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International Journal of Pharmaceutics 217 (2001) 25–31

international journal of pharmaceutics

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Enhanced pulmonary delivery of insulin by lung lavage fluid and phospholipids

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Received 5 June 2000; received in revised form 21 December 2000; accepted 22 December 2000

Abstract

Pulmonary delivery appears to be the most promising non-parenteral route of insulin administration. In this work, we investigated the enhancement of insulin absorption in the presence of phospholipids and lung lavage fluid in vivo and in vitro. In-vitro experiments of insulin uptake by type II cells showed a significantly enhanced absorption in presence of lavage fluid, compared to various buffer preparations. The same trend was obtained with in-vivo studies of tracheal instillation of insulin. The incorporation of phospholipids as absorption enhancers in 1,2-dipalmitoyl phosphatidylcholine (DPPC) dispersion was compared to blank liposomes. A significantly higher blood glucose decrease was observed with a DPPC–insulin physical mixture compared to liposome, suggesting a possible effect of the phospholipid chain physical state on the insulin in-vivo absorption. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: DPPC; Insulin; Lavage fluid; Pulmonary delivery; Type II cells

1. Introduction

Delivery of proteins and peptides by the pulmonary route has demonstrated a superior systemic bioavailability over other non-invasive alternative routes. Nevertheless, it is still considerably less efficient than the traditional injectable route due to the presence of a protective permeability barrier. Several studies in our laboratory have explored the role of absorption enhancers relative to pulmonary delivery of insulin. Bile salts have been found to be very effective absorption promoters for insulin absorption (Li et al., 1993). Moderate mucosal toxicity, however, still remains a concern.

The effect of blank (unloaded) liposomes on the pulmonary transport of insulin has also been examined (Liu et al., 1993). The results revealed that blank liposomes could improve insulin absorption significantly. This result suggests a possible inter-

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PII: S0378-5173(01)00588-9

action between the components of the liposomes and the pulmonary surfactants secreted at the surface of the alveolar membrane. The surfactant molecules can be extracted from the lungs by a lavage with a saline solution. Although it is difficult to isolate all the components of the lung lavage fluid, it has been shown to contain mainly phospholipids and about 10% of apoproteins, which play a major role in the formation of lattice structures (Suzuki et al., 1989).

Pulmonary surfactants consisting mainly of phospholipids and apoproteins have been demonstrated to enhance and regulate the uptake of lipids by type II alveolar cells in primary culture (Claypool et al., 1984). These surfactant constituents are known to reduce the surface tension in the alveolar surface, stabilize fluid balance in the lung, and defend against infections (Juers et al., 1973). The optimal function of lipids requires the presence of a few small proteins (Van Golde et al., 1988), which are considered to be important in surfactant spreading, adsorption and reutilization (Wright and Clements, 1987).

Pulmonary surfactants are synthesized by type II epithelial cells and transported intracellularly to lamellar bodies where they are packaged and stored along with cholesterol and surfactant proteins (Schmitz and Muller, 1991). Results from several studies suggest that the type II cells can internalize surfactant components and that the internalized material can be either reutilized or degraded.

So far, little research has been conducted to study the role of pulmonary surfactants in polypeptide pulmonary delivery. The purpose of this study, therefore, is to investigate the absorption enhancement mechanism of pulmonary surfactants in pulmonary peptide drug delivery by using the in-vitro type II cells and the in-vivo rat model. Different formulations of phospholipid and insulin were used to optimize the pulmonary delivery of insulin. First, mixtures of blank liposomes and insulin were prepared in lung lavage fluid to evaluate the effect of pulmonary surfactants on insulin absorption. Then, in order to study the effect of the specific structure of liposomes on the enhancement efficiency, a simple phospholipid dispersion was prepared in lung lavage fluid and saline solutions. The results may delineate the biochemical and structural aspects of surfactant molecules that will ultimately lead to the development of potentially effective, non-toxic and natural absorption promoters of macromolecules such as polypeptides.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoylphosphatidylcholine (DPPC), was purchased from Avanti Polar Lipid Inc. (Alabaster, AL). Cholesterol was purchased from Sigma Chemical Company (St. Louis, MO). Fisher Scientific (Fairlawn, NJ) supplied chloroform (spectral grade). Sterile 0.9% saline solution was obtained from Abbott Laboratories (North Chicago, IL). Eli Lilly and company (Indianapolis, IN) generously donated crystalline porcine zinc insulin (potency 26.3 U/mg). Buffer I and buffer II were prepared according to a published method (Dobbs et al., 1986). Both buffers contain 140 mM NaCl, 5 mM KCl, 2.5 mM sodium phosphate, 10 mM Hepes. In addition, buffer I includes 6 mM glucose and 0.2 mM EGTA; buffer II includes 2 mM $CaCl_2$ and 1.3 mM $MgSO_4.7H_2O$. Ringer solution prepared according to Joergensen (Joergensen and Bechgaad, 1993) contains 1.6 mM $NaHPO_4$, 0.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM KCl, 25 mM $NaHCO_3$, 112.4 mM NaCl, and 13 mM glucose.

2.1.1. Preparation of lavage fluid

Male Sprague–Dawley rats were anesthetized by an intraperitonial injection of a mixture of 90 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine. The lungs were lavaged three times by an instillation of 5 ml of 0.9% saline solution. The combined lavage fluid was then centrifuged at 1500 rpm for 8 min. at 4°C in order to remove the suspending cells (F0630 rotor, Beckman Model Centrifuge). The supernatant was stored in freezer and was used within 24 h of collection.

2.1.2. 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 lavage fluid or sterile saline solution was added. The pH of the solution was adjusted to the physiological value of 7.4 by the addition of 0.1 N NaOH.

2.1.3. Preparation of liposomes

Blank liposomes, which are empty liposomal vehicles, were prepared by the thin-film method (Bangham et al., 1965). The preparation procedure first involved solubilization of phospholipids and cholesterol in a molar ratio of 7:2 (total mass 50 mg) in 5 ml of chloroform. The solvent was then evaporated under vacuum at room temperature until the last traces of chloroform were removed. The thin-filmed flask was then kept under vacuum overnight. Three milliliters of saline solution were added to the thin-filmed flask containing several glass beads, and the flask was placed in a shaker bath at 45°C for 1 h. Then, 0.2 ml of insulin stock solution was added to prepare the final dispersion, containing 15.6 mg/ml of lipids and 0.0835 mg/ml of insulin (corresponding to 1 U/kg in the in-vivo experiment). Finally, the dispersion was homogenized by a Polytron PT 10/35 homogenizer (Brinkmann Instrument Inc., Westburg, NY) for 3 min. The system was then filtered (0.22 μm). The liposomes were characterized by an average hydrodynamic radius of 100 ± 50 nm, as measured by dynamic light scattering (DynaPro-801 TC).

2.1.4. Preparation of the DPPC–insulin physical mixture

First, 43.5 mg of DPPC were added to 3 ml of aqueous solution (sterile saline or lavage fluid). The dispersion was sonicated for 7 min. An aliquot (0.2 ml) of insulin stock solution was added to the DPPC dispersion and sonicated again for 2–4 min. The final dispersion contained 13.6 mg/ml of DPPC and 0.0835 mg/ml of insulin.

According to the phase diagram of the DPPC–water system (Guldbrand et al., 1982), the system, which has been referred as a DPPC–insulin physical mixture, consists of a dispersion of microcrystals of DPPC in the Lβ' phase (the structure is planar lamellar with chains in the crystalline state incorporating 17% water). The average hydrodynamic radius, as measured by dynamic light scattering, was observed to be 120 ± 70 nm for the DPPC dispersed phase, which is of the same order of magnitude as the liposomes. The main structural difference with the liposome is the state of the DPPC chains; in the liposome, the phospholipid hydrocarbon chains are in a fluid state, whereas in the dispersion, they are in a crystalline state (Guldbrand et al., 1982).

2.2. Methods

2.2.1. Isolation of type II cells

Freshly isolated type II cells were prepared by a published method (Dobbs et al., 1986). Pathogen-free male Sprague–Dawley rats, weighing 180–230 g, were anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine and heparin (400 U/kg body weight). The trachea was cannulated, and the inferior vena cava and bottom of the abdomen were cut open. The lungs were perfused with saline solution until the lobes were free of blood. The organs were then removed and lavaged to total lung capacity with phosphate-buffered saline of different compositions (twice with buffer II and 8 times with buffer I according to Dobbs et al., 1986) to remove the macrophages. The lung cavities were then filled with elastase solution (12 ml, 40 U/kg) for 20 min at 37°C. The trachea and large airways were then discarded. Each lung was minced with sharp scissors to a final size of 1 mm^3 in the presence of DNase I solution (4 ml, 0.025%) and 25% fetal bovine serum. The resulting tissue minces and cell suspension were filtered through cheesecloth and 70 mm nylon cell strainers (Becton Dickinson and company, Franklin Lakes, NJ). The cell suspension was then centrifuged at $130 \times g$ for 10 min. The cell pellets were resuspended gently with red blood cell lysis solution and centrifuged at $130 \times g$ for another 10 min. The cells were then redispersed with buffer II solution, added to IgG (500 mg/ml)-coated 100 mm bacteriologic plastic dishes, and placed in a 10% CO_2/air incubator at 37°C for 1 h. The plates were then removed form

the incubator carefully and tipped back and forth three times to free the settled type II cells but gentle enough to avoid detaching adherent macrophages and lymphocytes. The detached cells were centrifuged at $130 \times g$ for 10 min. The cell pellets were redispersed with the supernatant of initial lavage fluid or Ringer solution (Joergensen and Bechgaad, 1993).

2.2.2. Incubation conditions

A predetermined count of type II cells (average 2.2×10^7, $n = 3$) was suspended in 0.9 ml of supernatant of initial lavage fluid or Ringer solution in a 15 ml centrifuge tube at 37°C. After mixing with 0.1 ml of insulin alone or a liposome–insulin mixture, 0.1 ml samples were withdrawn at 0, 5, 10, 30 and 60 min and immediately stored on ice. The samples were then centrifuged at 4°C, 5000 rpm for 4 min in a Beckman TL-100 ultracentrifuge. An aliquot (60 μl) of the supernatant was collected and mixed with 60 μl of methyl alcohol. The insulin concentration was determined by an HPLC method and the percentage remaining insulin was calculated.

2.2.3. Pulmonary administration of insulin

The liposome–insulin dispersion was delivered to the rat lungs by a method described in our previous report (Li et al., 1993). Male Sprague–Dawley (SD) rats, weighing 170–230 g, were fasted for 18–24 h 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 of ketamine hydrochloride and 10 mg/kg of xylazine. Jugular vein cannulation was performed by inserting a 3 inch piece of Silastic® tubing, 0.047 inch o.d. (Dow Corning, Midland, MI). A 2 inch piece of PE-200 (Becton Dickinson, Parsippany, NJ) was used for tracheal cannulation. The insulin formulation (approximately 0.1 ml) was instilled into the lungs through a 4 inch long plastic tubing (PE-50) (Becton Dickinson, Parsippany, NJ) that had 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 was 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, IN). The percentage blood glucose remaining was plotted as a function of time. Then, the area above the percentage blood glucose remaining versus time curve (AAC) and below the 100% line was calculated by the linear trapezoidal method. Plasma immunoreactive insulin was quantitated by a double-antibody radioimmunoassay using RIA kit provided by Diagnostics Products Corporation (Los Angeles, CA). At least three experiments were performed for each system (parallel group study), and the data were analyzed with Student's t-test. The mean values with the standard errors have been reported.

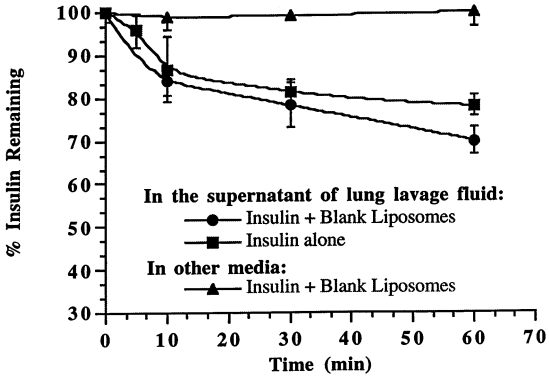

Fig. 1. Percentage insulin remaining as a function of time following incubation with type II cells in the absence and presence of liposomes in different media.

3. Results and discussion

3.1. Insulin uptake by the type II cells

Fig. 1 provides the percentage insulin remaining as a function of time following incubation with type II cells in the absence and presence of blank liposomes. Several incubation media, such as buffer I, buffer II and Ringer solutions have been employed. However, none of them exhib-

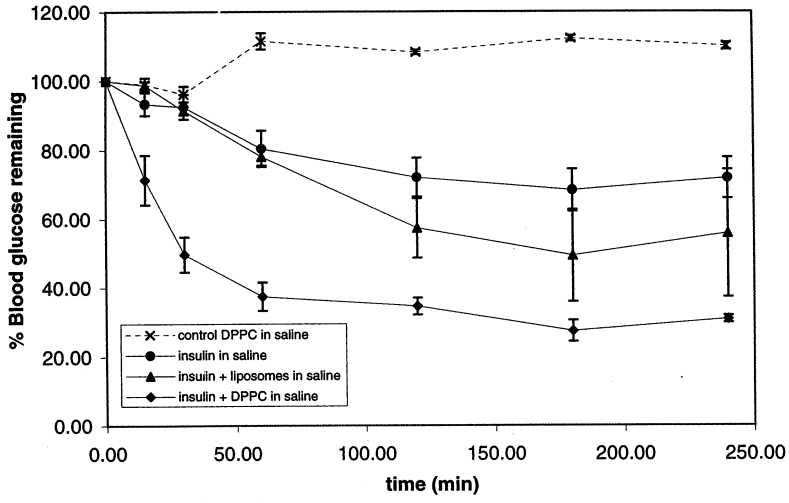

Fig. 2. Hypoglycemic effect observed in various formulations of insulin in saline.

ited measurable change in insulin concentration following incubation of insulin alone and in the presence of blank liposomes. These results indicate that insulin did not sediment in the presence of phospholipids under the experimental conditions, and a decrease in insulin concentration may be further attributed to an insulin uptake by the type II cells. When the supernatant of lung lavage fluid was used as the incubation medium, a significant insulin uptake by type II cells was noted in both formulations, i.e. for insulin alone and the insulin–liposome mixture. The rate of insulin uptake accelerated with increased incubation time. Moreover, insulin uptake by type II cells was greater in the presence of blank liposomes than with insulin alone. This is probably due to the fact that the incorporation of liposomes facilitated insulin uptake by type II cells.

This investigation also showed that insulin uptake by pulmonary cells occurred only when the supernatant of the rat-lung lavage fluid was employed as the incubation medium. It appears to be consistent with a previous report (Wright and Clements, 1987), in which liposome uptake by type II cells was noted to be a protein-regulated process. In-vivo studies of the effect of pulmonary surfactant on the insulin absorption in rats have been conducted to confirm these results.

3.2. In-vivo administration of insulin in liposome and DPPC dispersion. Effect of the lung lavage fluid

As illustrated in Fig. 2, instillation of insulin alone in saline at a dose of 1 U/kg resulted in a maximum blood glucose reduction of only 32% after 180 min, while an insulin–liposome mixture prepared with saline resulted in a maximal blood glucose depression of 50% after 180 min. The best result of hypoglycemia was obtained with the DPPC–insulin physical mixture in saline, generating a glucose reduction of 63% after 60 min and a maximum glucose reduction of 73% after 180 min. A control experiment showed that DPPC dispersion alone did not induce any decrease in the blood glucose. The area above the curve (AAC) calculated for a 4 h experiment for the DPPC–insulin physical mixture in saline ($14\,730 \pm 767$) is significantly higher ($P < 0.05$) than the AAC value of the insulin–liposome mixture (8136 ± 2037) and insulin control in saline (5581 ± 1250).

When lung lavage fluid was used, as can be seen in Fig. 3, to prepare the insulin–liposome mixture, a significant hypoglycemic effect occurred with maximum blood glucose reduction of 67% at 180 min. A control experiment showed that the lavage fluid alone did not induce any hypoglycemic effect. The AAC value calculated for a

4 h experiment for the formulation of the insulin–liposome mixture with lavage fluid (11 629 ± 1584) is not significantly different ($P > 0.05$) from the AAC value for the preparation of insulin–liposome mixture with saline (8136 ± 2037). With respect to the DPPC–insulin physical mixture, the result obtained in the presence of lavage fluid shows a maximum glucose reduction of 79% after 180 min, but only 34% reduction was obtained after 60 min. The area above the curve (AAC) was calculated to be 12 800 ± 917 for the insulin–DPPC dispersion mixture in lavage fluid, which is not significantly different from the insulin–DPPC physical mixture in saline. The hypoglycemic effects are summarized in Table 1. The decrease in glucose level corresponds to insulin absorption, as shown on Fig. 4 for insulin solution in lavage fluid, and the DPPC–insulin physical mixture in lavage fluid. As shown by the control experiment, DPPC alone did not have any effect on the insulin level. However, a statistically significant enhancement ($P < 0.05$) was not observed in the presence of DPPC.

In-vitro results suggested that pulmonary surfactant might be included as an absorption enhancer in the presence of liposome due to its effect on regulation and promotion of pulmonary cell functions. Therefore, attempts to isolate and characterize the active components responsible for the enhancement of insulin absorption should be investigated further. More experiments are necessary to confirm these results in vivo, for which a high variability in results has been obtained.

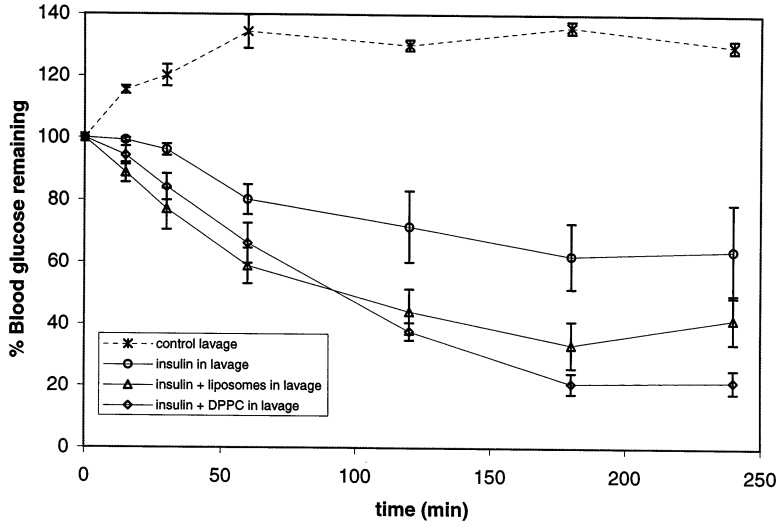

Fig. 3. Hypoglycemic effect observed in various formulations of insulin in lavage fluid.

Table 1
Comparison of hypoglycemic responses ($AAC_{0-240\ min}$) following pulmonary delivery of different insulin formulations (1 U/kg)

Formulation	Glucose reduction after 60 min (%)	Glucose reduction after 180 min (%)	($AAC_{0-240min}$) (%.min)
Insulin in saline	20 ± 5	32 ± 6	5581 ± 1250
Insulin in lavage	20 ± 5	38 ± 11	6055 ± 2040
Insulin + liposome in saline	22 ± 3	51 ± 13	8136 ± 2037
Insulin + liposome in lavage fluid	41 ± 6	67 ± 8	11 629 ± 1584
Insulin + DPPC in saline	63 ± 4	73 ± 3	14 730 ± 767
Insulin + DPPC in lavage fluid	34 ± 6	79 ± 3	12 801 ± 917

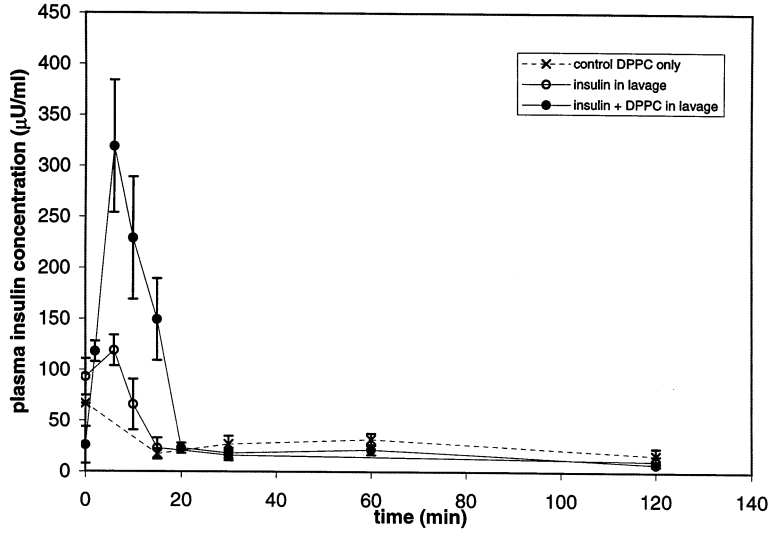

Fig. 4. Plasma insulin concentration following instillation of insulin in various formulations prepared with lavage fluid.

DPPC dispersions appear to be efficient absorption promoters in saline as well as in lavage fluid. Insulin molecules can bind to the external surface of phospholipid structures, in particular the liposome, and the degree of binding depends on the structure of the hydrocarbon chain (Wiessner and Hwang, 1982). The hydrophobic binding between insulin and phospholipids may be favored in the DPPC dispersion compared to the liposome, where the DDPC molecule is in the rippled state, which coincides with more rapid rotation of the molecule.

Acknowledgements

This work was supported in part by a grant from Hoechst-Marion-Roussel, Kansas City, MO.

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