liposomes containing all four lipid components. On the other hand, omission of cholesteryl sulfate or palmitic acid had little effect below 58°C. However, omission of cholesteryl sulfate resulted in a slight decrease in the phase transition temperature of liposomes,

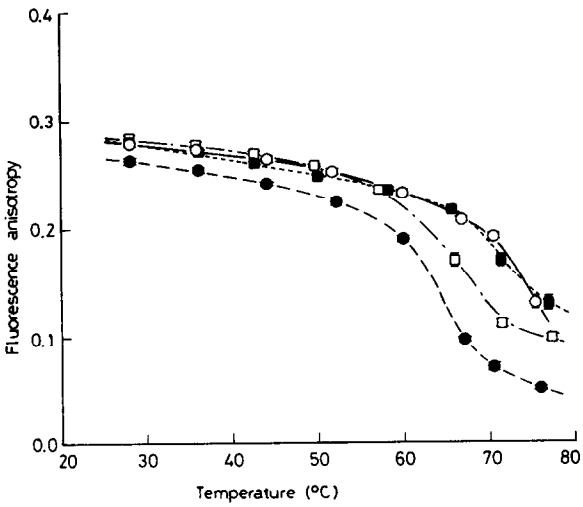

Fig. 1. Phase transition as monitored by temperature dependency of fluorescence anisotropy of DPH in liposomes composed of four stratum corneum lipid components (○), and those without cholesterol (●), without cholesteryl sulfate (□) and without palmitic acid (■). Data are means ± SD for four experiments.

but omission of palmitic acid had little effect on the phase transition (Fig. 1). Similar results were obtained with TMA-DPH instead of DPH (data not shown).

These results all indicate the possibility that cholesterol is the main component responsible for the solidity of stratum corneum lamellar lipid domains that causes poor transdermal penetration of drugs.

Effects of long-chain unsaturated fatty acids on the fluidity of stratum corneum lipids

Long-chain unsaturated fatty acids have been reported to enhance transdermal drug penetration (Cooper, 1984; Golden et al., 1987; Francoeur et al., 1990). These unsaturated fatty acids have been suggested to increase skin permeability by causing perturbation of stratum corneum lipids (Golden et al., 1987; Francoeur et al., 1990), although details of the mechanism involved are unknown. We examined the effects of additions of these fatty acids on the fluidities of both interfacial and central regions of the bilayer of stratum corneum lipid liposomes using DPH and its ionic derivatives. The results obtained at 37°C are shown in Table 2. Addition of the long-chain *cis*-unsaturated fatty acids, oleic acid and linoleic acid, decreased the fluorescence anisotropies of DPH and its ionic derivatives, although the decreases were less with DPH-PA than those with the other two probes. As shown for oleic acid, these unsaturated fatty acids increased the fluidity dose-dependently. The effects of oleic acid and linoleic acid were similar. These results indicate that *cis*-unsaturated fatty acids perturbed both the central and interfacial regions of the lipid bilayer. The *trans*-unsaturated fatty acid, elaidic acid, also decreased the fluorescence anisotropies of these probes, although slightly less than the *cis*-unsaturated fatty acids. However, the saturated fatty acid, stearic acid, did not decrease the fluorescence anisotropies of these probes, and, in fact, slightly increased that of DPH. These results on the effects of long-chain fatty acids on liposome fluidity are consistent

TABLE 2

Effects of additions of fatty acids on fluorescence anisotropies of DPH and its derivatives in stratum corneum lipid liposomes at 37°C

Probe	Fatty acid added	Mol% [a]	Fluorescence anisotropy
DPH	none		0.271 ± 0.003
	oleic	10	0.264 ± 0.002 [c]
		20	0.260 ± 0.002 [c]
	linoleic	20	0.259 ± 0.001 [c]
	elaidic	20	0.264 ± 0.001 [c]
	stearic	20	0.275 ± 0.003 [b]
TMA-DPH	none		0.285 ± 0.003
	oleic	10	0.276 ± 0.002 [c]
		20	0.269 ± 0.002 [c]
	linoleic	20	0.274 ± 0.001 [c]
	elaidic	20	0.276 ± 0.002 [c]
	stearic	20	0.286 ± 0.001
DPH-PA	none		0.287 ± 0.002
	oleic	10	0.281 ± 0.002 [c]
		20	0.276 ± 0.002 [c]
	linoleic	20	0.276 ± 0.003 [c]
	elaidic	20	0.280 ± 0.002 [c]
	stearic	20	0.287 ± 0.002

[a] Percent ratio of fatty acids, defining the total molarity of all four stratum corneum lipid components as 100%.
Data are means ± SD for eight experiments. Statistical significances of differences from control liposomes were determined by Student's *t*-test: [b] $P < 0.05$; [c] $P < 0.001$.

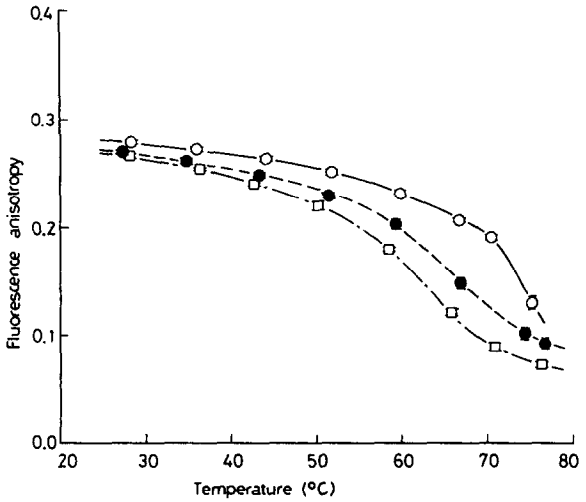

Fig. 2. Effects of oleic acid on phase transition of stratum corneum lipid liposomes monitored by temperature dependency of fluorescence anisotropy of DPH: control (○); with 10 mol% oleic acid (●); with 20 mol% oleic acid (□). Data are means ± SD for four experiments.

with the enhancing effects of the same long-chain fatty acids on transdermal drug penetration reported by others (Cooper, 1984; Golden et al., 1987).

The effects of *cis*-unsaturated fatty acids in increasing the fluidity of stratum corneum lipids were also demonstrated as dose-dependent effects in lowering the phase transition temperature of the lipids, as shown in Fig. 2 for oleic acid by a temperature-dependent change of fluorescence anisotropy of DPH. On addition of 10 or 20 mol% oleic acid, the fluorescence anisotropy decreased between 50 and 75°C. That is, gel to liquid-crystalline phase transition occurred near 66°C in the presence of 10 mol% oleic acid and 62°C in the presence of 20 mol% of the acid, although the phase transition was induced above 73°C in its absence. The results are consistent with DSC data on porcine stratum corneum (Golden et al., 1987), and suggest that the enhancing effects of long-chain unsaturated fatty acids on transdermal drug penetration are due at least in part to their extensive perturbation of lipid bilayers.

Discussion

The present results have demonstrated the possibility that cholesterol is a major component responsible for the solidity of stratum corneum lipid domains that contributes to the barrier properties of the skin. That is, we found that cholesterol decreased the fluidity at physiological temperature, at which lipid mixtures are in a gel state, as well as increasing the phase transition temperature (about 65°C in liposomes without cholesterol and above 73°C in liposomes of all four lipid components as mentioned above). These effects are different from its dual effects in mixtures of phospholipids and cholesterol: in lipid bilayers consisting of phospholipids and cholesterol, it causes liquefaction of lipids in the crystalline state and condensation of those in the liquid crystalline state (Demel and De Kruyff, 1976). Thus, cholesterol eliminates phase transition of phospholipids, although this effect depends on its concentration and the interaction between cholesterol and phospholipids seems very complex (Lentz et al., 1980).

The interaction between ceramides and cholesterol has been studied only recently (Abraham and Downing, 1991; Wiedmann and Salmon, 1991). The structures of ceramides are quite different from those of phospholipids, so their interaction with cholesterol differs from that of phospholipids with cholesterol. In fact, Wertz et al. (1986) reported that ceramides alone do not form liposomes, and cholesterol or cholesteryl sulfate is necessary to obtain liposomes containing ceramides. Therefore, ceramides must interact strongly with cholesterol and its analogs. Our findings that only cholesterol had a solidifying effect on lipids at 37°C, and that the fluorescence anisotropies of DPH and its derivatives did not increase in the absence of cholesterol even on increase in the concentration of cholesteryl sulfate (data not shown) indicate that ceramides must interact especially strongly with cholesterol. The condensed lipid state may involve hydrogen bonding between cholesterol and ceramides.

Long-chain *cis*-unsaturated fatty acids are reported to be effective for enhancing penetration of a variety of drugs (Walters, 1989). Although

their interaction with stratum corneum lipids appears to be important for their effects, details of the mechanism of their effects are unknown. Our results suggested that their actions involve extensive perturbation of lipid bilayers. However, the values obtained with our probes are means for the whole lipid bilayer. The bilayers of stratum corneum lipids may be heterogeneous, and the effects of unsaturated fatty acids may differ greatly in different lipid regions, as suggested by Francouer et al. (1990). However, since the localization of the fluorescent probes is unknown, we cannot predict it by the method used in this study. Conformational alteration of keratinized proteins by these fatty acids (Takeuchi et al., 1992) may also contribute to their effects in enhancing penetration of hydrophilic solutes.

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