Pulmonary Delivery of Free and Liposomal Insulin

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The effects of oligomerization and liposomal entrapment on pulmonary insulin absorption were investigated in rats using an intratracheal instillation method. The results indicated that both dimeric and hexameric insulins can be rapidly absorbed into the systemic circulation, producing a significant hypoglycemic response. Intratracheal instillation of insulin in two different oligomerized states has not resulted in any significant difference in the duration of hypoglycemic effect. However, the initial hypoglycemic response (first 10 min) obtained from intratracheal administration of 25 IU/kg hexameric insulin appears to be slower than that from the 25 IU/kg dimeric insulin, thereby suggesting that hexameric insulin may have a lower permeability coefficient across alveolar epithelium than the dimeric insulin. Intratracheal administration of insulin liposomes (dipalmitoylphosphatidyl choline:cholesterol, 7:2) led to facilitated pulmonary uptake of insulin and enhanced the hypoglycemic effect. Nevertheless, similar insulin uptake and pharmacodynamic response were obtained from both the physical mixture of insulin and blank liposomes and liposomally entrapped insulin.

KEY WORDS: insulin; insulin oligomerization; liposomes; pulmonary delivery.

INTRODUCTION

Pulmonary delivery of macromolecular protein and nonprotein compounds has attracted much attention of formulation scientists recently. This route of administration offers a number of advantages over the conventional gastrointestinal pathway, i.e., a large absorptive area, extensive vasculature, easily permeable membrane, and low extracellular and intracellular enzyme activity. The noninvasive nature of this pathway also makes it especially valuable for the delivery of large molecular protein drug candidates derived from recombinant DNA technology (1).

So far, little information is available regarding the primary factors determining the rate and extent of pulmonary drug absorption. It is believed, however, that factors such as molecular size, pH, charge, ions, surface activity, and solubility may play an important role in pulmonary absorption of peptides and proteins.

Pulmonary delivery of insulin, a model polypeptide compound with a molecular weight of 5.7 kD, was previously shown to produce a significant hypoglycemic effect following both instillation and aerosol mode of administration (2,3). Although the effect of insulin oligomerization on the nasal (4), enteral (5), and subcutaneous (6) insulin transport has been well demonstrated, such an influence has not yet been verified for the pulmonary pathway. In addition, other physicochemical and pharmacokinetic properties of insulin such as its rapid elimination from the central (blood) compartment also need to be taken into consideration prior to the successful development of any insulin delivery system.

Previous studies have revealed that intratracheal administration of a drug in liposomal form could produce a sustained effect (7–9). However, the influence of phospholipid on pulmonary uptake of insulin has not been investigated previously. It is probable that liposomes as an insulin carrier may facilitate insulin uptake in the lungs, thereby enhancing hypoglycemia.

This article describes an *in vivo* pulmonary absorption study aimed at clarifying how insulin self-association affects pulmonary absorption of insulin. Solutions containing insulin dimers (sodium insulin) and hexamers (zinc insulin) were administered to test the hypothesis that pulmonary absorption of hexameric insulin may be slower than that of dimeric insulin due to the size difference. Pulmonary delivery of insulin in phospholipid liposomes was also investigated in order to examine whether a prolonged hypoglycemic effect could be achieved by incorporating insulin inside lipid vesicles. Experiments were also conducted by combining insulin with blank liposomes. If the liposomal lipid is involved in rapid insulin uptake into the alveolar cells, then the mere presence of membrane lipid vesicles will enhance insulin uptake, thereby eliminating an expensive additional step of encapsulating insulin inside the lipid vesicle.

MATERIALS AND METHODS

Materials

Crystalline porcine-sodium insulin (25.9 IU/mg) and crystalline porcine-zinc insulin (26.3 IU/mg) were kindly donated by Eli Lilly and Company (Indianapolis, IN). Sterile 0.9% sodium chloride solution for intravenous use (Abbott Laboratories, North Chicago, IL) was used to dissolve insulin and to replace the blood volume taken during sampling. A sodium heparin solution for injection (1000 USP U/mL; Lyphomed; Rosemont, IL) was utilized with proper dilution. Cholesterol and 1,2-dipalmitoyl phosphatidylcholine (DPPC) were purchased from Sigma Chemical Company (St. Louis, MI). Certified-grade isopropyl ether and chloroform were obtained from Fisher Scientific (Fairlawn, NJ).

Preparation of Insulin Solution

Crystalline porcine-sodium and zinc insulin powders were stored in a desiccator kept at −20°C in a freezer. Crystalline insulin was first brought to room temperature, and an adequate amount of solid insulin was weighed into a 10-mL volumetric flask. In the case of zinc insulin, a minimal volume (0.1 mL) of 0.1 *N* HCl solution was added to solubilize the solid. Then the volume was brought to 10 mL by adding sterile saline solution. For sodium insulin, sterile saline solution was directly added to dissolve the solid.

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Preparation and Characterization of Sodium Insulin Liposomes

Sodium insulin liposomes were prepared by the reversed-phase evaporation method (9). The procedure involved was as follows: (a) 100 mg lipid (87 mg DPPC and 13 mg cholesterol) was dissolved in 5 ml chloroform/isopropyl ether (1:1) to which 5 ml insulin solution was added; (b) the system was then homogenized with a Polytron PT 10/35 homogenizer for 4 min; and (c) the organic solvent was removed by bubbling nitrogen at 50°C to obtain the milky insulin liposomes.

Liposomally entrapped insulin was then purified through removal of unentrapped insulin from the crude liposome product by the ultrafiltration method (10,11). An Amicon Model 3 minicell was used for this purpose with the help of a 20K-MWCO ultrafilter (Spectrum Medical Industries, Inc). The liposome thus produced was washed three times with a 0.9% USP saline solution. The filtrate was collected and the insulin concentration was subsequently measured by HPLC. The concentrated final liposomes were diluted properly with 0.9% saline and administered intratracheally to rats.

For entrapment efficiency calculations, total insulin concentration associated with the final liposome population was also determined by HPLC after dissolving 0.1 mL liposomes in 2-propanol (0.2 mL). The entrapment efficiency was expressed as the weight percentage of sodium insulin entrapped in the overall liposome population.

In Vivo Pulmonary Administration of Free and Liposomal Insulin

Male Sprague–Dawley rats, weighing 170–250 g, were fasted for 18–24 hr prior to an experiment. The rats were anesthetized with an intraperitoneal injection of a mixture of 90 mg/kg ketamine hydrochloride and 10 mg/kg xylazine. With the animal resting on its back on a board, the limbs were secured by taping them on the board. After the trachea had been exposed through a longitudinal incision along the ventral aspect of the neck, it was cut transversely, halfway through. Plastic tubing (PE-200) serving as a tracheal cannula was inserted through the tracheal incision caudally for a distance of about 0.3 in. After the surgery, the rat seemed to breathe normally. The right external jugular vein was cannulated with a 3-in. piece of Silastic tubing, 0.047-in. O.D. (Dow Corning, Midland, MI). A collar, made from PE-200 polyethlene tubing, was added onto the cannula in order to tie it with the jugular vein. Before insertion, the cannula was filled with heparinized saline. A 23-G needle, filed at the end to remove the bevel, was inserted into one end of the cannula. An aliquot blood sample (0.2 mL) was withdrawn from the jugular vein prior to the administration of insulin.

For intratracheal administration of free and liposomal insulin solution (6 IU/kg), an aliquot of the solution (0.1 mL) at room temperature was administered into the lungs through plastic tubing (PE-50) attached to a 1-mL syringe. For instillation, the tubing was inserted through the tracheal cannula to a depth where the tubing could not be further inserted. The solution was discharged rapidly over a period of 2–4 sec. Blood samples (0.2 mL) were withdrawn through the jugular vein at predetermined time intervals and then placed in heparinized Netelson capillary tubes (Scientific Products, McGaw Park, IL). Plasma samples were obtained by centrifuging the whole blood at 3500 rpm for 15 min and then stored in a −20°C freezer prior to analysis.

Experiments were performed using at least three rats in each group in order to afford meaningful comparisons. Differentiation of zinc and sodium insulin uptake kinetics requires the use of higher insulin concentrations to maintain zinc insulin in the hexameric form. A dose of 25 IU/kg in a volume of 0.1 mL was administered to rats for this purpose. Other dose studies (2 and 6 IU/kg) were also performed with sodium insulin solution for the construction of an insulin dose–hypoglycemic response relationship.

Measurement of Plasma Insulin and Blood Glucose

Plasma insulin concentrations were determined using a radioimmunoassay method. Coat-A-Count kits purchased from Diagnostic Products Corporation (Los Angeles, CA) were used to measure plasma insulin concentration. Blood glucose level was determined by Chemstrip bG and Accu-Chek IIm blood glucose monitor (Boehringer Mannheim Corporation, Indianapolis, IN). A drop of fresh blood sample (about 30 μl) obtained from the jugular vein was carefully discharged onto a Chemstrip stripe coated with a glucose-sensitive reagent. The change in color was determined by an AccuChek IIm blood glucose monitor, which gave a reading of glucose level in the blood. This analytical method determines blood glucose concentrations in the range of 10–500 mg/dL with a ±3% precision.

Data Analysis

The areas under the plasma insulin curve (AUC) and above the blood glucose curve (AAC) were calculated by the linear trapezoidal method. In the calculation of AUC, plasma insulin concentration was normalized by subtracting the endogenous insulin concentration at time 0. AAC, on the other hand, was calculated by subtracting the percentage glucose reduction from 100. The pharmacological availability (f) was calculated using the following equation:

$$f = \frac{\mathrm{AAC}_{0\text{–}240\ \mathrm{min\ it}}}{\mathrm{AAC}_{0\text{–}240\ \mathrm{min\ iv}}} \times \frac{\mathrm{Dose}_{\mathrm{iv}}}{\mathrm{Dose}_{\mathrm{it}}} \quad (1)$$

One-tailed Student t test was employed to examine any statistical significance of the data.

RESULTS AND DISCUSSION

A previous study from this laboratory has indicated that insulin is metabolized mainly to an equally bioactive metabolite (desB^1-Phe), most likely by an aminopeptidase present in the cytosolic fraction of the rat lung homogenate. Therefore, the metabolism in the lungs may not present a significant barrier to the absorption of biologically active insulin (12). To estimate the absolute bioavailability, a study involving the intravenous injection of insulin (0.2 IU/kg) via rat tail vein was performed. As reported previously from this laboratory (13) insulin follows a two-compartmental model behavior following intravenous bolus injection in rats. The plasma insulin concentration can be expressed as Eq. (2),

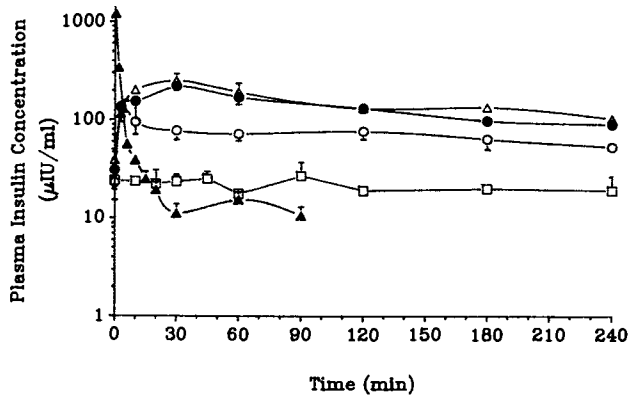

Fig. 1. Plasma insulin concentration profiles following intravenous administration of 0.2 IU/kg insulin (▲), intratracheal administration of 6 IU/kg insulin (○), intratracheal administration of blank liposomes (□), intratracheal administration of a mixture of 6 IU/kg insulin and blank liposomes (●), and intratracheal administration of 6 IU/kg liposomally entrapped insulin (△).

with the preexponential terms in μIU/mL and the exponential terms in min^{-1}.

$$C = 1735.78e^{-0.8401t} + 65.97e^{-0.06052t} \quad (2)$$

An *in vitro* study (11) utilizing isolated perfused rabbit lung as a model has compared the absorption pattern and pulmonary clearance of insulin following aerosol administration compared to the administration through intratracheal instillation. The results suggested that insulin perfusate concentrations obtained after an aerosol administration were comparable to that after intratracheal instillation. Based on this finding, the intratracheal instillation approach was chosen to examine the extent of pulmonary absorption of insulin.

Our *in vivo* absorption studies have revealed that insulin molecules can be rapidly taken up by the systemic circulation from both sodium and zinc salts, although at different rates. An intratracheal administration of sodium insulin (6 IU/kg) produced a peak plasma insulin concentration at

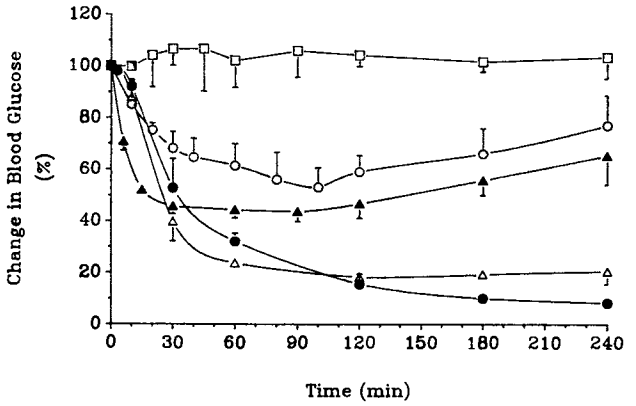

Fig. 2. Profiles of percentage change in blood glucose concentrations following intravenous administration of 0.2 IU/kg insulin (▲), intratracheal administration of 6 IU/kg insulin (○), intratracheal administration of blank liposomes (□), intratracheal administration of 6 IU/kg insulin and blank liposomes (●), and intratracheal administration of 6 IU/kg liposomally entrapped insulin (△).

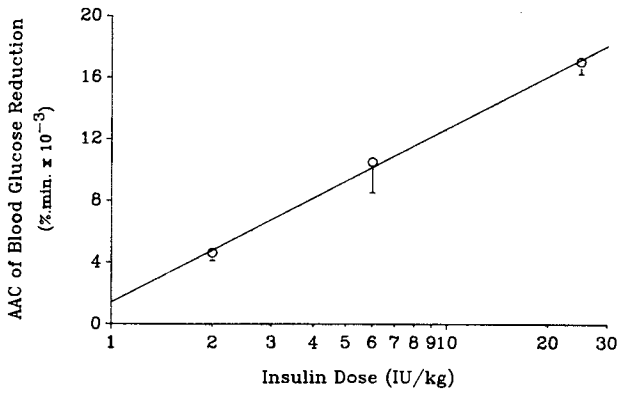

Fig. 3. Correlation between the extent of hypoglycemic response and the pulmonarily delivered sodium insulin dose.

about 3 min postadministration (Fig. 1). A significant hypoglycemic response was also observed following intratracheal administration of sodium insulin (Fig. 2). The hypoglycemic response appears to be dose dependent as shown in Fig. 3.

The initial phase (first 10 min) of insulin hypoglycemic response produced by insulin hexamers (Zn-insulin) appears to be somewhat slower than that by insulin dimers (Na-insulin) (Fig. 4). The magnitude of the blood glucose reduction at 10 min post intratracheal administration of 25 IU/kg sodium insulin is significantly greater than that after zinc insulin ($P < 0.05$). It is probable that insulin hexamers have a smaller diffusion coefficient and, as such, are transported slowly across the alveolar sacs into the systemic circulation. Another possible explanation for this similarity in the pharmacodynamic profiles may be the interchangeability among oligomer species such that new equilibria are rapidly established upon intratracheal administration of insulin solutions, thereby eliminating any differences between sodium and zinc insulins. The small differences in overall hypoglycemic action between insulin dimers and insulin hexamers also implied that the oligomerization may not be of clinical significance relative to pulmonary delivery. An intratracheal administration of 6 IU/kg insulin manifested a comparable lowering effect in blood glucose (maximum response and total response) as generated by an iv injection of 0.2 IU/kg insulin.

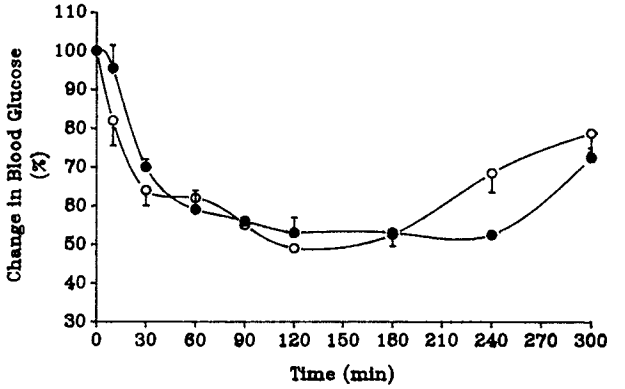

Fig. 4. Profiles of percentage change in blood glucose levels following intratracheal delivery of 25 IU/kg hexameric insulin (●) and 25 IU/kg dimeric insulin (○).

As a control study, a mixture of blank liposomes (DPPC:cholesterol, 7:2) and insulin solution was administered intratracheally to study the effect of lipid content on pulmonary absorption of insulin. The presence of liposomes containing 14 mg/mL lipid appeared to cause a considerable increase in pulmonary uptake of insulin compared to free insulin (Fig. 1 and Table I). The duration of the insulin hypoglycemic effect was also significantly enhanced (Fig. 2 and Table II).

Liposomally entrapped insulin was prepared using the reverse-phase evaporation method as described previously. The mean liposome size was 1.9 μm, with an insulin entrapment efficiency of 14%. The intratracheal administration of liposomally entrapped insulin (6 IU/kg) also enhanced pulmonary absorption of insulin as shown in Fig. 1 and Table I. The average absolute bioavailability of insulin following intratracheal administration of liposomal insulin is 30.3% (Table I), being twice that of free insulin. A significant increase in hypoglycemic response was also observed (Fig. 2).

In order to compare the relative effectiveness of the three formulations in lowering blood glucose levels, the area above the percentage glucose–time curve, maximum percentage of glucose reduction, time for maximum glucose reduction, and pharmacological availability values were compiled in Table II. When pharmacological availabilities were compared statistically, a significant difference ($P < 0.05$) was noted between insulin alone in saline and in combination with blank liposomes. A similar difference also exists between insulin in saline and liposomally entrapped insulin. However, no statistically significant difference ($P > 0.05$) was found between liposomally entrapped insulin and insulin with blank liposomes. Therefore, a need for entrapping insulin inside the liposomal core may not be a necessary prerequisite to achieve enhanced delivery.

The mechanism of absorption enhancement may be due to the fact that the lungs contain surfactants dispersed on the surface of alveolar cells. The surfactants consist mainly of DPPC, with a very small portion of lung surfactant protein molecules A, B, and C, which undergo a rapid turnover at the interface between the surfactant layer and the membrane of alveolar cells (15). The addition of exogenous DPPC may accelerate the surfactant turnover process in the alveolar cells, leading to enhanced penetration of insulin molecules into the systemic circulation. A previous study (16) indeed indicated that pulmonary absorption of terbutaline can be markedly affected by liposome size, cholesterol content, and phospholipid composition. This study reported that a liposome formulation containing a higher amount of DPPC did not prolong the absorption time of terbutaline in the lungs. These results are consistent with the present study. The presence of liposomes (DPPC:CH, 7:2) did not increase the residence time of insulin molecules in the lungs (Table I). While the mean residence times (MRTs) of all three formulations were not significantly different, the time of peak insulin concentration increased in the presence of liposomes. The long mean absorption time (MAT) may imply that insulin clearance in lungs is slow, probably due to tissue binding.

The literature concerning pulmonary delivery of liposomes is quite confusing and full of inconsistency. Morimoto and Adachi (17) reported that 50% of ^{14}C-DPPC molecules were retained in the lungs for 24 hr after intratracheal administration of ^{14}C-DPPC liposomes. However, the authors failed to clarify further whether ^{14}C-DPPC molecules retained in the lungs were present in the intact liposomal form. A previous report (16) indicated that the pharmacokinetic profile of a liposomally entrapped drug is dependent largely on the liposome composition. Their results suggested that the elimination rate of DPPC liposomes should be much faster than previously expected. The half-life of terbutaline disappearance from liposomes containing 95% DPPC and 5% dipalmitoyl phosphatidylglycerol (DPPG) was similar (1.4 hr) to that of free terbutaline (1.3–1.4 hr). Our results have shown that liposomally entrapped insulin can be absorbed by the systemic circulation over a prolonged period of time as compared to free insulin alone. However, no significant difference in the rate of absorption was observed between liposomally entrapped insulin and a physical mixture of blank liposomes and insulin solution. This evidence seems to suggest that prolonged absorption is not attributable solely to the entrapment of insulin in liposomes. Previous studies have also shown that insulin molecules can bind to the surface of liposomes (18,19).

In summary, both hexameric and dimeric insulin can be rapidly absorbed into the systemic circulation through the rat lungs, producing a significant hypoglycemic response. The larger molecular size of hexameric insulin seemed to cause a minor delay in the initial pulmonary absorption of insulin as evidenced from the hypoglycemic responses of zinc and sodium insulin. The extent of hypoglycemic response obtained from the intratracheal administration of insulin was dose dependent. The intratracheal administration of a mixture of liposomes (DPPC:cholesterol, 7:2) and insulin resulted in an improved insulin absorption in the rat lungs. A similar result was also achieved by the intratracheal administration of liposomally entrapped insulin. The mechanism for enhanced pulmonary absorption of liposomal insulin is not yet clear. The similar extent of absorption in the presence of blank and entrapped liposomes suggests possible specific interactions of the phospholipid (DPPC) with the surfactant layer or even the alveolar membrane. Whether DPPC is cotransported with insulin via endocytotic pathways also remains to be studied.

Table I. Pharmacokinetic Parameters of Insulin Delivered Intratracheally in Different Formulations at a Dose of 6 IU/kg[a]

Formulation	Bioavailability [mean % (range)]	t_p (min)	MRT (min)	MAT (min)
Sodium insulin solution	14.7 (11.2–18.2)	3	115	112
Sodium insulin solution + blank liposomes	26.2 (21.2–31.2)	30	100	97
Liposomally entrapped sodium insulin	30.3 (23.5–37.1)	30	112	109

[a] t_p, peak time; MRT, mean residence time; MAT, mean absorption time.

Table II. Pharmacodynamic Parameters Related to the Hypoglycemic Effects of Insulin Administered Intravenously and Intratracheally[a]

Route of administration	Formulation	Insulin dose (U/kg)	$AAC_{0-240\ min}$ (% · min)	bG_{max} (% gluc.)	t_{max} (min)	f' (%)
iv	Insulin in saline	0.2	11,616.4 ± 1,332.3	56.3	90	100
it	Insulin in saline	6	8,216.5 ± 1,171.7	47.0	100	2.36 ± 0.33
it	Insulin + blank liposomes	6	17,614.5 ± 451.2	91.8	>240	5.05 ± 0.13
it	Liposomally entrapped insulin	6	17,259.7 ± 420.0	81.8	120	4.95 ± 0.12

[a] iv, intravenous; it, intratracheal; AAC, area above the curve of percentage glucose remaining versus time; bG_{max} = 100 − minimum percentage of glucose level at t_{max}; f', pharmacological availability.

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