Dipalmitoylphosphatidylcholine Liposomes with Soybean-Derived Sterols and Cholesterol as a Carrier for the Oral Administration of Insulin in Rats

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Dipalmitoylphosphatidylcholine (DPPC) liposomes with a soybean-derived sterol mixture (SS) or cholesterol (Ch) were examined as a carrier for the oral administration of insulin in rats. Four kinds of liposomes were prepared: liposomes containing SS or Ch (molar ratio of DPPC/X=7:2 or 7:4, X=SS or Ch), respectively. The pharmacological availability was greater and the lag time for the glucose reduction was later in the order of DPPC/SS (7:4)>DPPC/SS (7:2)>DPPC/Ch (7:4)≫DPPC/Ch (7:2)-liposomes. This order appears to correspond well with that of the rigidity of the liposomal membrane. In particular, DPPC/SS (7:4)-liposomes reduced blood glucose levels for up to 21 h in rats after oral administration. The highest absolute pharmacological availability was 31.6% at a dose of 20.0 IU/kg of DPPC/SS (7:4)-liposomes among the liposomes in comparison to intravenous administration.

Key words dipalmitoylphosphatidylcholine; liposome; soybean-derived sterol; cholesterol; availability; insulin

Insulin is a peptide widely used for the treatment of diabetic disease. However, most peptides and proteins are not effective when administered orally, and must be administered by injections because they are degraded by proteolitic digestion in the gastrointestinal tract when taken orally.

Liposomes have been extensively explored as carriers for improving the delivery of various therapeutic drugs.[1-4] When a liposomes-entrapped substance is administered, it could have different pharmacokinetics than that of its free, non-entrapped substance.[5] Liposomes are suitable as a drug carrier to protect insulin from enzymatic degradation and to maintain sustained release. Therefore, many studies have reported on insulin entrapped in liposomes for oral administration.[6-7]

We have reported that a soybean-derived sterol mixture (SS) stabilized the dipalmitoylphosphatidylcholine (DPPC) liposomes to a greater extent than cholesterol (Ch), which is usually used as a stabilizer, *in vitro*[8] and *in vivo*.[9,10] The SS is a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%), as shown in Fig. 1.

In this study, we examined the degree and duration of the hypoglycemia produced upon the oral administration of DPPC/SS- and DPPC/Ch-liposomes entrapping insulin to rats.

MATERIALS AND METHODS

Materials DPPC, Ch and cryatalline bovine pancreas insulin (27.0 IU per mg; crystalline; zinc content; approx. 0.5%) were purchased from Sigma Chemical Co. (St. Louis, MO). SS was generously supplied by Ryukakusan Co., Ltd., (Tokyo, Japan). All other chemicals used were of reagent grade.

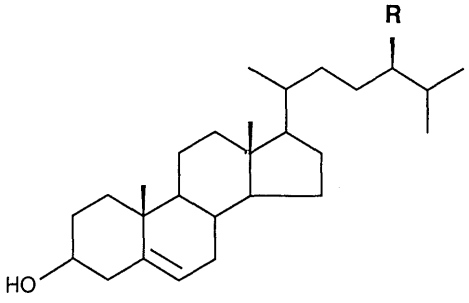
Male Wistar rats weighing 200—250 g, purchased from Saitama Experimental Animal Supply (Saitama, Japan), were used in all experiments.

Preparation of Liposomes Multilamellar vesicle liposomes (MLV) were prepared according to a standard method[11] as described in a previous study.[8] 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. Then pH 7.4 phosphate buffered saline (PBS) was added. The obtained lipid film (70 μmol DPPC) was then hydrated in 3 ml of insulin PBS solution (30 IU/ml). It was mixed by vortexing, followed by sonication in a bath-type sonicator (Honda Electronics, W220R, Tokyo, Japan) and centrifugation at 9500 × *g* for 5 min to remove large particles and to form a homogeneous size.

Four kinds of liposomes were prepared: liposomes containing SS and Ch (molar ratio of DPPC/X=7:2 or 7:4, X=SS, Ch), respectively. The size of liposomes was determined using a Nicomp 370 Submicron Particle Analyzer (Pacific Scientific, CA, U.S.A.).

The concentration of DPPC in the liposomes was measured by enzymatic assay using a Phospholipid B-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Determination of Insulin Entrapped in Liposomes The liposome-entrapped insulin (insulin/liposomes) was separated from free insulin by ultracentrifugation at 100000 × *g* for 20 min at 4 °C. The process was repeated three times



R= CH_3 : campesterol
R= C_2H_5 : sitosterol
R= C_2H_5 and Δ^{22} : stigmasterol
R= CH_3 and Δ^{22} : brassicasterol

Fig. 1. Chemical Structures of Soybean-Derived Sterols (SS)

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by making a suspension of the precipitate in 1 ml of PBS. A 0.3 ml portion of liposome fraction or free insulin fraction as a standard was shaken with 0.09 ml of chloroform to disrupt the liposomes. After centrifugation at 3000 rpm for 5 min, 0.02 ml of the aqueous phase containing insulin was injected into HPLC. The HPLC method was described by Nakazawa and Nagase.[12]

Animal Experiments Male Wistar rats (weighing 200—250 g) were fasted for 24 h before administration of the drug. The serum glucose level at zero was taken as a 100 percent glucose level. Insulin/liposomes after centrifugation at $9500 \times g$ (500 μl/200 g body weight, 15.5—28.3 IU/kg) were administered. A 500 μl portion of PBS and a dose of 80 IU/kg of insulin in 500 μl PBS (insulin solution), respectively, were given orally as a control. Approximately 5 min before administration, a 400 μl blood sample was taken from the jugular vein. Aliquots 400 μl of blood samples were taken at 1, 2, 3, 4, 6 and 21 h after administration ($n=3$). Serum was separated by centrifugation at 3000 rpm for 2 min and was kept frozen until analysis. The area under the glucose reduction–time curve (AUC) was calculated by the trapezoidal rule from 0 to 21 h. In this experiment, the areas above baseline levels were not included in the counting.

Analytical Method The serum glucose level was determined by the glucose oxidase method using a glucose B-Test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Data Analysis The absolute pharmacological availability (F) of the *p.o.* dose of insulin/liposomes was calculated from the following equation:

$$F=(AUC_{po}/\text{dose}_{po})/(AUC_{\text{iv}}/\text{dose}_{\text{iv}})$$

where AUC_{po} and AUC_{iv} are the individual area under the serum glucose levels of each rat administered insulin/liposomes (dose_{po}) orally and of the free insulin solution administered intravenously, respectively. The AUC_{iv} of the free insulin solution dose (0.5 IU/kg) was fit for a good linear relationship between the AUC and dose, which Morishita *et al.* reported.[13]

Statistical Analysis Data from the animal experiments using rats were compared using analysis of variance and the Student's t-test. A p value of 0.05 was considered significant.

Table 1. The Liposomal Characteristics of DPPC/SS and DPPC/Ch-Liposomes

Liposome composition	Size (nm)[a]	Entrapped insulin[b] (g/mol phospholipid)	Retention of insulin (%)[c]
DPPC/Ch (7:2)	83.6— 96.9	8.1	23.4
DPPC/Ch (7:4)	106.9—108.4	8.2	21.5
DPPC/SS (7:2)	139.6—157.1	3.3	17.9
DPPC/SS (7:4)	106.2—111.6	8.0	33.6

a) Size of liposomes was measured by nicomp. *b, c*) The mean value for duplicate measurements by HPLC.

RESULTS AND DISCUSSION

Characterization of Liposomes We have reported that SS stabilizes DPPC-liposomes (DPPC/SS (7:2)-liposomes) entrapping calcein by measuring the leakage of calcein *in vitro*[8] and *in vivo*.[9] Qi *et al.*[10] have also reported that the stabilizing effect was the greatest at DPPC/SS (7:4)-liposomes prepared by reverse phase evaporation vesicle (REV). Therefore, we used DPPC/SS (7:2, 7:4)-liposomes and DPPC/Ch (7:2, 7:4)-liposomes as carriers for the oral administration of insulin.

The liposome composition, size and entrapped insulin content are summarized in Table 1. Each size of DPPC/SS (7:2)-, DPPC/SS (7:4)-, DPPC/Ch (7:2)- and DPPC/Ch (7:4)-liposomes was 139—157 nm, 106—111 nm, 83—97 nm and 106—108 nm, respectively. The retention of insulin in DPPC/SS-liposomes increased with SS content, but the retention in DPPC/Ch-liposomes decreased with Ch content. The retention of insulin (%) appeared to be high, irrespective of MLV. This may be because insulin is adsorbed to the surface of liposomes using ultracentrifugation for the separation method of free insulin from liposomal insulin.

Liposome Composition Table 2 and Fig. 2 show the effect of liposome composition on serum glucose levels *vs.* time profiles, respectively. The serum glucose levels after the oral administration of PBS and insulin solution with and without liposomes at a dose of 80 IU/kg were higher than the initial level during the experimental period (Fig. 2a). This increase is considered to be due to stress by blood sampling under non-anesthetized conditions.[13] After oral administration of a mixture of DPPC/SS (7:4)-liposomes and insulin solution, the serum glucose level did not change. DPPC/SS (7:4)-liposomes were selected, since they are the most stable[10] and are effective in the intestinal absorption of liposomes containing insulin, which is

Table 2. Effect of Liposome-Entrapped Insulin on Serum Glucose Levels in Rats

Liposome composition	Dose (IU/kg)[a]	Percentage of glucose level at time (h)[b]						
		0	1	2	3	4	6	21
i.v.[c]								
Insulin solution	0.5	100±5.3	63.7±5.8	89.9±7.4	111.9±14.9	116.5±9.0	111.7±6.0	90.2± 3.2
p.o.								
DPPC/Ch (7:2)	23.8	100±6.3	105.6±4.8	124.2±6.7	111.3± 6.2	107.7±6.0	110.8±5.8	112.8± 2.6
DPPC/Ch (7:4)	15.5	100±3.3	98.0±1.4	99.8±4.4	95.0± 9.3	89.7±4.0	90.8±0.6	88.0± 2.8
DPPC/SS (7:2)	28.3	100±4.4	103.0±2.4	102.2±1.4	89.7± 5.2	90.0±4.1	81.5±8.2	70.1±10.7
DPPC/SS (7:4)	20.0	100±1.3	101.7±2.6	107.0±6.2	100.8± 4.1	104.8±8.5	92.7±8.7	63.4± 7.2

a) Insulin dose was measured by HPLC. *b*) $n=3$, mean±S.E. *c*) Percentage of glucose level was 30.8±5.2 at 0.5 h after i.v. injection used for calculating AUC_{iv}.

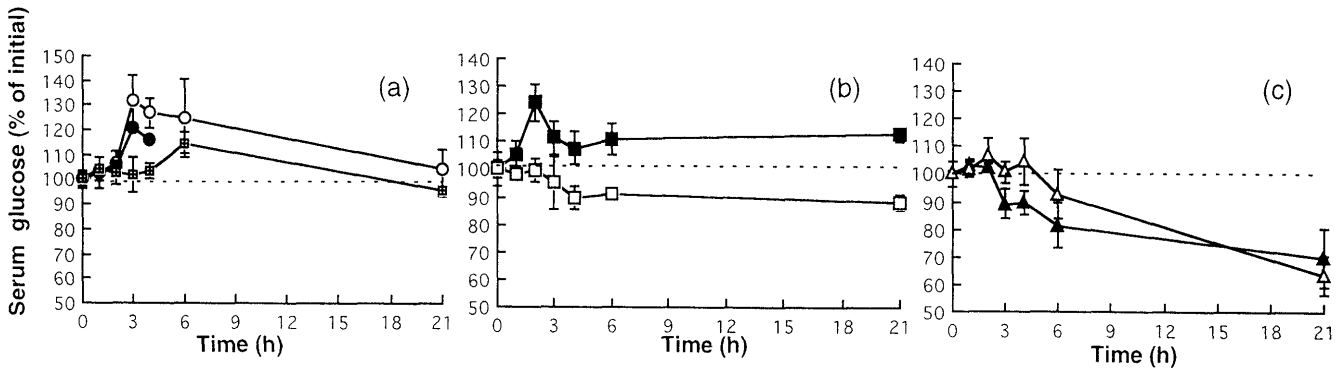

Fig. 2. Effect of the Oral Administration of PBS, Insulin Solution, and a Mixture of Insulin Solution and Liposomes (DPPC/SS or DPPC/Ch (7:2 or 7:4))

Each value represents the mean±S.E. of three rats. (a) PBS (●), insulin solution at a dose of 80 IU/kg (⊞), insulin solution and DPPC/SS (7:4)-liposomes mixture at a dose of 80 IU/kg (○). (b) DPPC/Ch (7:2)-insulin/liposomes (■), DPPC/Ch (7:4)-insulin/liposomes (□). (c) DPPC/SS (7:2)-insulin/liposomes (▲), DPPC/SS (7:4)-insulin/liposomes (△).

described in the following section.

Weingarten *et al.*[14] reported the stability of free insulin compared with insulin entrapped in liposomes. Free insulin was denatured by digestive enzymes such as pepsin, trypsin and α-chymotrypsin. Insulin in the mixture of insulin solution and liposomes may be denatured by enzymes, so that insulin was not absorbed.

Figures. 2b and c show the effect of the composition of insulin/liposomes on serum glucose levels. The glucose level of DPPC/SS (7:2, 7:4)-liposomes decreased to 70.1% and 63.4% of initial serum glucose levels, respectively, at 21 h. On the other hand, the glucose levels of DPPC/Ch (7:2)-liposomes did not decrease, but that of DPPC/Ch (7:4)-liposomes decreased to 88.0% at 21 h.

Insulin/liposomes were administered at a dose of almost 20 IU/kg within a range from 7.9 to 16.7 mg/kg of lipid concentration; therefore, each insulin concentration in liposomes was different. The insulin/liposomes for oral administration contained free insulin and insulin entrapped in liposomes even after centrifugation at $9500 \times g$. The total insulin dose in each insulin/liposome combination is expressed as the following: 23.8 IU/kg for DPPC/Ch (7:2), 15.5 IU/kg for DPPC/Ch (7:4), 28.3 IU/kg for DPPC/SS (7:2) and 20.0 IU/kg for DPPC/SS (7:4) (Table 2). However, free insulin was not effective in decreasing the serum glucose level (Fig. 2a).

We also compared filtrated and un-filtrated DPPC/SS (7:2)-insulin/liposomes through a polycarbonate membrane (Nucleopore, U.S.A.) of 0.1 μm pore size for intestinal absorption. The size of the filtrated liposomes was less than 100 nm, whereas the un-filtrated ones showed an average size of 139—157 nm (Table 1). The un-filtrated DPPC/SS (7:2)-insulin/liposomes showed about 70.0% initial serum glucose levels at 21 h (Fig. 2c), whereas the filtrated ones were 60.8% (data not shown). The filtrated liposomes were better absorbed than the un-filtrated ones. Wiessner *et al.*[15] reported that an excess of insulin enhanced the aggregation of liposome entrapping insulin. This result suggested that the size of insulin/liposomes affected the absorption of insulin/liposomes, and/or excess free insulin decreased it.

Lag Time and Duration of Reduction of Blood Glucose Level DPPC/SS (7:2, 7:4)- and DPPC/Ch (7:4)-insulin/liposomes reduced blood glucose levels for up to 21 h (Table 2). Hashimoto and Kawada[16] reported that positively charged liposomes entrapping insulin (phosphatidylcholine/cholesterol/stearylamine=7:2:2.25) showed a blood glucose reducing effect which lasted for 8 to 21 h after oral administration to rats. Our data correspond well with their results.

Table 3. Comparison of the Hypoglycemic Effect Obtained from DPPC/SS-, DPPC/Ch-Liposomes Administered Orally to Rats

Liposome composition	Dose (IU/kg)	*AUC* (% glucose reduced)	Availability (*F* %)	
i.v.				
Insulin solution	0.5	79.9±10.8	98.8±13.4	
p.o.				
DPPC/Ch (7:2)	23.8	11.8± 9.4	0.8± 0.6	
DPPC/Ch (7:4)	15.5	212.8±56.8	21.9± 5.8	*
DPPC/SS (7:2)	28.3	419.3±85.0	24.1± 4.9	
DPPC/SS (7:4)	20.0	392.8±70.7	31.6± 5.7	

$n=3$, mean±S.E. * $p<0.05$.

We have already reported[9] the blood residence study of DPPC/SS (7:2)-reverse-phase evaporation vesicle entrapping calcein (calcein/REV), which shows that the elimination half lives value was 147.2 min. At most, the blood circulation time of DPPC/SS (7:2)-calcein/REV should be less than about 300 min. However, the decrease in glucose level continued from 6 to 21 h after administration. One reason may be that the liposomes penetrate continuously through intestinal mucosae. The lag time from the start to the decrease in glucose level is smaller in the following order after administration of liposomes: DPPC/Ch (7:4) > DPPC/SS (7:2) > DPPC/SS (7:4), except for DPPC/Ch (7:2), which did not effectively decrease the glucose level (Table 2). The size and dose of DPPC/Ch (7:4)- and DPPC/SS (7:4)- were not significantly different. The difference in lag time might be correlated with the penetration rate across the intestinal mucosae.

Pharmacological Bioavailability Table 3 summarizes the pharmacological availability (*F*) of insulin/liposomes. *F* value was greater in the order of DPPC/SS (7:4) > DPPC/SS (7:2) > DPPC/Ch (7:4) ≫ DPPC/Ch (7:2)-liposomes. *F* values after the oral administration of DPPC/

SS (7:2, 7:4)-insulin/liposomes were about 24.1% and 31.6%, respectively.

On the other hand, DPPC/Ch (7:2, 7:4)-insulin/liposomes showed significantly different F values: about 0.8% and 21.9%, respectively. DPPC/SS (7:2, 7:4)-insulin/liposomes showed significantly high AUC and F values compared with DPPC/Ch (7:2)-insulin/liposomes, but those of DPPC/SS (7:2) and DPPC/SS (7:4) were not significantly different. Liu *et al.*[17] reported that the absolute bioavailability was 30.3% following the intratracheal administration of DPPC/Ch (7:2)-REV entrapping insulin. Our experimental data, using oral administration, compared with their bioavailability.

Liposomes[18,19] and microparticles[20] are taken up by rat Peyer's patches following intraluminal and oral administration. The mechanism of the intestinal absorption of liposomes is not clear, but it may be that; a) liposomes are denatured by bile salt and digestive enzymes, b) liposomes penetrate through intestinal mucosae (ex. blood, Peyer's patch, lymph). We have already reported that DPPC/SS-liposomes were stable in the blood circulation,[9] and that the stabilization of liposomes correlated with the rigidity of the liposomes according to the measurement of the fluorescence anisotropy.[8] The rigidity of liposomal membranes is higher at 37 °C, in the order of: DPPC/SS (7:4) > DPPC/SS (7:2) ≥ DPPC/Ch (7:2),[8,21] and correlates with a short lag time, a long duration of the reduction of blood glucose level and high F values. The rigidity of liposomal membranes might be an important factor in the stability at the surface of the intestinal mucosae and/or in penetration through it. Further investigations of these points are continuing.

This study shows that insulin entrapped in multilamellar liposomes was effectively absorbed across the intestinal mucosae when administered orally without an enhancer, but the mechanisms of uptake of the liposomes are still under investigation.

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