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Absorption Enhancement of Intrapulmonary Administered Insulin by Various Absorption Enhancers and Protease Inhibitors in Rats

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Abstract—The effects of absorption enhancers and protease inhibitors on the pulmonary absorption of insulin were examined by means of an in-situ pulmonary absorption experiment. Absorption enhancers used in this study were sodium glycocholate, linoleic acid-surfactant mixed micelles and *N*-lauryl-β-D-maltopyranoside whereas aprotinin, bacitracin and soybean trypsin inhibitor were used as protease inhibitors. The absorption of insulin from the lung was evaluated by its hypoglycaemic effect. In the absence of these additives, a slight hypoglycaemic effect was obtained following intrapulmonary administration of insulin. However, we found significant and continuous hypoglycaemic effects after the insulin administration with these additives. *N*-Lauryl-β-D-maltopyranoside and bacitracin appeared to be more effective for enhancing the pulmonary absorption of insulin than the other adjuvants. These findings suggest that the use of these two adjuvants would be a useful approach for improving the pulmonary absorption of insulin.

Nasal (Hirai et al 1981a), buccal (Ishida et al 1981), rectal (Nishihata et al 1983), vaginal (Okada et al 1982), conjunctival (Yamamoto et al 1989) and pulmonary routes (Yoshida et al 1979) have been investigated for systemic delivery of peptides and proteins, since the oral absorption of these compounds is typically poor. Among these, the pulmonary route would seem to be a promising alternative for delivering these drugs, since a number of drugs which are poorly absorbed from enteral and other sites are well absorbed from the lung, due to the large surface area of the alveolar epithelium and the short air to blood pathway (O'Hagan & Illum 1990). Indeed, the absorption of inulin, dextran and insulin is observed after intrapulmonary administration, although they are poorly absorbed from the gastrointestinal tract (Wigley et al 1971; Enna & Schanker 1972a). However, the bioavailability of these drugs from the pulmonary route is still poor when compared with the parenteral route. Therefore, adjuvants such as absorption enhancers and protease inhibitors are required to enhance the pulmonary absorption of these macromolecular drugs.

For the oral, nasal and rectal routes, various absorption promoters such as surfactants, bile salts, chelating agents and fatty acids, and protease inhibitors such as aprotinin, bacitracin and soybean trypsin inhibitor have been used for improving the absorption of poorly absorbable drugs (Lee & Yamamoto 1990). However, few studies have been carried out on the adjuvant effect of these additives on the pulmonary absorption of poorly absorbable drugs including peptides and proteins. Niven & Byron (1990) reported that adjuvants such as oleic acid, oleyl alcohol and Span 85 can produce an increase in the transfer rate of disodium fluorescein, a model compound, from the airway of the rat isolated lung. In our previous studies, *N*-lauryl-β-D-maltopyranoside (LM), linoleic acid-surfactant mixed micelles (MM), sodium glycocholate and sodium caprate were effective for enhancing the pulmonary absorption of fluorescein isothiocyanate-labelled dextrans (FDs), while a slight enhancing effect was obtained following the administration of FDs with EDTA and sodium salicylate (Ohtani et al 1991; Morita et al 1993). Concerning peptide absorption from the lung, Okumura et al (1992) reported that the pulmonary absorption of insulin was enhanced in the presence of various adjuvants such as glycocholate, surfactin, Span 85 and nafamostat.

In the present study, the effects of various absorption enhancers on pulmonary absorption of insulin were investigated in rats. We also examined the adjuvant effect of various protease inhibitors on the pulmonary absorption of insulin.

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Materials and Methods

Materials

Insulin, sodium glycocholate and aprotinin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Bacitracin, soybean trypsin inhibitor (STI) and glucose B-test Wako were obtained from Wako Pure Chemical Industries Co., Japan. Linoleic acid of high purity grade (> 99%) was kindly supplied by Nippon Oil & Fats Co. (Tokyo, Japan). HCO60 was a gift from Nikko Chemical Co. (Tokyo, Japan). *N*-Lauryl-β-D-maltopyranoside (LM) was kindly supplied by Japan Fine Chemical Co., Japan. All other chemicals and solvents were of reagent grade quality.

Preparation of test solution

Dosing solutions containing insulin were prepared in isotonic phosphate buffer (PBS) at pH 7·4 to yield a final concentration of 10 units mL^{-1}. In some experiments, the dosing solutions were added with absorption enhancers or protease inhibitor. These additives were dissolved in PBS at pH 7·4. A solution of LA-HCO60 mixed micelles (MM) was

prepared by dispersing linoleic acid and HCO60 (molar ratio of linoleic acid:HCO60, 30:4) in PBS, followed by sonication at 40 W for 5 min in an ice-bath using an Ohtake model 5202 sonicator (Ohtake Seisakusho Co., Tokyo, Japan). The fatty acid was neutralized with aqueous 1 M NaOH before dispersion.

Animal experiments

Absorption of drugs from rat lung was investigated according to the method of Enna & Schanker (1972a, b). Male Wistar strain rats (Japan SLC, Inc., Hamamatsu, Japan), 240–300 g, were anaesthetized with sodium pentobarbitone (32 mg kg^{-1}) given by intraperitoneal injection. Animals were fasted for about 16 h before experiments but allowed free access to water. After the animal had been secured on its back on an animal board, the trachea was exposed through a longitudinal incision along the ventral aspect of the neck. The trachea was then cut transversely, halfway through, between the fourth and fifth tracheal rings caudal to the thyroid cartilage. A section of polyethylene tubing (i.d., 1·5 mm, o.d., 2·5 mm) of length 2·5 cm, which served as a tracheal cannula, was inserted through the tracheal incision caudally for a distance of 0·6 cm so that 1·9 cm of the cannula protruded from the trachea. The incision in the skin was then closed with wound clips after drawing the skin up close to the sides of the cannula.

Body temperature was maintained at 37±1°C by heat from a 40 W incandescent lamp in a reflector suspended over the animal at a distance of about 25 cm during the experiment. Rectal temperature was monitored continuously using a thermistor probe and thermometer.

One hundred microlitres of drug solution at 37°C was injected into the lungs through an obtuse needle of a calibrated 100-μL syringe (Microliter no. 710, Hamilton Co.). For the injection, the needle was inserted through the tracheal cannula to a depth of 2·5 cm below the tracheal incision. Then, at the distance of insertion, the tip of the syringe needle was located 1–2 mm above the bifurcation of the trachea. With the syringe thus positioned, the solution was injected over a period of 1–2 s, with the rat being maintained at an angle of 80°. Immediately thereafter, the tubing was withdrawn completely and 45 s after administration the animal was returned to an angle of 10°. The animal was maintained under light anaesthesia for the remainder of the experimental period.

In some experiments, insulin solution (0·1 units) was intravenously administered by bolus injection to determine the pharmacological availability in each experiment.

Analytical methods

The pulmonary absorption of insulin was estimated by its hypoglycaemic effect. For determination of the glucose concentration in plasma, 200 μL blood was taken from the left femoral artery periodically after dosing, centrifuged at 10000 rev min^{-1} for 3 min, and the plasma samples were collected. The glucose concentrations in plasma were determined by a Glucose Test Wako (Wako Pure Chemical Industries, Co., Osaka, Japan). A decrease in the plasma glucose concentration (D%) was calculated by a modified method of Hirai et al (1981a) from the following equation:

$$D\% = \left(1 - \frac{AUC_{0\to240}}{100\% \times 240\ min}\right) \times 100$$

Results

Fig. 1 shows the relationship between dose of intrapulmonary insulin and the decrease in plasma glucose concentration (D%). There is a linear relationship between these two parameters over the range of 0–5 units, suggesting that the D% values can be used as an index of insulin absorption from the lung.

No significant changes in the glucose concentrations were observed when PBS was administered to the lung. Fig. 2 shows concentration-time profiles of glucose in plasma after intrapulmonary administration of insulin in the presence of various absorption enhancers. In the absence of these

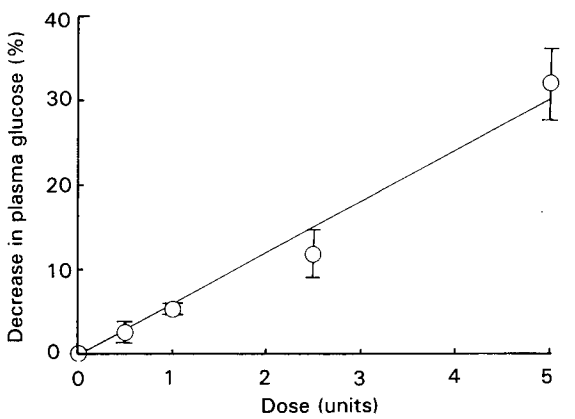

FIG. 1. Relationship between dose of intrapulmonary insulin and decrease in plasma glucose concentration (D%). Error bar represents s.e.m. for four or five rats.

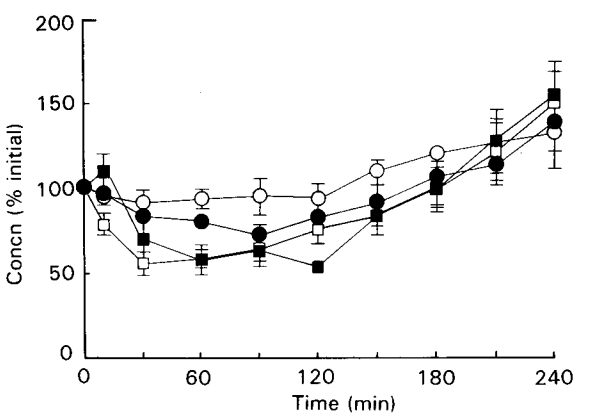

FIG. 2. Concentration-time profiles of glucose in plasma after intrapulmonary administration of 1 unit insulin in the presence of absorption enhancers. ○ Control, ● 10 mM linoleic acid-HCO60 mixed micelles, □ *N*-lauryl-β-D-maltopyranoside, ■ 10 mM sodium glycocholate. The glucose concentration was expressed as percentage value at time zero. Error bar represents s.e.m. for four rats.

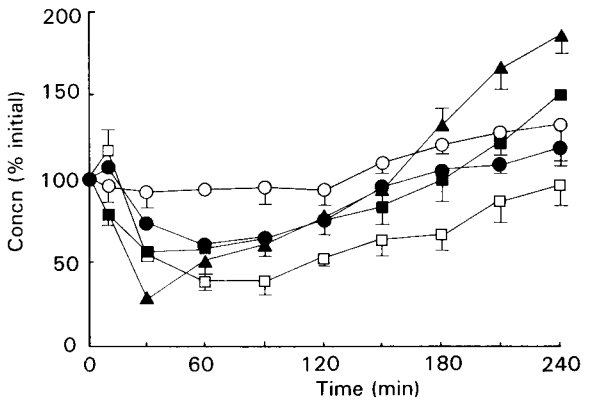

FIG. 3. Concentration-time profiles of glucose in plasma after intrapulmonary administration of 1 unit insulin in the presence of varying concentrations of *N*-lauryl-β-D-maltopyranoside. ○ Control, ● 1, □ 5, ■ 10, ▲ 20 mM. The glucose concentration was expressed as percentage value at time zero. Error bar represents s.e.m. for four or five rats.

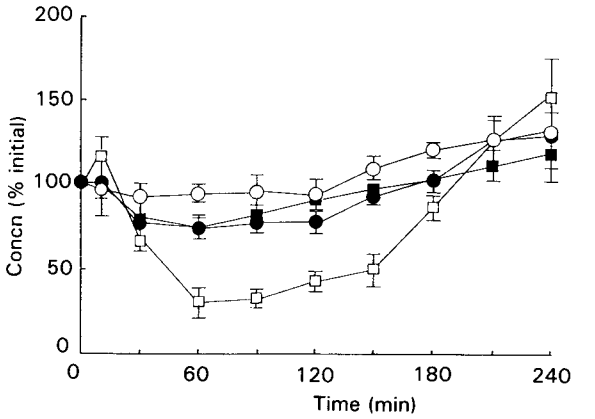

FIG. 5. Concentration-time profiles of glucose in plasma after intrapulmonary administration of 1 unit insulin in the presence of varying concentrations of bacitracin. ○ Control, ■ 5, ● 10, □ 20 mM. The glucose concentration was expressed as percentage value at time zero. Error bar represents s.e.m. for four or five rats.

Table 1. Effect of various absorption enhancers on the pulmonary absorption of insulin.

Enhancers	Concn	D%	PA%
Control	—	5·3±0·6	11·9
Sodium glycocholate	10 mM	22·1±1·4	50·0***
MM	10 mM	13·2±3·0	29·9**
LM	1 mM	17·4±3·2	39·3**
	5 mM	37·5±2·9	85·0***
	10 mM	22·7±4·7	51·5**
	20 mM	24·0±4·0	54·4*

PA%: Pharmacological availability %

$$= \frac{D\%_{\text{pul.}}}{D\%_{\text{i.v.}}} \times \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{pul.}}} \times 100$$

The D% values are expressed as mean ± s.e. of 4–5 rats. $*P<0.01$, $**P<0.05$, $***P<0.001$ compared with control.

Table 2. Effect of various protease inhibitors on the pulmonary absorption of insulin.

Protease inhibitor	Concn	D%	PA%
Control	—	5·3±0·6	11·9
Aprotinin	10 mg mL^{-1}	10·4±1·5	23·6*
STI	10 mg mL^{-1}	13·3±1·5	30·1*
Bacitracin	5 mM	10·4±1·3	23·6**
	10 mM	17·5±2·2	39·7*
	20 mM	35·9±3·5	81·2***

PA%: as for Table 1. The D% values are expressed as mean ± s.e. of 4–5 rats. $*P<0.01$, $**P<0.05$, $***P<0.001$ compared with control.

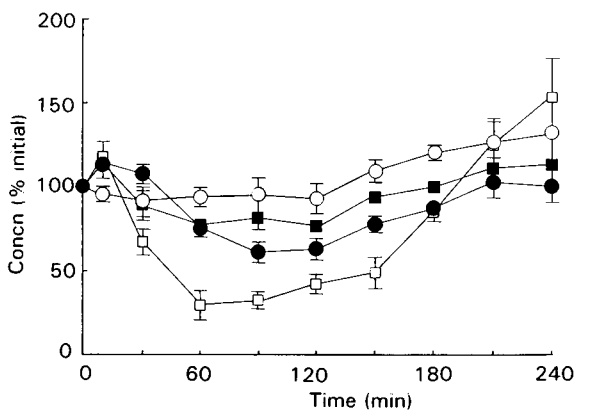

FIG. 4. Concentration-time profiles of glucose in plasma after intrapulmonary administration of 1 unit insulin in the presence of protease inhibitors. ○ Control, ● 10 mg mL^{-1} soybean trypsin inhibitor, □ 20 mM bacitracin, ■ 10 mg mL^{-1} aprotinin. The glucose concentration was expressed as percentage value at time zero. Error bar represents s.e.m. for four rats.

additives, a slight hypoglycaemic effect was obtained following intrapulmonary administration of insulin. In the presence of absorption enhancers, the plasma glucose concentration reached a minimum of 47·7–70·0% of base line within 120 min of solution administration. The glucose concentration returned to base line within 210 min.

The time course of glucose concentrations in plasma after intrapulmonary administration of insulin solutions containing varying amounts of LM is displayed in Fig. 3. The plasma glucose level was influenced by the amount of LM coadministered with insulin and a maximal effect was noted when insulin solution containing 5 mM LM was administered in the lung. The decrease in plasma glucose level and pharmacological availability were 5·3 and 11·9% in the absence of absorption enhancers but in their presence reached 13·2–37·5 and 29·9–85·0%, respectively (Table 1).

The concentration-time profiles of glucose in plasma after intrapulmonary administration of insulin in the presence of various protease inhibitors are displayed in Fig. 4. In the presence of protease inhibitors, the plasma concentration of glucose reached a minimum of 24·0–66·7% of base line within 90 min of solution administration. At this concentration of these protease inhibitors, bacitracin was much more

effective for enhancing the pulmonary absorption of insulin than aprotinin or STI. Fig. 5 indicates the time course of glucose concentration in plasma after intrapulmonary administration of insulin solutions containing varying amounts of bacitracin. The plasma glucose level decreased with increasing the amount of bacitracin. The decrease in plasma glucose level and pharmacological availability were improved by the addition of these protease inhibitors, especially 20 mM bacitracin (Table 2).

Discussion

In the present study, it was observed that a slight hypoglycaemic effect was obtained following intratracheal administration of insulin without absorption enhancer, indicating that insulin is absorbed from the lung to some extent in the absence of the additives. This result was in agreement with the previous reports of Wigley et al (1971), Yoshida et al (1979), and Okumura et al (1992), who demonstrated pulmonary absorption of insulin without these adjuvants. Therefore, the lung is permeable to macromolecular compounds such as insulin which are poorly absorbed from the intestine and thus offers a potential route of administration for systemically-acting peptides and proteins.

Our present data demonstrated that absorption enhancers were effective for enhancing the pulmonary absorption of insulin in rats. The reason why these absorption enhancers were used in the present experiment is that a significant increase in the pulmonary absorption of fluorescein isothiocyanate-labelled dextrans was found by the addition of these adjuvants, as reported previously (Ohtani et al 1991; Morita et al 1993). The result that glycocholate improved the pulmonary absorption of insulin is consistent with the report of Okumura et al (1992).

The mechanisms whereby the pulmonary absorption of insulin was improved by these adjuvants are not still understood. In the case of sodium glycocholate, it was reported that bile salts enhance the permeability by removing the epithelial cells, which constitute a major permeability barrier (Hersey & Jackson 1987). In addition, Gordon et al (1985) suggested that bile salts interact with cell membranes to form a reverse micelle which acts as a channel to increase permeation by the test compounds. Further, it was demonstrated by Hirai et al (1981b) that bile salts may promote insulin transport across the nasal mucosa by retarding insulin degradation by leucine aminopeptidase, a proteolytic enzyme. Similarly, our previous studies demonstrated that the hydrolysis of insulin in various mucosal homogenates of rats was inhibited by sodium glycocholate (Yamamoto et al 1990, 1992). It may be speculated that these mechanisms may be related to the enhancement effect of sodium glycocholate on the absorption of insulin from the lung, although these mechanisms were mainly investigated in the gastrointestinal tract and nasal route.

MM is known to improve the absorption of a number of poorly absorbable drugs such as heparin, streptomycin, gentamicin, bleomycin, interferon and carboxyfluorescein from the gastrointestinal tract (Muranishi 1985, 1990). Our previous results suggest that the increase in membrane permeability caused by the fatty acid is associated with the disorder of the interior of the membrane and interaction between the incorporated fatty acid and polar head group of the phospholipid (Muranushi et al 1981). More recently, it was found that a sulphydryl-related substance is involved in the permeability-enhancing effect of oleic acid (Murakami et al 1988). Therefore, it may be considered that MM increased the pulmonary absorption of insulin by some of these mechanisms in the same manner as the intestine, unless oleic acid has a different action to the pulmonary mucosa.

LM, an alkylsaccharide, has recently been found to lower surface tension and to have absorption enhancing activity in the gastrointestinal tract (Murakami et al 1992). In our previous studies, rectal absorption of carboxyfluorescein, FDs and insulin was improved by coadministration of LM and the maximal effect was noted at 10 mM LM (Murakami et al 1992). Concerning the pulmonary absorption studies, it was demonstrated in our laboratory that LM is one of the most effective enhancers for improving the pulmonary absorption of FDs (Ohtani et al 1991). In this study, we found a significant hypoglycaemic effect after the pulmonary administration of insulin with LM. These findings indicate that LM is a suitable absorption enhancer for enhancing the pulmonary absorption of macromolecules. In addition, it was observed that there exists an optimal concentration of LM (5 mM) for enhancing the pulmonary absorption of insulin. This parabolic absorption-enhancing effect of LM may be associated with the micelle formation above the critical micelle concentration (the critical micelle concentration of LM is 0·16 mM).

The present research demonstrated that various protease inhibitors were also effective for improving the pulmonary absorption of insulin. In our pilot studies, insulin hydrolysis was clearly inhibited by the addition of these proteolytic inhibitors to lung homogenate. Consequently, it may be considered that these protease inhibitors may reduce the activity of various proteolytic enzymes and improve the stability of insulin in the lung homogenate, thereby enhancing the pulmonary absorption of insulin. At present, we have not determined which proteolytic enzymes are present in the pulmonary tissue and which enzymes are responsible for the hydrolysis of insulin in the pulmonary homogenate. However, it could be speculated that at least there may exist aminopeptidase-like, trypsin and chymotrypsin-like substances in the pulmonary tissue, since bacitracin inhibits the activities of aminopeptidase (Raehs et al 1988), while the activities of trypsin and chymotrypsin were reduced by the addition of aprotinin and STI (Saffran et al 1988). Unlike our data, Okumura et al (1992) reported that bacitracin did not affect the bioavailability of insulin after intratracheal administration and they concluded that the proteolytic enzyme activity of the lung in degrading insulin is less than that of rectal and subcutaneous tissues. This result was inconsistent with our present finding that bacitracin remarkably increased the absorption of insulin from the lung. This discrepancy may be attributed to the concentration of bacitracin used in each experiment, since Okumura et al (1992) used 1 mM bacitracin for enhancing the pulmonary absorption of insulin, whereas 20 mM was used in our experiment.

In conclusion, it was suggested that the lung may become a useful route for administration of insulin, a systemically acting drug. In addition, sodium glycocholate, MM, LM and

bacitracin may be suitable adjuvants for improving the pulmonary absorption of insulin. Local toxicity of these agents needs to be assessed.

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