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RESEARCH PAPER

The Relationship Between the Rigidity of the Liposomal Membrane and the Absorption of Insulin After Nasal Administration of Liposomes Modified with an Enhancer Containing Insulin in Rabbits

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ABSTRACT

The relationship between the rigidity of the liposomal membrane and the absorption of insulin after nasal administration of liposomes modified with an enhancer containing insulin was investigated for the nasal delivery of peptide drugs in rabbits. The rigid liposomal membrane makes liposomes stable, protecting insulin from enzymatic degradation. Soybean-derived sterol (SS) or its sterylglucoside (SG) was used as an enhancer. Dipalmitoylphosphatidylcholine (DPPC) liposomes modified with SG had increased fluidity of the hydrophobic group of the liposome bilayer compared with the liposomes modified with cholesterol (Ch) or SS, as shown by measurements of the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5,-hexatriene (DPH); however, the fluidity of the polar group of the liposome bilayer was decreased according to measurements of steady-state fluorescence anisotropy of dansylhexadecylamine (DSHA) at 37°C. These findings suggest that the fluidity of the hydrophobic group of the liposome bilayer is responsible for the increase of liposomal leakage and instability of the liposomes. When insulin was administered nasally to rabbits as a solution, no hypoglycemic effect was observed. The administration of insulin contained in DPPC/SG (7/4, mole) liposomes with high fluidity caused a high glucose reduction of long duration (8 hr). DPPC/SS and DPPC/Ch (7/4)

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liposomes with low fluidity caused low glucose reductions. These results demonstrated that liposomes modified with SG can be useful as carriers of insulin administered nasally.

INTRODUCTION

The nonparenteral routes that have been investigated for insulin delivery include nasal (1,2) and buccal routes since most peptides and proteins are not effective when administered orally. The nasal route is considered to be the most promising (3); therefore, several dosage forms (i.e., liquid, powder, and liposomal) for nasal absorption have been examined.

Liposomes are suitable as a drug carrier to protect insulin from enzymatic degradation and to maintain sustained release. However, insulin present alone in liposomes could not penetrate through nasal mucosa in vitro (4). We previously demonstrated that soybean-derived sterylglucoside (SG) and its sterol (SS) are effective enhancers for the nasal absorption of insulin liquid and powder dosage forms in rabbits (5). If liposomes modified with enhancer are used, the part of the enhancer projected from the liposomes may attack the nasal mucosa, and then the insulin concentrated in the liposomes may penetrate the nasal mucosa effectively. However, an enhancer disturbs the lipid structure of the nasal mucosa and also decreases the rigidity of the bilayer of liposomes. Therefore, the nasal absorption of liposomal insulin modified with SG may depend on the effectiveness of enhancer and the stability of liposomes.

In the intestinal absorption of liposomal insulin, the stability of the liposomes used is an important factor. SS stabilized L-α-dipalmitoylphosphatidylcholine (DPPC) liposomes to a greater extent than cholesterol (Ch), which is usually used as a stabilizer (6–8). In these cases, SS and Ch may decrease the drug-releasing ability of liposomes since the bilayer stability of liposomes and permeability across the nasal mucosa should always be balanced carefully. The stable liposomes modified with SS were absorbed orally to rats effectively (9). Therefore, we examined the nasal absorption of liposomal insulin in rabbits in terms of the rigidity of the liposomal membrane using liposomes modified with various molar ratios of SS, Ch, and SG.

MATERIALS AND METHODS

Materials

DPPC, Ch, and crystalline bovine pancreas insulin (27.0 IU per mg, crystalline; zinc content ~0.5%) were purchased from Sigma Chemical Company (St. Louis, MO). The SS and SG were generously supplied by Ryukakusan Company (Tokyo, Japan). All other chemicals used were reagent grade. The SG is a mixture of the glucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) (Fig. 1). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and dansylhexadecylamine (DSHA) were purchased from Sigma Chemical Company.

Preparation of Liposomes

Multilamellar vesicle liposomes (MLVs) were prepared according to a standard method (10) as described in a previous study (6). Briefly, the appropriate lipids were dissolved in chloroform and dried under reduced pressure. Insulin was dissolved in 0.2 ml of 0.01 N HCl, and the solution was neutralized by adding an equal volume of 0.01 N NaOH. Phosphate-buffered saline (PBS) (pH 7.4) was then added. The obtained lipid film (70 μmol DPPC) was then hydrated in 3 ml of the insulin-PBS solution (90 IU/ml). It was mixed by vortexing, followed by sonication in a bath-type sonicator (Honda Electronics, W220R, Tokyo) and centrifugation at 9500 *g* for 5 min to remove large particles and to form liposomes of homogeneous size.

DPPC liposomes with Ch, SS, or SG were composed of DPPC/Ch, DPPC/SS, or DPPC/SG liposomes at the

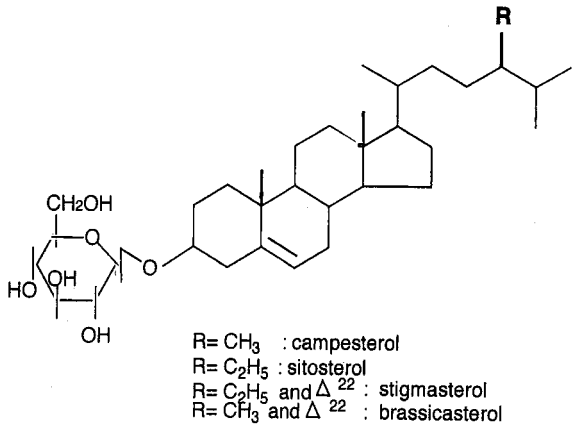

Figure 1. Chemical structures of soybean-derived sterylglucosides (SG).

molar ratio of 7/2 or 7/4, respectively. The mean size of the liposomes was 106–111 nm, and the insulin entrapment efficiency of the liposomes was 21–33 % (9). The insulin activity of the physical mixture solution of insulin solution and empty DPPC/SG (7/4) liposomes was adjusted to 37.4 IU/ml. DPPC/SG (7/4) liposome suspension was freeze-dried (SG-FD), and 11.721 mg of SG-FD-DPPC/SG (7/4) liposomes were resuspended in 3 ml of PBS (SG-Sus-FD).

Fluorescence Anisotropy Measurements

The precise method of fluorescence anisotropy measurement was previously reported (6). The fluorescence anisotropy of DPH in liposomes was measured at 20°C–50°C, and that of DSHA in liposomes was measured at 37°C. The excitation and emission wavelengths used for DPH were 357 and 430 nm, respectively, and those for DSHA were 350 and 480 nm, respectively. The effect of temperature on the fluorescence anisotropy of DPH was measured at a heating rate of 1.25°C/min over the range 25°C–35°C and 0.83°C/min over the range 35°C–47.5°C.

Administration Methods

Female Japanese rabbits weighing between 2.5 and 3.0 kg (Saitama Experimental Animal Supply Co., Saitama, Japan) were fasted for 24 hr before the administration of the drug. A polyethylene tube with an inner diameter of 0.58 mm and a length of 10.0 cm (PE50, Becton Dickinson Co., Lincoln Park, NJ) was fitted to the top of a syringe and inserted into the nose of the rabbit (11). A 250-μl dose of the liposome suspension was loaded into the syringe and administered through the tube into the rabbit's nasal cavity.

Pharmacological Bioavailability

The pharmacological bioavailability D of the intranasal (i.n.) dose of liposomal insulin was calculated with the following equation:

$$D = (AUC_{in}/dose_{in})/(AUC_{iv}/dose_{iv})$$

where AUC_{in} and AUC_{iv} are the individual areas under the serum glucose level curves of each rabbit administered liposomes containing insulin ($dose_{in}$) intranasally and that of the free insulin solution administered intravenously (i.v.), respectively. The AUC_{iv} of the free insulin solution dose (0.5 IU/kg, $dose_{iv}$) was fitted for a good linear relationship between the AUC and dose, as Yamamoto et al. reported (11).

Statistical Analysis

Data from the animal experiments were compared using analysis of variance and Student t test. A p value less than .05 was considered significant.

RESULTS AND DISCUSSION

Fluorescence Anisotropy of 1,6-Diphenyl-1,3,5-Hexatriene and Dansylhexadecylamine

Steady-state fluorescence anisotropy studies were performed using two different types of fluorescent probes: DPH, which partitioned deep in the hydrophobic interior of the liposome, and DSHA, which partitioned at the surface of the bilayer. The fluorescence anisotropy is proportional to the microviscosity. To determine the effects of SS, Ch, and SG on the fluidity of liposomes, the fluorescence anisotropy of DPH in the DPPC/X = 7/2 liposomes (mole, X = SS, Ch, or SG) and DPPC/X = 7/4 liposomes (X = SS, Ch, or SG) was determined. As shown in Fig. 2, the fluidity parameters for DPH appear to be related to the packing density of the bilayer (i.e., the acylchain orientation order).

Based on the fluorescence anisotropy data of DPH in liposomes, SG decreased the anisotropy of DPH in liposomes more so than did Ch and SS. Ch and SS each stabilized the DPPC liposomes by van der Waals force, but SG destabilized the liposomes since the fluorescence anisotropy values of the DPPC/SG liposomes were low. This finding corresponds well with the fact that lipo-

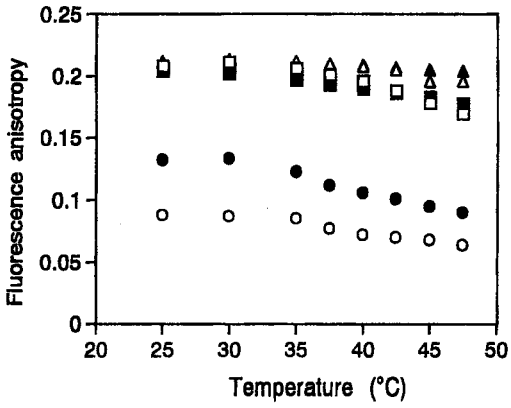

Figure 2. Temperature dependence of fluorescence anisotropy of DPH in liposomes. △, DPPC/SS (7/2) liposomes; ▲, DPPC/SS (7/4) liposomes; □, DPPC/Ch (7/2) liposomes; ■, DPPC/Ch (7/4) liposomes; ○, DPPC/SG (7/2) liposomes; ●, DPPC/SG (7/4) liposomes.

somes modified with SG have an increased release of the compound contained in the liposomes compared with liposomes modified with SS or Ch (6,7).

Figure 3 shows the results of the change in the fluorescence anisotropy of DSHA, representing the lipid fluidity of the polar head group of DPPC. When the SG was increased from 0 to 36.4 mol% (DPPC/SG = 7/4), the fluorescence polarization value increased from 0.02 to 0.06. SG may decrease the fluidity, that is, increase the microviscosity of the polar head group of DPPC and thus disturb its movement. In contrast, SS increased the fluidity at 36.4 mol%.

The microviscosity of the hydrophobic group and that of the polar group of the liposome bilayer was decreased, that is, the fluorescence polarization was decreased. The glucose residue of SG entering the polar part of lipids may increase the microviscosity of the polar group of the bilayer (Fig. 3), but the sterol group of SG may decrease the microviscosity of the hydrophobic part of the bilayer in liposomes (Fig. 2) since the packing state of the sterol group of SG may be changed by its glucose residue that projects out of the liposomes.

We reported that, with the use of DPPC liposomes containing SS, Ch, and SG (molar ratio 7:1.2), SS was closely packed in the DPPC lipid layer, whereas DPPC liposomes containing SG released more of the entrapped contents than those containing SS and Ch after incubation under physiological conditions (6). The results of the present study suggest that SG fluidizes (i.e., decreases the microviscosity of) the highly ordered hydrocarbon region deep in the liposome bilayer, but caused a decrease in the fluidity (i.e., increased the microviscosity) in the region near the surface of the bilayer. This finding suggests that the fluidity of the hydrophobic group of the bilayer is responsible for the increase of liposomal leakage and instability of the liposomes, that is, the hydrophobic group of the bilayer is responsible for the rigidity of the liposomal membrane and the stability of liposomes in biological fluids.

Nasal Absorption

We previously reported that SG is a more effective enhancer of the nasal absorption of insulin in suspension and powder dosage forms than is SS (5).

Figure 4 shows the effects of the compositions of liposomes containing insulin and insulin solution on the serum glucose levels at a dose of 7 IU/kg. After the nasal administration of insulin solution, the serum glucose level did not change. The DPPC/SG (7/4) liposomes were more effective than the DPPC/SS and DPPC/Ch (7/4) liposomes. The levels in the animals administered the DPPC/SS (7/4) and DPPC/Ch (7/4) liposomes decreased to 69.9% and 74.6% of the initial serum glucose levels, respectively, at 1 hr, whereas the levels in the animals treated with DPPC/SG (7/4) liposomes decreased to 63.6% at 1 hr, and the reduction effect lasted for 8 hr.

The *D* values of the DPPC/SG (7/4), DPPC/SS (7/4), and DPPC/Ch (7/4) liposomes were about 13.3%, 5.1%, and 3.0%, respectively. The DPPC/Ch or DPPC/SS (7/4) liposomes, with highly rigid liposomal membranes

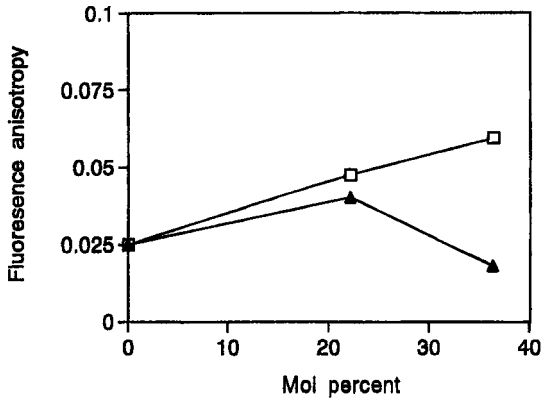

Figure 3. Fluorescence anisotropy of DSHA as a function of SS and SG in liposomes at 37°C. The mean values of duplicate measurements are shown. ▲, DPPC/SS liposomes; □, DPPC/SG liposomes (7/2 or 7/4).

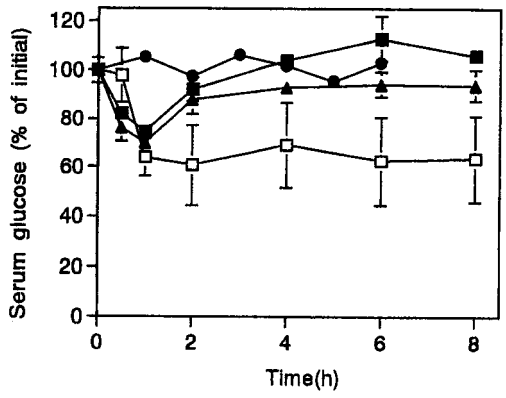

Figure 4. Serum glucose level after the nasal administration of insulin solution or liposomes (DPPC/SS, Ch, or SG = 7/4) at a dose of 7 IU/kg to rabbits. Each value represents the mean ± SE (n = 3 or 4). ▲, DPPC/SS (7/4) liposomes; ■, DPPC/Ch (7/4) liposomes; □, DPPC/SG (7/4) liposomes; ●, insulin solution.

Table 1

Comparison of Hypoglycemic Effect Obtained with DPPC/SS, DPPC/Ch, and DPPC/SG Liposome Suspensions and Powder After Nasal Administration in Rabbits

Preparation DPPC/*X*	Dose (IU/kg)	$AUC_{0-6\ hr}$ (% hr Glucose Reduced)	Availability (*F*, %)	
Intravenous insulin solution	0.5	102.0 ± 2.6		
Intranasal				
SS (7/4)	7.0	72.5 ± 7.1	5.1 ± 0.5	[b]
Ch (7/4)	7.0	42.9 ± 5.1	3.0 ± 0.36	[b]
SG (7/4)	7.0	190.0 ± 81.8	13.3 ± 5.7	
SG (7/4) mix[a]	5.0	160.1 ± 32.5	15.7 ± 3.2	[b]
SG-FD (7/4) powder	2.0	98.7 ± 21.5	24.2 ± 5.3	[b]
SG-Sus-FD (7/4)	2.0	72.5 ± 5.2	17.8 ± 1.3	[b]

$n = 3$ or 4, mean ± SE.
[a]Mix: the mixture of empty DPPC/SG (7/4) liposomes and insulin solution.
[b]$p < .05$.

(Fig. 2, Table 1), showed low absorption of insulin after nasal administration. The DPPC/SG (7/4) liposomes, with less rigid liposomal membranes, showed higher absorption of insulin.

Ando et al. (13) reported that SG showed a higher degree of enhancement of the permeation of insulin through the nasal mucosa, with some effect on lipids in the nasal mucosa. Therefore, the low rigidity of liposomal membranes might be an important factor in the release of insulin from liposomes since liposomes do not release insulin instantly (4) and have a high affinity for lipids the in nasal mucosa (13).

We previously reported that DPPC/SS and DPPC/Ch (7/4) liposomes are effective carriers for the oral administration of insulin compared with DPPC/SG (7/4) liposomes (9), and their high rigidity of the liposomal membranes correlates with the long duration of the reduction of the blood glucose level and the high *D* value of DPPC/SS and DPPC/Ch (7/4) liposomes. This finding suggested that liposomes as a carrier for the oral administration of insulin may need highly rigid liposomal membranes to protect liposomes from lumenal movement. Weingarten et al. (14) reported the stability of free insulin compared with liposomal insulin, and free insulin was denatured at the surface of the intestinal mucosa by gastrointestinal enzymes. Therefore, high rigidity of liposomal membranes (i.e., the stability of liposomes) might be an important factor in the lumen when liposomes are taken orally.

In contrast, liposomes used as a carrier for nasally administered insulin may not need rigid liposomal membranes, but rather may need the quick release of the drug from the liposomes and/or an enhancer for the nasal mucosa.

Effects of Various Preparations of Liposomes

Figure 5 shows the effects of various preparations of DPPC/SG (7/4) liposomes on the serum glucose levels over time. Four kinds of preparations were prepared and administered to groups of rabbits: DPPC/SG (7/4) liposomes, the physical mixture of insulin solution and empty DPPC/SG (7/4) liposomes (Mix), an SG-Sus-FD-DPPC/SG (7/4) liposome suspension (SG-Sus-FD), and the powder of DPPC/SG (7/4) liposomes (SG-FD).

The glucose level in the Mix- and SG-FD-treated groups decreased to 52.8% and 72.3% of the initial glucose levels, respectively, at 1–2 hr. The level of the SG-Sus-FD group decreased to 86.0% of the initial glucose level at 4 hr. The same dose of insulin could not be given to each group since the activities of insulin in the various formulations were different.

Table 1 summarizes the *D* values of the various preparations. The *D* value after the nasal administrations of SG-FD, Sus-FD, and Mix were about 24.2%, 17.8%, and 15.7%, respectively. The insulin solution was not absorbed, but the insulin solution with SG was absorbed

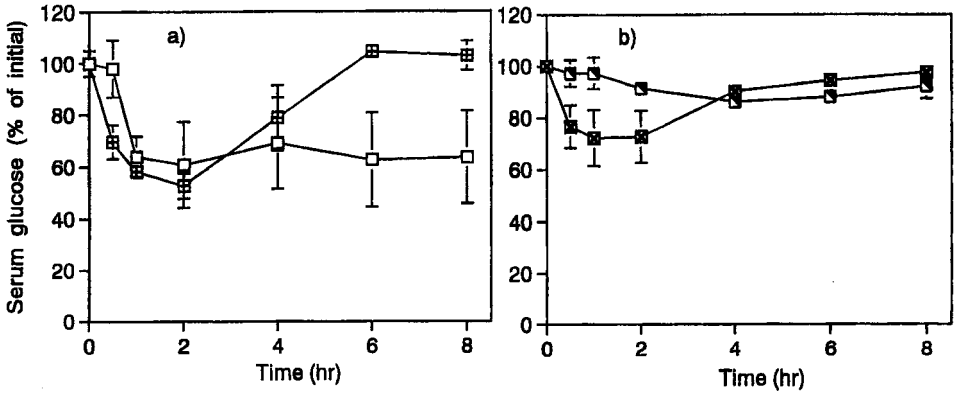

Figure 5. Serum glucose level after the nasal administration of □ DPPC/SG (7/4) liposomes at a dose of 7 IU/kg, ⊞ the physical mixture of DPPC/SG (7/4) empty liposomes and insulin solution (Mix) at a dose of 5 IU/kg, ⊠ freeze-dried powder of DPPC/SG (7/4) liposomes (SG-FD) at a dose of 2 IU/kg, and ◪ a suspension of SG-FD (SG-Sus-FD) at a dose of 2 IU/kg. Each value represents the mean ± SE (n = 3 or 4).

(5). The use of the DPPC/SG (7/4) liposomes resulted in a long-term reduction of the glucose level compared with the other liposome preparation (Fig. 5a). One of the reasons for this difference may be that the transiently high insulin concentration induces a strong pharmacological effect that prolongs the decrease in the glucose level since SG in insulin liquid and powder dosage forms enhances the insulin absorption from the nasal mucosa more than SS and Ch do (13). This hypothesis may be supported by the finding that the release of calcein from DPPC/SG liposomes containing calcein as a model drug was higher than for DPPC/SS or DPPC/Ch liposomes (7). In addition, DPPC/SG (7/4) liposomes might adhere to the nasal mucosa more than other liposomes do and thus releases insulin in a sustained manner.

Significant differences of D values between the Mix and liposomes were not observed. This suggests that insulin outside the empty liposomes in the Mix may be absorbed. Liu et al. (15) reported that an intratracheal instillation of a physical mixture of insulin and that of liposomes containing insulin produced similar pharmacodynamic responses. Our data correspond well with their results.

In the present study, the correlation between the enhancement of fluidity of liposomes induced by SG and the enhancement of the transport of insulin across the nasal mucosa is remarkable. The enhancement of nasal absorption depends on both the partitioning tendency and diffusivity of the permeant; it is expected that lipid fluidization of nasal mucosa by SG would increase both the partitioning tendency and the diffusivity for a permeant. The results of the present study may indicate that SG enhances the nasal absorption mainly, or at least in part, by fluidizing the lipids in the nasal mucosa as it does in liposomes.

CONCLUSIONS

The nasal administration to rabbits of DPPC/SG (7/4) liposomes, which showed high fluidity at 37°C, caused a high serum glucose reduction, and the reduction effect lasted for 8 hr. In contrast, DPPC/SS and DPPC/Ch (7/4) liposomes, which showed low fluidity, caused low serum glucose reductions. These results demonstrated that liposomes modified with SG can be useful as a carrier of insulin administered nasally.

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