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RESEARCH PAPER

Double Liposomes: Hypoglycemic Effects of Liposomal Insulin on Normal Rats

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ABSTRACT

The biopharmaceutical characteristics of double liposomes (DLs) containing insulin were examined, and the usefulness of DLs in combination with aprotinin is discussed. Encapsulation of insulin was influenced by lipid composition, and the highest efficiency was observed with positively charged liposomes. Insulin encapsulated in liposomes, especially in DLs, was protected from enzymatic proteolysis. A portion of insulin molecules was adsorbed on the surface of the membrane when liposomes were prepared using a lipid with a positive charge and was degraded by enzymes. Remarkable hypoglycemic effects were observed after intragastric administration of DLs containing insulin at a dose of 20 IU/kg to normal male Wistar rats. The highest mean relative efficacy to administration was obtained with insulin-loading DLs containing aprotinin as a protease inhibitor. These results suggest that DLs are applicable as an oral dosage form for peptide drugs such as insulin etc., especially in combination with protease inhibitors.

Key Words: Double liposomes; Glassfilter method; Insulin; Hypoglycemic effects; Protease inhibitor; Oral administration.

INTRODUCTION

Diabetes patients currently number over 135 million people worldwide, and the number is still increasing. The goal of therapy for diabetes is protection of patients from complications through maintenance of blood glucose levels within a range of reference values. In general, the secretion of insulin from Langerhans' islands is facilitated under hyperglycemia and is restrained under hypoglycemia. Diabetes

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is classified into insulin-dependent diabetes mellitus (IDDM), which is caused by absolute lack of insulin, and noninsulin-dependent diabetes mellitus (NIDDM), which is caused by dysfunction of insulin. Insulin is a peptide consisting of 51 amino acids. Insulin is administered by subcutaneous injection because it is readily degraded enzymatically after oral administration. If this problem could be overcome, oral administration of insulin would have a number of significant advantages over SC administration: e.g., i) circumvention of pain due to injection; ii) simplicity of intake; and iii) better patient compliance as a consequence of these improvements. Therefore, there have been a number of studies dealing with oral administration of insulin.[1–3] The objective of the present study was to investigate the hypoglycemic effects of novel double liposomes containing insulin in conjunction with aprotinin as a protease inhibitor.

Liposomes consist of lipid bilayer vesicles[4,5]; the vesicles' interior space is separated from the surrounding solution because small molecules have only limited permeability through the bilayer. They are biocompatible systems for carrying various drugs, either in their internal aqueous space, in the lipid bilayers, or are bound to the surface. Therefore, these liposomes have been used as a drug carrier[5–7] and a vector for gene delivery.[8,9] A novel type of liposome, designated here as double liposome (DL), contains a few small liposomes[10] (illustrated in Fig. 1), which could be prepared by filtering inner liposomes prepared in advance through a glass filter coated with the lipid layer. The microphotograph of DLs prepared by the glassfilter method was shown in the previous report.[10] Double liposomes are thought to protect the inner liposomes against several enzymes similar to multivesicular vesicles.[11–13] Therefore, such DLs were thought to be more effective than ordinary liposomes. In this study, drug loading, stability, and biopharmaceutical characteristics of novel DLs containing insulin prepared by the glassfilter method were investigated.

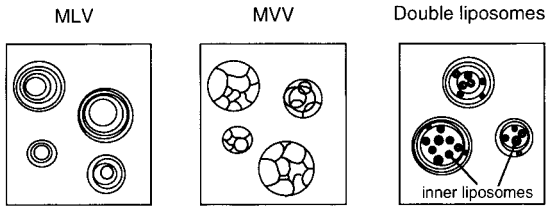

Figure 1. Illustrations of multilamellar vesicles (MLV), multivesicular vesicles (MVV), and double liposomes (DLs).

MATERIALS AND METHODS

Materials

Hydrogenated soybean phosphatidylcholine (H-soyaPC) was a kind gift from NOF Co. Ltd. (Tokyo, Japan). Stearylamine (SA), phosphatidylserine (PS), pepsin, trypsin, aprotinin, and insulin were purchased from Sigma Chemical Company (St. Louis, MC). Calcein (CF) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All reagents were of the highest grade available and were used without further purification.

Animals

Wistar rats (male, 180–200 g) were purchased from Saitama Experimental Animal Supply Co. (Saitama, Japan). Three animals were used in each group to examine in vivo hypoglycemic effects ($n = 3$). All experiments in the present study conformed to the Guidelines for Animal Experimentation of Hoshi University.

Preparation of Insulin-Loading Double Liposomes

DLs were prepared using the glassfilter method with small modification.[10] That is, 26 μM H-soyaPC was dissolved alone or with 2.6 μM SA or PS as lipids with electrical charges in chloroform. The mixture was infiltrated into a G4 glassfilter (pore size: 10–16 μm) and chloroform was evaporated with a gentle stream of nitrogen gas at room temperature. Calcein or insulin dissolved in phosphate-buffered saline (PBS, pH 7.4) (CF: 5 mM, insulin: 5 mg/1 mL of 0.01 N HCl + 1 mL of 0.01 N NaOH + 3 mL of PBS) was poured into the glassfilter formed on the lipid layer and hydrated for 10 min. The glassfilter was soaked in a waterbath and sonicated for 30 min at 60°C. Then, 3 mL of buffer solution was passed through the filter repeatedly by alternately pressing syringes connected to both sides of the filter to form the liposomes. Double liposomes were prepared by filtering a suspension of liposomes prepared using a G4 filter with or without aprotinin into a G3 filter (pore size: 40–100 μm) coated with a similar lipid layer.

Encapsulation efficiency of CF was determined as follows: Four mL of PBS was added to 1 milliliter of a suspension of liposomes containing CF, the suspension was centrifuged (1800 rpm, 10 min), and the

supernatant was removed. This process was repeated twice. To destroy the liposomes, 1 mL of 10% Triton X-100 was added to the pellet. The solution was measured spectrophotometrically at 495 nm. Encapsulation efficiency (%) was calculated by the following formula:

$$\text{Encapsulation efficiency (\%)} = \text{Elemental drug in pellet} / \text{Elemental drug used} \quad \text{(a)}$$

For insulin-loading liposomes, analysis was performed using high-performance liquid chromatography (HPLC) according to the method of Nakazawa and Nagase.[14] This procedure was carried out using a Shimadzu LC-6A apparatus equipped with an YMC-Pack ODS-AP 302 reversed-phase column (4.6 mm in inner diameter × 150 mm in length) and an SPD-6A UV detector (Shimadzu) set at 220 nm at room temperature. The mobile phase was a mixture of aqueous phase (0.7 mL of trifluoroacetate dissolved in 690 mL of pure water containing 0.1 mol NaCl) and acetonitrile (69:31, v/v). The flow rate was 1.0 mL/min.

Stability

The release of insulin from DLs was investigated as follows: Two milliliters of a suspension of liposomes containing insulin was centrifuged at 1800 rpm for 10 min, and 2 mL of Japanese Pharmacopoeia XIV (JP XIV) first fluid (pH 1.2) was added to the pellet. After incubation with stirring at 50 strokes/min for 1 h at 37°C, the samples were centrifuged (1800 rpm, 10 min), and then the supernatant (2 mL) was taken as the sample solution. Six milliliter of JP XIV second fluid (pH 6.8) was poured into the pellet, and the suspension was incubated with stirring at 100 strokes/min for 1 h at 37°C. At selected time intervals, 1 mL of the suspension was taken, and the sample solution was analyzed by HPLC as described above.

The stability of liposomes was also examined in pepsin and trypsin. Liposomes were added to 5 mL of pepsin solution (in 0.1 M glycine buffer, pH 1.3, 5.0 U/mL) or saline (5 mL), and incubated with stirring at 50 strokes/min at 37°C for 1 h. After centrifugation, 10% Triton X-100 (0.2 mL) was added to the pellet. Finally, insulin concentration was measured described above. Similarly, liposomes were added to 5 mL of trypsin solution (in 0.2 M potassium dihydrogenphosphate buffer, pH 7.8, 7500 IU/mL) or saline (5 mL). The subsequent procedures were carried out as described above, except incubation was performed with stirring at 100 strokes/min. Protective effects of liposomes on enzymatic degradation were calculated as the percentage of native insulin detected by HPLC.

Hypoglycemic Effects

After removal of free insulin by centrifugation, liposomal insulin suspended in PBS at a dose of 20 IU/kg was administered into the stomach of rats fasted for 16 h. The group without treatment was employed as a control. At predetermined time points, blood samples (0.2 mL) were withdrawn from the jugular vein without anesthesia. Sera were obtained after centrifugation (3000 rpm, 2 min). These samples were frozen and preserved at −20°C before analysis. Glucose concentration in sera was measured by the glucose-oxidase method using a Glucose B-test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical Analysis

The significant difference was checked by Student's *t*-test using statistical software. The data were evaluated to be significantly different when the *p*-value was less than 0.05.

RESULTS

Encapsulation Efficiency

Table 1 summarizes the encapsulation efficiency of drugs and the particle size of liposomes. The particle size was determined by microscopy. The diameters of ordinary liposomes prepared using a G3 or G4 glassfilter were ca. 8 and 2 μm, respectively. Double liposomes were similar in size to ordinary liposomes prepared using a G3 filter. Liposomes prepared using the glassfilter were confirmed to be MLV.[10] Encapsulation efficiency of CF in DLs was greater than that in ordinary liposomes. As shown in Table 1, the encapsulation efficiency of CF was lower than that of insulin. Further, in both cases (double and ordinary liposomes), the encapsulation efficiency of insulin was in the following order: SA (+) > no addition (±) > PS (−).

Table 1. Encapsulation efficiencies of drugs and particle size of liposomes.

Drug[a]	Lipid composition[b] (glassfilter)	Encapsulation efficiencies (%)	Particle size (μm)
Calcein	H-soyaPC (G3)	9.4 ± 0.5	9.1 ± 0.3
	H-soyaPC (DL[c])	13.8 ± 0.5^{f}	9.3 ± 0.3
Insulin	H-soyaPC (G3)	17.8 ± 0.3	7.6 ± 0.4
	H-soyaPC (G4)	21.4 ± 0.9^{e}	2.1 ± 0.3
	H-soyaPC/SA (G3)	27.3 ± 1.7^{d}	8.8 ± 0.4
	H-soyaPC/PS (G3)	5.7 ± 1.0^{f}	7.9 ± 0.2
	H-soyaPC (DL[c])	20.1 ± 0.8^{d}	8.3 ± 0.3
	H-soyaPC/SA (DL[c])	22.9 ± 1.6^{d}	8.0 ± 0.3
	H-soyaPC/PS (DL[c])	7.6 ± 1.0^{f}	9.0 ± 0.1

[a]5 mM calcein and 1 mg/mL of insulin were used, respectively.
[b]Total lipid concentration: 52 μM.
[c]DL, double liposomes were prepared using a G3 glassfilter after using a G4 glassfilter.
Each value represents the mean ± S.D. ($n = 3$–5).
[d]: $p < 0.05$, [e]: $p < 0.01$, [f]: $p < 0.001$ vs. corresponding H-soyaPC (G3).

Stability

Figure 2 shows release characteristics of insulin from ordinary and double liposomes. In JP XIV first fluid, the release from liposomes prepared with addition of SA to the lipid tended to be higher than that from liposomes consisting of H-soyaPC alone.

Figure 3 shows the stability of liposomal insulin against protease. In pepsin solution, proteolysis of liposomes prepared with addition of SA was greater than that of liposomes consisting of H-soyaPC alone. Also, the stability of liposomes in trypsin was similar to that of liposomes in pepsin solution.

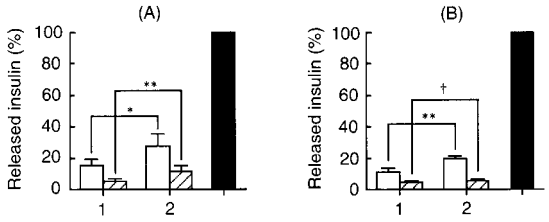

Figure 2. Release of insulin from ordinary and double liposomes in the JP XIV first and second fluid. (A) Ordinary liposomes prepared using a G3 glassfilter, (B) Double liposomes. Open, hatched, and closed bars represent the release in the first fluid, second fluid, and Triton X-100, respectively. **1** and **2** indicate H-soyaPC alone, H-soyaPC + SA, respectively, †: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$ vs. H-soyaPC alone.

Hypoglycemic Effects

Figure 4 shows the changes in blood glucose level after oral administration of liposomes containing insulin at a dose of 20 IU/kg to normal rats. Blood glucose level immediately before administration was taken to be 100%. Blood glucose level was markedly reduced by insulin-loading DLs. Further, the groups treated with DLs exhibited better hypoglycemic effects than those treated with ordinary liposomes. Figure 5 shows the changes in blood glucose level after oral administration of liposomal insulin prepared using a G4 glassfilter. The groups treated with liposomal insulin prepared using G4 glassfilters showed little hypoglycemic effect although they showed lower blood glucose level than controls.

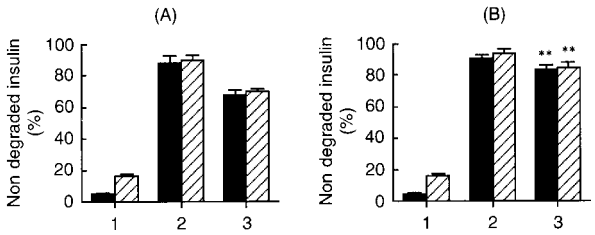

Figure 3. Stability of insulin encapsulated in liposomes against enzymatic degradation. (A) Ordinary liposomes prepared using a G3 glassfilter, (B) Double liposomes. Closed and hatched bars represent pepsin and trypsin, respectively. **1**, **2,** and **3** indicate free insulin, H-soyaPC alone, and H-soyaPC + SA, respectively. **: $p < 0.01$ vs. ordinary liposomes.

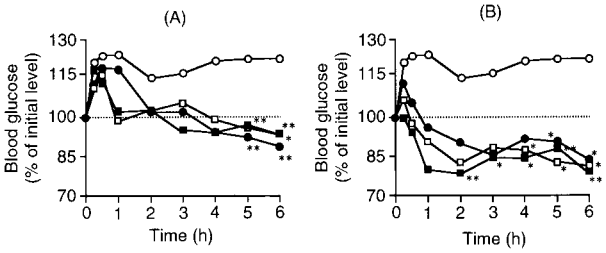

Figure 4. Changes in blood glucose level after oral administration of liposomal insulin at a dose of 20 IU/kg to normal rats ($n=3$). (A) Ordinary liposomes prepared using a G3 glassfilter, (B) Double liposomes. Open and closed circles represent control and H-soyaPC alone, respectively. Open and closed squares represent H-soyaPC with SA and with aprotinin, respectively. *: $p<0.05$, **: $p<0.01$ vs. control.

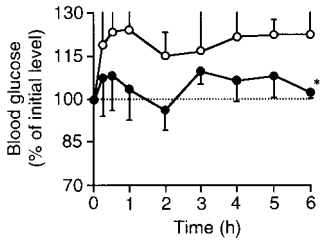

Figure 5. Changes in blood glucose level after oral administration of liposomal insulin at a dose of 20 IU/kg to normal rats ($n=3$). Open and closed circles represent control and liposomes prepared using G4 glassfilters, respectively. *: $p<0.05$ vs. control.

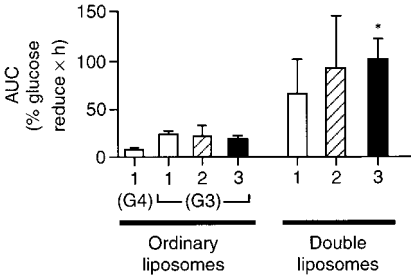

Figure 6. Hypoglycemic effects of ordinary and double liposomes after oral administration to normal rats ($n=3$). **1**, **2**, and **3** indicate H-soyaPC alone, H-soyaPC + SA and H-soyaPC + aprotinin, respectively. *: $p<0.05$ vs. ordinary liposomes.

The areas in the plasma glucose reductive area under time curve concentration (AUC) based on Figs. 4 and 5 are shown in Fig. 6. The curve was calculated as the area of the curves below the baseline, 100%, using the trapezoidal method. The curve in DLs prepared with H-soyaPC alone, those with added SA, and those with added aprotinin were 2.8-, 4.2-, and 5.2-fold higher than those of ordinary liposomes, respectively. Table 2 shows the efficacy of oral administration of liposomal insulin relative to intravenous administration of insulin. The efficacy of oral administration of liposomal insulin was calculated from the cumulative percent of the changes based on Figs. 4 and 5. The relative efficacies of DLs were high; especially those with aprotinin were 5.5%, while those of ordinary liposomes were unexceptionally less than 1.5%.

DISCUSSION

Although the detailed in vivo efficacies of DLs are discussed in the previous paper[18], the detailed in vitro properties, especially their unique structures, are needed to clarify. Encapsulation efficiency of drugs with high molecular weight and good aqueous solubility is low compared with drugs with low molecular weight.[15] Encapsulation efficiency of insulin was thought to be low because insulin has a high molecular weight. Therefore, the effects of addition of lipid with an electric charge were examined. The results suggest that the charge of liposomes had a marked effect on encapsulation efficiency of insulin due to the negative charge of insulin in PBS.

In JP XIV first fluid, insulin encapsulated in liposomes prepared with addition of SA to the lipid showed a slight higher release than that encapsulated in liposomes consisting of H-soyaPC alone (Fig. 2). It was suggested that insulin adsorbed on the surface of liposomes due to the static electrical compatibility between insulin and SA was released. Insulin encapsulated in liposomes, especially in DLs, was considered to be protected from enzymatic proteolysis because free insulin was almost completely degraded (Fig. 3). Weingarten et al. reported that insulin entrapped in liposomes prepared using noncharged lipid was stable against pepsin, trypsin, and chymotrypsin.[16] That is, a part of insulin molecules was adsorbed on the surface of membrane when liposomes were prepared using a lipid with a positive charge, and was degraded by enzymes. The results of the present study (Fig. 3) were compatible with these observations. Further, Morishita et al. reported the usefulness of addition of an enzyme inhibitor; i.e., microspheres containing protease inhibitor due to suppression of the proteolysis by trypsin and chymotrypsin.[1]

Table 2. Efficacy of oral administration of liposomal insulin as percentage of IV insulin efficacy.

Treatment	Efficacy relative to IV (%) Normal rats
Ordinary liposomes H-soyaPC alone (G4)	0.39
Ordinary liposomes H-soyaPC alone (G3)	1.28
H-soya PC containing SA	1.20
H-soya PC containing AP	1.06
Double liposomes H-soyaPC alone	3.56
H-soyaPC containing SA	5.01
H-soyaPC containing AP	5.49

Each value represents the mean ($n = 3$).

Patel et al. reported that insulin entrapped in liposomes was transferred to the systemic circulation after partial degradation in the digestive tract, and was detected in both normal and diabetic animals.[17] Double liposomes shows markedly better hypoglycemic effects than ordinary liposomes due to the controlled release of insulin[10] and to protection from enzymatic degradation. Further, the hypoglycemic effects tended to be manifested earlier by insulin-loading liposomes in combination with aprotinin as a protease inhibitor than by those without aprotinin [Fig. 4(B)]. These findings suggested that insulin released in the upper regions of the small intestine was protected by aprotinin from proteolysis by trypsin and chymotrypsin, and that a portion of the liposomes were retained around the ilea loop that contains a relatively small amount of enzymes, and insulin was released from liposomes in this region. Moreover, the results shown in Fig. 5 implied that the hypoglycemic effects were attributed to DLs, and not to inner liposomes prepared using the G4 glassfilter. Double liposomes were effective as an oral delivery vehicle because the relative efficacy was 3.4% when liposomal insulin was administered at a dose of 20 IU/kg as reported earlier.[2] These findings suggested that novel DLs prepared by the glassfilter method enable peptide or protein drugs to be administered orally. Because normal rats were used in this study to examine the in vivo hypoglycemic effects of DLs, further studies are required to investigate the hypoglycemic effects on diabetic rats.

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