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Pharmacodynamics of insulin in polyethylene glycol-coated liposomes

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Abstract

To reduce the injection frequency and toxicity of intravenously administered protein drugs, it is necessary to develop safe and sustained injectable delivery systems. In this study, to evaluate liposomes as safe and sustained injectable delivery systems of proteins, we chose insulin as a model protein drug and tested its incorporation efficiency and pharmacodynamics in various liposomes with and without polyethylene glycol (PEG)-derivatized phospholipid. The liposomes coated with PEG showed 3-fold higher efficiency of insulin incorporation than did the liposomes without PEG. Moreover, among the liposomes coated with PEG, dipalmitoylphosphocholine (DPPC) liposomes showed higher incorporation efficiency than did dimyristoylphosphocholine (DMPC) liposomes. For pharmacodynamic study, insulin (2 IU/kg) was administered in various formulations, such as insulin alone in phosphate-buffered saline and insulin in the DPPC liposomes with and without PEG, to streptozotocin-treated diabetic rats. The pharmacodynamics of insulin alone, however, could not be measured due to the immediate death of rats caused by hypoglycemic shock. In contrast, all the rats treated with liposomal insulin survived, probably by the sustained release of insulin from liposomes. Pharmacodynamics of liposomal insulin showed that PEG-coated liposomes induced the lowest level of blood glucose—the nadir—1 h later than did the liposomes without PEG. These results indicate that PEG-coated liposomes could be developed as a relatively safe and sustained injectable delivery system for insulin with improved incorporation efficiency. Moreover, it is suggested that the liposomes coated with PEG might have a potential as safe injectable delivery systems for other protein and peptide drugs.

Keywords: Incorporation; Insulin; Liposomes; Pharmacodynamics; Polyethylene glycol

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1. Introduction

Recently, with the advance of biotechnology, an increasing number of proteins and peptides have been developed as therapeutic drugs. Due to the low bioavailability after oral delivery, these drugs are usually administered by the parenteral route. However, parenteral administration has some limitations. First, the short biological half-lives of protein and peptide drugs result in an inconveniently high dosing frequency. Second, the high blood concentrations of some protein drugs right after intravenous administration could cause significant host toxicity (Debs et al., 1990). To reduce the injection frequency and toxicity of intravenously administered protein drugs, it would be necessary to develop safe and sustained injectable protein delivery systems.

Liposomes have been studied as sustained drug delivery systems (Blume and Cevc, 1990). Liposomes have advantages over other delivery systems, since these are biodegradable, non-toxic and non-immunogenic. However, the rapid uptake of liposomes by the reticuloendothelial system (RES) after injection has limited the application of liposomes to those drugs whose target sites are located in the RES (Couvreur et al., 1991).

Recently, liposomes coated with polyethylene glycol (PEG) have been developed to reduce the distribution of encapsulated drugs to the RES, enhancing the delivery of drugs to non-RES target sites (Lasic et al., 1991). Furthermore, liposomes with PEG-derivatized lipids have been reported to be stable in vitro (Blume and Cevc, 1990) and in the circulation (Gabizon and Martin, 1997), which might contribute to the sustained release of encapsulated drugs. Several reports showed that liposomes with PEG-derivatized lipids increased the half-lives of various drugs. Gabizon et al. (1993) showed that the circulation time of doxorubicin was prolonged by liposomes containing a PEG-derivatized phospholipid. Woodle et al. (1992) reported that the systemic delivery of a peptide drug, vasopressin, was prolonged by liposomes with PEG-derivatized lipid. Although pharmacokinetic studies have been reported for various drugs entrapped in liposomes with PEG-derivatized phospholipids, there is limited knowledge on the pharmacodynamics of protein drugs administered in liposomes coated with PEG.

In this study, to evaluate liposomes as safe and sustained injectable delivery systems for proteins, insulin was chosen as a model of protein drug since it is one of the most widely used protein drugs, has a short half-life (30 min) after intravenous administration (Owens, 1986), and might cause hypoglycemic shock. We formulated insulin in various liposomes, including PEG-coated liposomes, and examined the pharmacodynamics of liposomal insulin. Furthermore, considering the relatively high cost of insulin, we studied the impact of lipid components on the incorporation efficiency of insulin in liposomes.

2. Materials and methods

2.1. Materials

Dimyristoylphosphocholine (DMPC), dipalmitoylphosphocholine (DPPC), cholesterol (CH) and streptozotocin were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphoethanolamine–polyethylene glycol 2000 (DSPE–PEG) was from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Porcine insulin (26.1 units/mg) was kindly supplied from Green Cross Co. (Seoul, Korea). Glucose-E kit was obtained from International Reagent Co. (Kobe, Japan). All other reagents were of reagent grade and used without further purification.

2.2. Preparation and physicochemical characterization of insulin-incorporating liposomes

2.2.1. Preparation

Liposomes containing insulin were prepared with various lipid compositions using the method previously described (Kim et al., 1994; Kim and Jeong, 1995). Various lipids dissolved in chloroform were mixed, and the organic phase was removed under reduced pressure (360 mmHg). A dried thin film of the desired lipid composition, as shown in Table 1, was dissolved in the mixture of organic solvents (diisopropyl ether:chloroform =

2:1), and added with insulin in 0.145 M phosphate-buffered saline (PBS). The solution was then vortexed and sonicated in a bath type sonicator at 37°C for 3 min to form a water-in-oil emulsion. The organic solvents were removed under reduced pressure until a suspension of liposomes was obtained. The liposomes were then extruded three times through a 0.2-μm polycarbonate membrane filter (Nucleopore®; Costar, MA, USA), loaded onto a Sepharose CL-4B column (1 × 30 cm) to remove the liberated insulin and stored at 4°C.

2.2.2. Determination of incorporation efficiency

The incorporation efficiency of insulin was determined by insulin-to-phospholipid ratios after incorporation. The amounts of insulin were calculated by the Lowry method using the lysates of liposomes in 0.5% sodium deoxycholate. The concentrations of phospholipids were determined by the Stewart assay (Stewart, 1959) with minor modification. In brief, aliquots of liposomes were dried in a rotary evaporator, dissolved in 3 ml of chloroform in triplicate, and added with 3 ml of 0.1 M ammonium ferrothiocyanate. Then, the mixture was vortexed vigorously for 15 s and centrifuged at 1000 rpm for 10 min. The lower layer was collected and the optical density was measured at 485 nm. The concentrations of phospholipid in test samples were calculated from the standard curve.

2.2.3. Particle size determination

The sizes of liposomes were examined by photon correlation spectroscopy. Liposomes were placed in a disposable cuvette and photon counts were measured in a photon correlator at 25°C. A laser particle analyzer (LPA-3000, Otsuka Electronics, Japan) was used for the size distribution data.

Table 1
Lipid composition (molar ratio) of various liposomes

DMPC:DSPE:PEG (9:0.8)	DPPC:CH:DSPE–PEG (9:0.8)
DMPC:CH:DSPE–PEG (8:1:0.8)	DPPC:CH:DSPE–PEG (8:1:0.8)
DMPC:CH:DSPE–PEG (7:2:0.8)	DPPC:CH:DSPE–PEG (7:2:0.8)
DMPC:CH:DSPE–PEG (6:3:0.8)	DPPC:CH:DSPE–PEG (6:3:0.8)
DMPC:CH (6:3)	DPPC:CH (6:3)

2.2.4. Stability of liposomes

The physical stability of liposomal insulin kept at 4°C was evaluated by monitoring particle size changes at designated time intervals.

2.3. Pharmacodynamic study

Male Sprague-Dawley rats weighing 240–310 g were supplied from the Experimental Animal Breeding Center of Seoul National University (Seoul, Korea). The animals were fed with commercial rodent chow (Samyang Co., Seoul, Korea) and tap water ad libitum. Diabetes was induced by tail vein injection of a freshly prepared solution of streptozotocin (65 mg/kg) in 0.1 M citrate buffer (pH 4.5). After 5–6 days, the induction of diabetes was tested by measuring the weight and blood glucose level of the rats (Sato et al., 1991). Before administration of insulin in various dosage forms, the diabetic rats were fasted overnight, and both femoral artery and femoral vein were catheterized with polyethylene tubing (PE-50, Becton Dickinson, NJ, USA) under light anesthesia. A single dose of insulin (2 IU/kg) was given to each rat in PBS or liposomal formulations via femoral venous cannula. Blood samples were collected from femoral arterial cannula at each time point, centrifuged to obtain plasma and stored at −20°C until the analysis of glucose by the following procedure. The level of glucose in plasma was determined using the Glucose-E kit. The mixture of glucose oxidase (24 units/ml) and 4-aminoantipyrine (0.1 mg/ml) was reconstituted with *p*-hydroxybenzoic acid solution (1.66 mg/ml). Then 3 ml of the reconstituted solution were added to 20 μl of plasma sample and standard solution, respectively. The resulting mixture was incubated at 37°C for 10 min and the optical density was read at 540 nm. The concentrations of glucose in the plasma samples were calculated from the standard curve. All samples were tested in duplicate. Plasma glucose level–time profiles were illustrated and pharmacodynamic parameters were determined. All results are expressed as

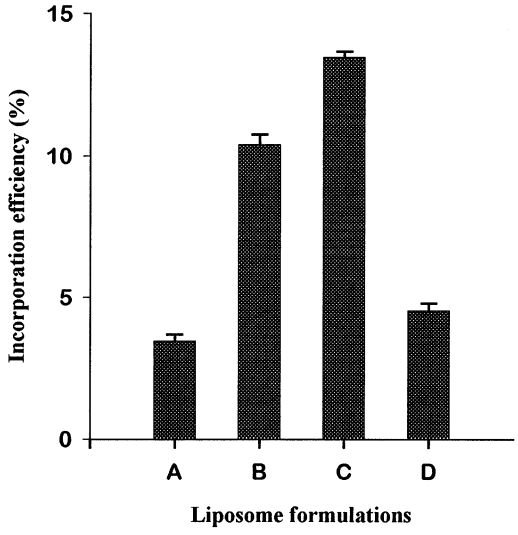

Fig. 1. Effect of lipid components on the incorporation efficiency. (A) DMPC:CH (molar ratio 6:3); (B) DMPC:CH:DSPE–PEG (6:3:0.8); (C) DPPC:CH:DSPE–PEG (6:3:0.8); (D) DPPC:CH (6:3).

the mean ± standard deviation (SD). Student's unpaired t-test was used to evaluate significance ($p < 0.05$).

3. Results and discussion

3.1. Effect of lipid components on incorporation efficiency

Given the high cost of insulin, it would be important to elucidate the impact of each lipid component on the incorporation efficiency and to formulate insulin in the liposomes showing the highest incorporation efficiency. The lipid compositions of various liposomes tested are shown in Table 1. PEG-derivatized lipids influenced the efficiency of insulin incorporation. In the liposomes containing either DMPC or DPPC, the presence of DSPE–PEG increased the incorporation efficiency about 3-fold (Fig. 1). Other lipid components of liposomes also affected the incorporation efficiency of insulin. As the lipid molar ratio of CH increased, the efficiency of insulin incorporation enhanced significantly (Fig. 2). CH is known to increase the rigidity of the liposomal membrane. Thus, it appears that the incorporation-enhancing effect of CH might have resulted from the reduced release of insulin from more rigid liposomes containing CH during the incorporation process (Kim and Han, 1995). Although liposomes composed of either DMPC or DPPC consistently showed enhanced incorporation efficiency of insulin in the presence of CH- and PEG-derivatized lipids, DPPC-based liposomes showed higher efficiency of incorporation than DMPC-based liposomes (Fig. 2), indicating that the carbon chain length of phospholipids may play a role in the incorporation of insulin.

3.2. Size of insulin-incorporating liposomes

The size distribution of liposomes was affected by the incorporation of PEG-derivatized lipid. The liposomes composed of DPPC, CH and DSPE–PEG showed a smaller average diameter and narrower size distribution compared with the liposomes composed of DPPC and CH, as shown in Fig. 3 (91.2 ± 8 nm versus 161.6 ± 75 nm).

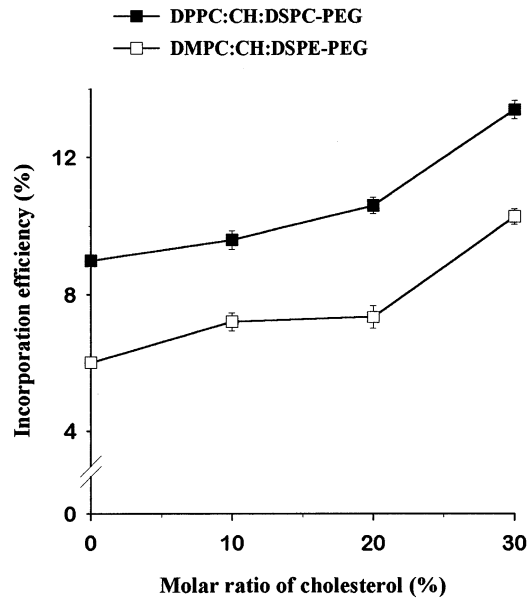

Fig. 2. Effect of cholesterol on incorporation efficiency. Liposomes were composed of either DMPC:CH:DSPE–PEG (molar ratio x:y:0.8) or DPPC:CH:DSPE–PEG (x:y:0.8). The sum of x and y was 9. The values of x and y varied from 9 to 6 and from 0 to 3, respectively.

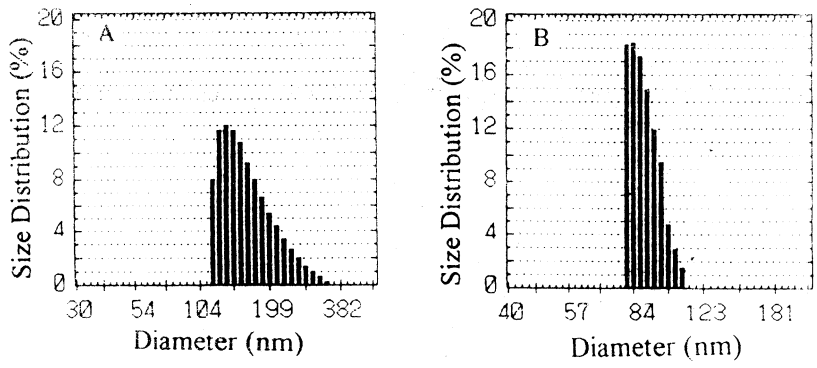

Fig. 3. Size distribution of liposomes. (A) Liposomes composed of DPPC:CH (6:3); (B) liposomes composed of DPPC:CH:DSPE–PEG (6:3:0.8).

Although the mechanism by which DSPE–PEG influenced the size distribution of liposomes is not clear, it is possible that DSPE–PEG increased the polarity of the liposomal surface, preventing the aggregation of extruded liposomes.

3.3. Stability of liposomes

The physical stability of liposomal insulin kept at 4°C was evaluated by monitoring particle size changes for 30 days. Liposomes without DSPE–PEG were flocculated and aggregated after a few days. On the other hand, the physical appearance and the mean diameter of liposomes with DSPE–PEG were not significantly changed during the storage period.

3.4. Pharmacodynamics of liposomal insulin in diabetic rats

The body weight and plasma glucose level of rats were determined to check the induction of diabetes. After treatment with streptozotocin, rats showed a slightly decreased body weight and more than a 3-fold increase of plasma glucose level than before treatment (Table 2). The increased glucose level of streptozotocin-treated rats indicates that these rats could be used as model animals of diabetes.

The pharmacodynamics of insulin was studied after the administration of insulin in free form or in liposomes of different compositions. Among the liposomes of various compositions shown in Table 1, DPPC-based liposomes were selected, since they showed higher incorporation efficiency of insulin than DMPC-based liposomes. Two kinds of liposomes were used in the pharmacodynamic study: one composed of DPPC:CH (molar ratio 6:3), the other a PEG-coated liposome made of DPPC:CH:DSPE–PEG (molar ratio 6:3:0.8). Plasma glucose level was used as an indicator of insulin pharmacodynamics. However, the pharmacodynamics of insulin alone could not be measured, since all the diabetic rats did not survive longer than 30 min after the administration of free insulin (2 IU/kg) in PBS. The high mortality of free insulin-treated rats might be due to hypoglycemic shock. In contrast to the rats treated with insulin alone, the rats administered with liposomal insulin showed no mortality at the same dose, implying that insulin might be released from liposomes in a sustained pattern, thus preventing the sudden decrease of plasma glucose level to the toxic range.

Table 2
Body weight and plasma glucose level of the rats ($n = 5$) before and after streptozotocin treatment

	Before treatment	After treatment
Body weight (g)	288 ± 31	266 ± 31
Plasma glucose level (mg/dl)	181 ± 30	593 ± 7

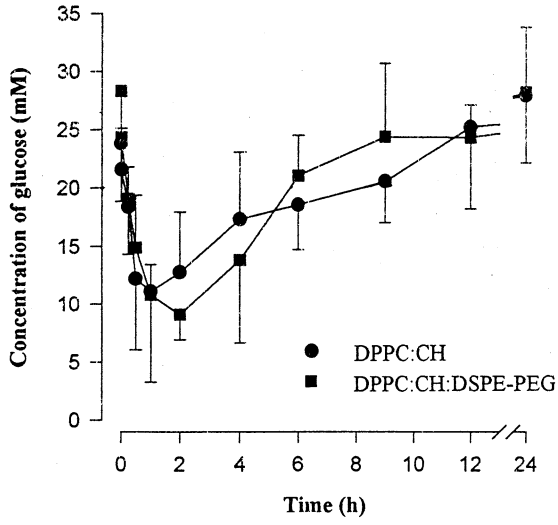

Fig. 4. Plasma concentrations of glucose after intravenous administration of insulin in various formulations. Streptozotocin-treated rats were administered with insulin (2 IU/kg) in PBS, or in liposomes composed of DPPC:CH (molar ratio 6:3) or DPPC:CH:DSPE–PEG (6:3:0.8). Each point represents the mean ± SD ($n \geq 4$). Plasma concentrations of glucose after the administration of insulin alone in PBS are unavailable due to the immediate death of all rats.

Plasma glucose level–time profiles and pharmacodynamic parameters are shown in Fig. 4 and Table 3. Two liposomes with and without DSPE–PEG did not show significant difference in the plasma glucose levels at the nadir (C_{nadir}).

Table 3
Pharmacodynamic parameters of liposomal insulin

Parameter	Liposome composition	
	DPPC:CH (molar ratio 6:3)	DPPC:CH:DSPE–PEG (molar ratio 6:3:0.8)
C_{basal} (mM)	28.81 ± 4.69	28.29 ± 3.86
C_{nadir} (mM)	11.09 ± 7.81	9.07 ± 2.16
C_{delta} (mM)	17.64 ± 4.35	19.31 ± 3.85
T_{nadir} (h)	1	2
$AUC_{0\rightarrow 24h}$ (mM h)[a]	537.75 ± 50.32	539.19 ± 101.14

[a] $AUC_{0\rightarrow 24h}$ was calculated by the trapezoid method over 24 h.

The C_{nadir} value alone, however, may not be a suitable parameter for the measurement of insulin pharmacodynamics since the basal level of blood glucose may vary between animals and experiments. Thus, we also measured other parameters such as the basal level (C_{basal}), the concentration difference between the C_{basal} and C_{nadir} glucose level (C_{delta}), the time to reach the nadir (T_{nadir}) and the area below basal glucose level ($AUC_{0\rightarrow 24h}$). Table 3 shows that these parameters were not significantly different between the two liposomes except T_{nadir}.

Liposomes without DSPE–PEG showed the nadir at 1 h after the administration, whereas liposomes coated with DSPE–PEG reached it at 2 h. T_{nadir} of liposomes coated with DSPE–PEG increased 2-fold compared with that of liposomes without DSPE–PEG. It is thought that the difference in T_{nadir} between the liposomes might be contributed by the more sustained release of insulin from PEG-coated liposomes and the higher stability of PEG-coated liposomes in the circulation (Gabizon and Martin, 1997) than liposomes without DSPE–PEG.

4. Conclusion

It is concluded that liposomes might have a potential to be developed as safe and sustained injectable insulin delivery systems. Of various liposomes, PEG-coated DPPC liposomes might have advantages over other liposomes, based on the higher incorporation efficiency, narrow size distribution and stability in vitro and in vivo. Moreover, it is suggested that PEG-coated liposomes might be further developed as safe and sustained injectable delivery systems of other peptide and protein drugs.

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