Effects of Phospholipid Chain Length, Concentration, Charge, and Vesicle Size on Pulmonary Insulin Absorption

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Received May 18, 1995; accepted September 23, 1995

Purpose. Non drug loaded lipid vesicles have been investigated as promoters of pulmonary insulin absorption.

Methods. Physical mixtures of liposomes with insulin were delivered intratracheally to rats by direct instillation method at an insulin dose level of 1 U/kg.

Results. The overall hypoglycemic response, represented by area above the curve (AAC), correlated linearly with the lipid concentration for both the neutral and charged liposome-insulin preparations. The strongest response was observed with the positively charged liposomes followed by negatively charged and neutral liposome-insulin mixtures. Further toxicological studies indicated that charge-inducing agents, i.e., stearylamine and dicetylphosphate, can cause apparent disruption of pulmonary epithelial cells. From the difference of overall hypoglycemic response (AAC) among various formulations, it appears that the stronger hypoglycemic effect following positively charged liposome-insulin mixture is due to the membrane destabilizing effect of stearylamine. Optimum hypoglycemic effect was observed with a medium acyl-chain lipid (C10). The cumulative hypoglycemic response appeared to correlate inversely with the acyl carbon number of the phospholipid component from C10 to C18. The overall hypoglycemic effect does not appear to change within the liposomal size range of 0.1 μm - 1.98 μm, indicating that insulin absorption following intratracheal instillation is independent of the vesicle size within the range studied.

Conclusions. Phospholipid promoted insulin pulmonary absorption is significantly dependent on the concentration, charge and acyl chain length of the phospholipids.

KEY WORDS: insulin; pulmonary delivery; phospholipid; liposomes; mucotoxicity; stearylamine; dicetylphosphate.

INTRODUCTION

Absorption enhancement of proteins and peptides by non-invasive routes has attracted much attention in the pharmaceutical area. Pulmonary administration of these macromolecules has been reported to produce better systemic absorption than other mucosal pathways (1). However, the alveolar-capillary barrier generally limits penetration of large molecules such as proteins and polypeptides (2). The possibility of utilizing liposomes to achieve a variety of therapeutic objectives including enhancing the therapeutic index and reducing the side effect has generated renewed interest especially in the pulmonary drug delivery area.

A previous study from our laboratory demonstrated that encapsulation of insulin into the liposome vesicles may not be a necessary prerequisite for enhanced insulin uptake (3). In addition, the external binding of insulin to the surface of liposomes, resulting from interaction with either polar or nonpolar regions of the insulin molecules (4), could lead to an over-estimation of true encapsulation efficiency. Few studies have been performed to examine the physical mixtures of proteins with empty liposomes as potential pulmonary drug delivery system. In addition, many parameters determining the rate and extent of drug uptake following pulmonary delivery of a physical mixture of drugs with liposomes need to be characterized in a systematic manner. It is, therefore, the purpose of this investigation to identify possible factors controlling pulmonary absorption of insulin in the presence of liposomes.

MATERIALS AND METHODS

Materials

1,2-distearoylphosphatidylcholine (DSPC), 1, 2-dipalmitoylphosphatidylcholine (DPPC), 1, 2-dimyristoylphosphatidylcholine (DMPC), 1, 2-dilauroylphosphatidylcholine (DLPC), 1, 2-dicaprylphosphatidylcholine (DCPC), 1, 2-dicapryloylphosphatidylcholine, 1, 2-dibutyroylphosphatidylcholine were purchased from Avanti Phospholipid Inc. (Alabaster, Alabama). Stearylamine (SA) and sodium dicetylphosphate (DCP) (Sigma Chemical Company, St. Louis, Missouri) were utilized to make positively charged and negatively charged liposomes, respectively. Cholesterol, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) kits were purchased from Sigma Chemical Company (St. Louis, Missouri). Chloroform (spectral grade) was supplied by Fisher Scientific (Fairlawn, New Jersey). Sterile saline solution (Abbott Laboratories, North Chicago, IL) was used to dissolve insulin and to replace the blood volume taken during sampling.

Methods

Preparation of Insulin Solution

Zinc insulin powder (26.3 U/mg) was solubilized with a minimal volume of 0.1 N HCl solution to which sterile saline solution was added. The pH of the solution was subsequently adjusted to a physiological value of 7.4 by the addition of 0.1 N NaOH.

Preparation and Characterization of Liposomes

A physical mixture of insulin solution and unloaded liposomes, composed of phospholipids of different acyl chain length and electric charge, were prepared by the thin-film method (5). The preparation procedure first involved solubilization of the weighted contents which were composed of phospholipids, cholesterol and/or charge inducing agents in a molar ratio of 7:2:0.5 (total mass = 50 mg) in 5 ml chloroform. The organic solvent was then evaporated under vacuum at room temperature until the last traces of solvent were removed. The thin-filmed flask was then kept under vacuum overnight. 3 ml saline solution was added to the thin-filmed flask with several glass beads. Then the flask was placed in

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0724-8741/96/0100-0076$09.50/0

a shaker bath at 50°C for 1 hour. For DSPC, a temperature of 58 °C was used in order to go above the phase transition temperature. The pH was adjusted carefully to 7.4 since stearylamine and sodium dicetylphosphate are basic and acidic compounds respectively. Then 0.2 ml of insulin stock solution was added to prepare the final suspension which contains 15.6 mg/ml of lipids and 1 U/kg of insulin (equivalent to 0.0835 mg/ml). Finally, the suspension was homogenized by a Polytron PT 10/35 homogenizer (Brinkmann Instrument Inc., Westburg, New York) for 3 minutes. The median diameter of liposomes were measured using Microtrac particle size analyzer (Leeds and Northrup, North Wales, Pennsylvania). To study the effect of liposome size on the hypoglycemic response, liposomes with 0.1 μm and 0.4 μm vesicle sizes were obtained using LiposoFast ™-Basic polycarbonate membranes (Avestin, Inc. Ottawa, ON, Canada) with pore sizes of 100 nm and 400 nm, respectively.

Pulmonary Administration of Insulin

The liposome-insulin suspension was delivered to the rat lungs by a method described in our previous report (6). Male Sprague-Dawley (SD) rats, weighing 170-230 g, were fasted for 18-24 hours prior to an experiment while water was allowed ad libitum. The rats were anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg ketamine hydrochloride and 10 mg/kg xylazine. Jugular vein cannulation were performed for each rat by inserting a 3-inch piece of Silastic® tubing, 0.047 inch o.d. (Dow Corning, Midland, Michigan). A 2-inch piece of PE-200 (Becton Dickinson, Parsippany, New Jersey) was used for tracheal cannulation purpose. The liposome formulation (approximately 0.1 ml) was instilled into the lungs through a 4-inch long plastic tubing (PE-50)(Becton Dickinson, Parsippany, New Jersey) that has been attached to the needle of a calibrated 1.0-ml syringe. The tubing was inserted slowly through the tracheal cannula until the bifurcation site is reached and then the preparation was discharged slowly. Blood glucose levels were determined by Chemstrip bG® testing strips in an AccuChek IIm® Blood Glucose Monitor (Boehringer Mannheim Corporation, Indianapolis, Indiana).

Mucotoxicity Study

Stearylamine or dicetylphosphate, without other phospholipids and cholesterol, was dissolved in chloroform and prepared by the liposome-forming procedure described previously. In the last step, sterile saline was added and the pH was adjusted to 7.4. SD rats with body weight 230-250 g were administered pulmonarily with the suspension of stearylamine or dicetylphosphate. As a control, 0.9% saline solution was also delivered to three rats. After a 4 hour-exposure, the rat lungs were lavaged by instilling 8 ml saline solution once. The lavage fluid was centrifuged at 1000 rpm for 10 minutes at 4 °C in order to remove cells. The supernatant was utilized in the measurement of ALP and LDH enzyme activities (detailed assay procedures available with assay kits).

Data Analysis

The percent blood glucose remaining was plotted as a function of time. Then the area above the % blood glucose remaining versus time curve (AAC) and below the 100% line was calculated by the linear trapezoidal method.

RESULTS AND DISCUSSION

Figure 1A depicts the effect of liposomal charge on the hypoglycemic response following pulmonary delivery of liposome-insulin mixtures. The total lipid concentration was maintained at 15.6 mg/ml in the liposomal preparations. Positively charged liposomes resulted in significant blood glucose depression with the maximum effect reached at 3 hr post instillation. The negatively charged preparation displayed a slightly stronger effect than neutral liposomal preparation, although not being statistically different. Neutral liposomes, therefore, appears to be a weak absorption enhancer to pulmonarily delivered insulin.

To demonstrate whether the synergistic effect in absorption promotion could be due to the charge-inducing agents incorporated in the liposomes, suspensions of these agents with insulin were then instilled pulmonarily. As shown in Fig 1B, both stearylamine and dicetylphosphate significantly enhanced hypoglycemic response of insulin, compared to in-

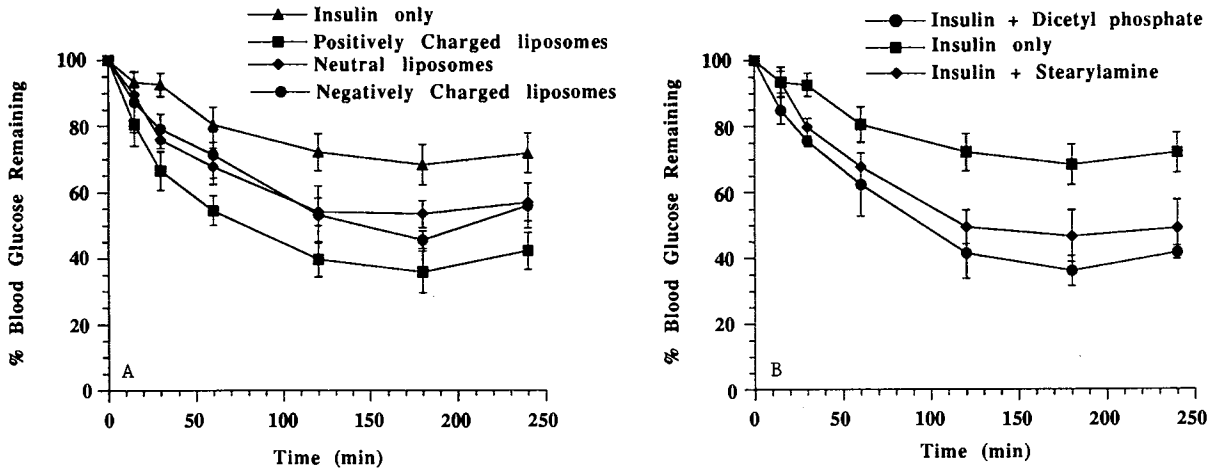

Fig. 1. Hypoglycemic effect following pulmonary delivery of 1 U/kg insulin. (A) with differently charged blank liposomes. Lipid concentration = 15.6 mg/ml. Values represent means ± SE (n = 4–9). (B) With charge-inducing agents. Values represent means ± SE (n = 3–4).

sulin alone. In order to compare the cumulative hypoglycemic effect of different formulations in a quantitative manner, the areas above % blood glucose remaining vs. time curves ($AAC_{0\text{-}240min}$) were obtained and listed in Table I. Also included in the table are differences in the two AAC values ($\Delta AAC_{0\text{-}240min}$) in each group. The difference between the AACs of neutral liposome-insulin mixture and insulin alone was found to be 2516 (%.min). The AAC difference between positively charged liposome-insulin mixture and insulin with stearylamine was 2555 (%.min). The two net AAC values appeared to be similar in magnitude, indicating that the additionally achieved absorption enhancing effect is due to the incorporation of the charge inducing agent only, i.e., stearylamine. On the contrary, the negative charge-inducing agent displayed a weaker effect when incorporated in liposomes with a ΔAAC of 1937%.min. The reason for this observation is still unclear. However, it is very likely due to the fact that, dicetylphosphate may directly perturb the pulmonary membrane and may cause more irritation to the epithelial cells. In negatively charged liposomes, dicetylphosphate is incorporated into the liposomes. Insulin (pI = 5.4), being also negatively charged at pH 7.4, may repulse the negatively charged liposomes. Therefore, the head of phospholipid in the liposomes may be twisted which may reduce the contact of the dicetylphosphate with the pulmonary membrane.

The dependence of cumulative hypoglycemic response ($AAC_{0\text{-}240min}$) on the total concentration of neutral, negatively, and positively charged liposomes is illustrated in Fig. 2. The AACs are linearly related to the lipid concentration for all three types of blank liposome-insulin preparations studied. Increases in lipid concentration caused progressive enhancement in the hypoglycemic effect. This result seems to support a previous observation (7) where pulmonary absorption of liposomally encapsulated carboxyfluorescein was found to be lipid dose-dependent. The rate of carboxyfluorescein absorption was greatest in the presence of highest level of phospholipids. In the case of positively charged insulin-liposome preparations (DPPC:Chol:Stearylamine = 7:2:0.5, molar ratio), even stronger responses were observed with increasing lipid and charge-inducing agent concentrations while the fraction of total charged species in the preparation was kept constant. Negatively charged liposome-insulin formulations (DPPC:Chol:Dicetylphosphate = 7:2:0.5, molar ratio) did not result in a much stronger response

Table I. A Comparison of Cumulative Hypoglycemic Responses ($AAC_{0-240min}$) Following Pulmonary Delivery of Different Insulin (1 U/kg) Formulations

Formulation	$AAC_{0-240min}$ (% · min)
Insulin alone	6437.3 ± 1019.9
Insulin + neutral liposomes	8993.0 ± 1010.1
Insulin + stearylamine	9775.4 ± 1086.9
Insulin + positively charged liposomes	12291.8 ± 1066.7
Insulin + dicetylphosphate	11573.4 ± 1302.4
Insulin + negatively charged liposomes	9636.3 ± 1383.1

Concentration of lipids in liposomes = 15.6 mg/ml.
Values represent means ± SE (n = 3–8).

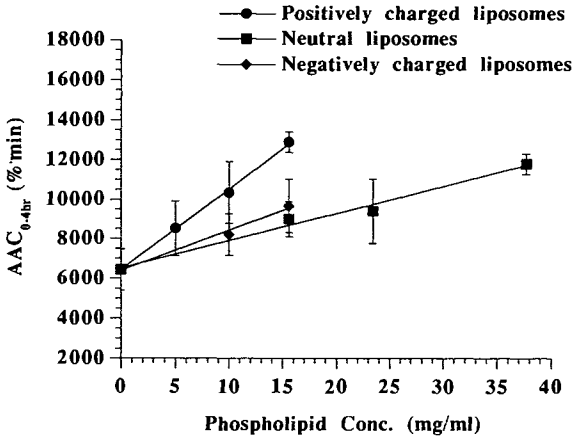

Fig. 2. Cumulative hypoglycemic effect (AAC) as a function of phospholipid concentration following pulmonary delivery of neutral and charged liposome-insulin (1U/kg) mixtures. Values represent means ± SE (n = 4).

than the neutral preparation although exhibiting a slightly higher effect. From the slope differences of the three profiles, it appears that the enhanced insulin pharmacodynamic response depends on both lipid concentration and electrical charge of the preparation.

Figure 3 describes the relationship between the lipid acyl chain length and the cumulative hypoglycemic response following intratracheal instillation of neutral liposome-insulin mixtures. A much stronger hypoglycemic effect was observed with a medium acyl chain lipid (C10) (lipid:cholesterol = 7:2, total lipid concentration = 15.6 mg/ml). The cumulative hypoglycemic response ($AAC_{0\text{-}240min}$), appeared to correlate inversely with the ascending acyl carbon number of the lipid from C10 to C18. However, in the range of C4-C8, the hypoglycemic effect increased with the ascending homologous series. This could be related to the fact that the phospholipids with lower acyl chain length are water-soluble and unable to form bilayer membrane vesicles. Increases in the carbon number of phospholipids also lead to increases in phase transition temperature considerably (8), making the phospholipids structurally more rigid. Above the phase tran-

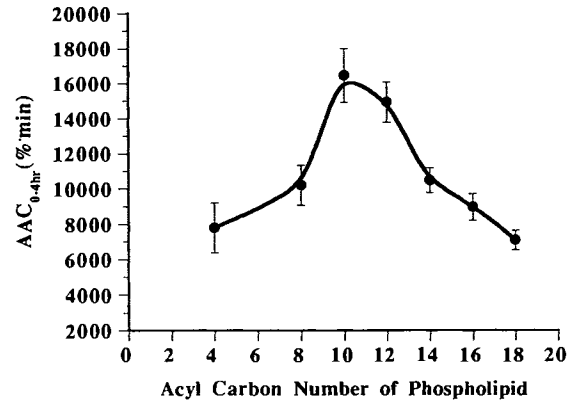

Fig. 3. Overall hypoglycemic effect following pulmonary delivery of physical mixtures of 1 U/kg insulin and liposomes with phospholipids of different acyl chain length. Values represent means ± SE (n = 3).

sition temperature, phospholipids are known to transform from a gel-like to a liquid-like state. Our results illustrated that the liquid state of liposomes delivered into the lungs is of significant importance. In conclusion, liposome bilayer formation with medium chain phospholipids appears to be an important feature for effective absorption of insulin in the lungs.

As illustrated in Fig. 4, mixing 1 U/kg of insulin with neutral liposomes (DPPC:Chol = 7:2, molar ratio) of three different sizes, i.e., 1.98 μm, 0.4 μm and 0.1 μm respectively, resulted in similar overall hypoglycemic effects. A conclusion can be drawn that the insulin absorption following intratracheal instillation is independent of the blank liposomal size in this range. The reason for this observation is still unclear. A previous study (9) reported that the absorption of liposome-encapsulated [^{14}C] sucrose following intraperitoneal administration was also independent of size. From this results, it may be concluded that liposomal particle size is not a critical factor in pulmonary insulin absorption following liposomal deposition in the lower airspace of the lungs.

Adams et al. (10) have previously reported that both stearylamine and dicetylphosphate are toxic to the central nervous system. To address the toxicity of these agents to the lungs, the activities of two marker enzymes in the lavage supernatant were measured. The activities of the membrane-bound alkaline phosphatase following saline, stearylamine, and dicetylphosphate washes were 70.1±2.8, 105.0±1.2 ($P<0.01$ vs. saline) and 78.6±7.9 U/L ($P>0.1$ vs. saline) at 25 °C. The activities of cytosolic lactate dehydrogenase following saline, stearylamine and dicetylphosphate treatments were 49.4±6.3, 62.9±6.5 ($P<0.05$ vs. saline), and 68.2±7.8 U/L ($P<0.05$ vs. saline) respectively at 25 °C. All values are expressed as means±SD (N = 3-4). Based on this information, it can be concluded that stearylamine and dicetylphosphate mediated insulin absorption enhancement may be related to the alteration in the rat pulmonary tissues.

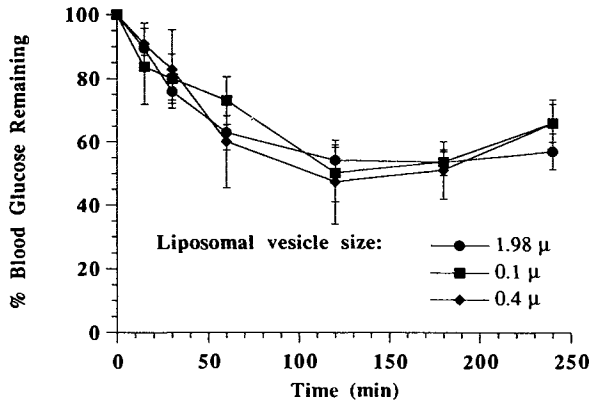

Fig. 4. The relationship between liposome vesicle size and pharmacodynamic response following pulmonary delivery of 1 U/kg insulin-liposome mixtures. Values represent means ± SE (n = 3–4).

In conclusion, the feasibility of using liposomes as pulmonary absorption promoters has been evaluated in this work. Enhancement of insulin hypoglycemic response was found to depend markedly on electric charge and acyl chain length of the phospholipid component. The size of liposomes, on the other hand, plays no significant role. It must be emphasized that the absorption enhancement by charge inducing agents may be linked to their mucotoxic nature. Long-term toxicity studies need to be performed before charged liposomes can be recommended as pulmonary delivery promoters for peptides and proteins.

ACKNOWLEDGMENTS

This work was supported by a grant from Zeneca Pharmaceuticals group. Instrumentation support was obtained in part by a grant from NIH Grant NS 25284 and in part by a Biomedical Research Support Grant RR 05586.

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