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Proteolytic enzymes as a limitation for pulmonary absorption of insulin: in vitro and in vivo investigations

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Abstract

In vitro biodegradation of insulin in lung cytosol and subcellular pellets of normal and diabetic rats was investigated. Rat lung was homogenized and subcellular fractions were isolated by ultracentrifugation. Degradations of [^{125}I]-insulin after incubation with lung cytosol or subcellular pellets was determined using the trichloroacetic acid method. The results show that insulin is highly degraded in cytosol and subcellular pellets. Cytosolic insulin degradation was strongly inhibited by bacitracin or sodium cholate. The degradation of insulin in the lung cytosol from diabetic rats was significantly less than from normal rat. The lung protease activity reached a maximum at pH 7.4. Enzyme inhibitors like bacitracin and sodium cholate noticeably enhanced the relative pharmacological bioavailability of insulin when given intratracheally with insulin to normal rats. Acidic insulin solutions (pH 3.0) had more pronounced hypoglycaemic effects than neutrol solution (pH 7.0). These in vitro and in vivo results suggest that the proteolytic enzymes in the lung limit pulmonary delivery of insulin. The coadministration of protease inhibitors would be a useful approach for improving the pulmonary absorption of insulin.

Keywords: Insulin; Lung; Proteolytic enzymes; Degradation; Pulmonary route; Intratracheal administration; Rat

1. Introduction

Pulmonary delivery of insulin has attracted much attention from pharmaceutical researchers around the world (Liu et al., 1993 Yamamoto et al., 1994b; Edwards et al., 1997; Robert, 1997). This route of administration offers a number of advantages over the conventional gastrointestinal pathway including large surface area for absorption, extensive vasculature, a very thin epithelial barrier and easily permeable membrane. Previous studies have shown that pulmonary administration of an aerosol or solution form of insulin to animals and humans can induce effective hypoglycaemia and increase serum insulin concentrations (Okumura et al., 1992; Laube et al., 1993; Jendle and Karlberg, 1996). To enhance pulmonary absorption of insulin, it is necessary to investigate

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the enzyme barriers and the enzyme activities in the lung. Literature has shown that insulin degradation is considerable when incubated with cultured rat type II alveolar cells (Hsu and Bai, 1998) or lung homogenates (Liu et al., 1992).

The purpose of the present study was to evaluate the degradations of insulin by proteolytic enzymes in the lung of normal and diabetic rat in vitro. In vivo experiments estimating the enhancement of hypoglycaemic effect by some enzyme inhibitors were also conducted to support the results of in vitro experiments.

2. Materials and methods

2.1. Materials

Crystalline porcine zinc insulin (26.3 U/mg), was purchased from Xuzhou Biochemical plant (People's Republic of China) and bacitracin was purchased from Server (Germany). Alloxan, sodium cholate and Tris–HCl were purchased from Sigma (St. Louis, MO, USA). The insulin radioimmunoassay kit was obtained from the China Institute of Atomic Energy. The reagents for blood glucose assay were purchased from Sigma. All other chemicals and solvents were of analytical grade.

2.2. Preparation of subcellular fractions

Male Sprague–Dawley rats weighing 170–200 g, were randomly divided into two groups, a control group and a group intravenously injected with 40 mg/kg alloxan. One week later, rats with blood glucose over 250 mg/dl were chosen as diabetic rats for experiments. According to the procedure of Liu et al. (1992), all rats were anaesthetized with an overdose of sodium pentobarbital (150 mg/kg) by intraperitoneal injections. The lungs were surgically isolated and washed with ice-cold Tris–HCl buffer (pH 7.4, 0.1 M) containing 1 mM EDTA. Lung homogenates were prepared with a glass homogenizer. The resulting mixture was centrifuged for 10 min at 3500 rpm and 4°C.The supernatant was collected and centrifuged for 40 min at 35,000 rpm and 4°C in a Beckman L8-70M ultracentrifuge. The final supernatant was used as cytosol, and the precipitate fraction was used as subcellular pellets, a mixture of organelles.

To compare the stability of insulin in alveolar epithelium and intestine epithelium, intestine epithelial cell cytosol was also prepared. Briefly, rats were anaesthetized as already described, and the intestine was surgically isolated and exposed with a longitudinal incision. The exposed intestine was rinsed three times with ice-cold Tris–HCl buffer to remove gastrointestinal fluid and lumenal contents. The epithelial tissue was surgically collected and homogenized. The cytosol was prepared from the homogenate by the method described.

2.3. Degradation of insulin in normal and diabetic rat lung

Degradations of insulin in cytosol and subcellular pellets were determined using the trichloroacetic acid (TCA) method. The degradation of insulin was examined utilizing the solubilization of degradation products of [^{125}I]-insulin to 5% TCA as described previously (Duckworth et al., 1972), while intact insulin was precipitated. The study used 30 pM labelled insulin (from radioimmunoassay kit) plus large amounts of non-labelled insulin. We assumed that the proteolytic enzymes cannot distinguish between labelled and non-labelled insulin. Briefly, the assay system consisted of 50 μl 0.4 U/ml insulin, 50 μl 30 pM [^{125}I]-insulin and 100 μl cytosol or subcellular pellets for each reaction tube. As the control, 100 μl Tris–HCl buffer or intestine epithelial cytosol was used. The mixture was incubated at 37°C. Periodically, triplicate samples were taken from the water bath and mixed with 200 μl 10% TCA solution, and the resulting mixture was then centrifuged at 3500 rpm for 10 min. The supernatant containing the soluble insulin fragments was discarded and the radioactivity of the precipitate was counted with a γ counter.

2.4. Effects of inhibitors and pH on insulin degradation

The effect of pH on insulin degradation in lung

cytosol was determined at 37°C using acetate buffer at pH 3.6 or 4.5, phosphate buffer at pH 6.6, or Tris–HCl buffer at pH 7.4 or 8.5. Degradation was terminated after 30-min incubation by the addition of 200 μl 10% TCA solution. Bacitracin or sodium cholate was dissolved in 0.4 U/ml insulin, and added to a final concentration of 2.5 mM. The degradation of insulin by lung cytosol in the presence of inhibitors was determined by the method already described.

2.5. Intratracheal administration of insulin

Male Sprague–Dawley rats, 300–400 g, fasted overnight before the experiment, were anaesthetized with 40 mg/kg sodium pentobarbital. The animals were secured on their backs on a board and their tracheas were surgically exposed, and 100 μl insulin solution (1.0 and 0.5 U/kg; pH 7.0 or 3.0) with or without inhibitors was administered into the lung. Phosphate-buffered saline solution without insulin was intratracheally administered as a control. Insulin solution (0.5 U/kg; pH 7.0) was subcutaneously administered to determine the relative pharmacological bioavailability (Fr). Blood samples (0.3 ml) were collected by clipping the tail tip at predetermined time intervals. Blood glucose levels were measured immediately by the glucose oxidase method.

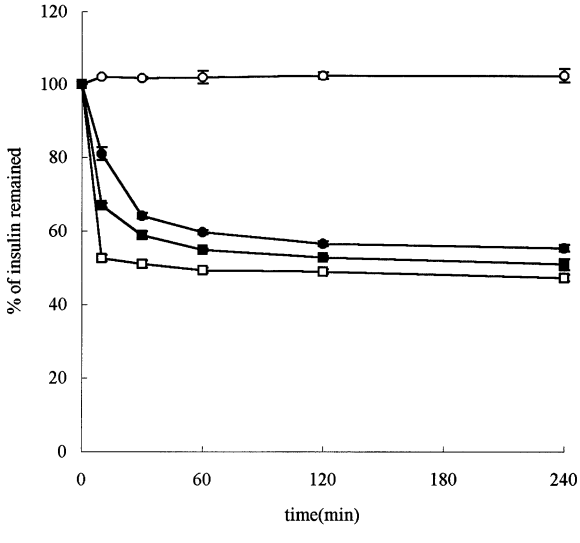

Fig. 1. Degradation of insulin in lung cytosol or subcellular pellets of normal rat: (○) Tris buffer, (■) lung cytosol, (●) subcellular pellets, (□) intestine epithelial cytosol. Each point represents the mean ± S.D. of three experiments.

2.6. Data analysis

The area above the blood glucose curve (AAC) was calculated by the trapezoidal method. The relative pharmacological bioavailability (Fr) was calculated by the following equation (Ritschel and Ritschel, 1984):

$$Fr = (AAC_{i.t.}/AAC_{s.c.})/(dose_{s.c.}/dose_{i.t.})$$

The subscripts i.t. and s.c. refer to intratracheal and subcutaneous administrations, respectively.

Student's *t*-test was used to determine statistical significance.

3. Results

When insulin was incubated at 37°C with normal rat lung cytosol and subcellular pellets, it lost its ability to be precipitated by TCA. Fig. 1 shows insulin degradation over a 4-h incubation period. As shown in Fig. 1, lung cytosol or subcellular pellets significantly decreased the concentration of intact insulin. No degradation was observed when insulin was incubated with Tris–HC1 buffer. These results indicate that the proteolytic enzymes are present in the lung and strongly degrade insulin.

We also incubated insulin with intestinal epithelial cytosol for comparison as a control. As seen in Fig. 1 and Table 1, insulin was significantly degraded within 10 min in the intestine epithelial cytosol group. The time during which more than 60% insulin remained ($T_{60\%}$) of lung cytosol and intestine cytosol were 27 and 9.1 min, respectively, indicating that proteolytic enzymes in the lung were less active than in the intestine.

Insulin was incubated in both normal and diabetic rat lung subcellular fractions (Fig. 2). Degradations were much greater in both lung cytosol and subcellular pellets of normal rat than in diabetic rat ($P < 0.01$).The $T_{60\%}$ value of insulin in

Table 1
A comparison of $T_{60\%}$ values of insulin degradation in various fractions of rat lung[a]

	$T_{60\%}$ (min)
Normal lung cytosol	27.0 ± 2.5
Normal lung subcellular pellets	60.1 ± 3.4*
Diabetic lung cytosol	118 ± 2.8*
Diabetic lung subcellular pellets	>240**
Normal intestine epithelial cytosol	9.1 ± 1.5*

[a] Results are expressed as the mean ± S.D. of three experiments.
* $P<0.01$ versus normal lung cytosol.
** $P<0.01$ versus normal lung subcellular pellets.

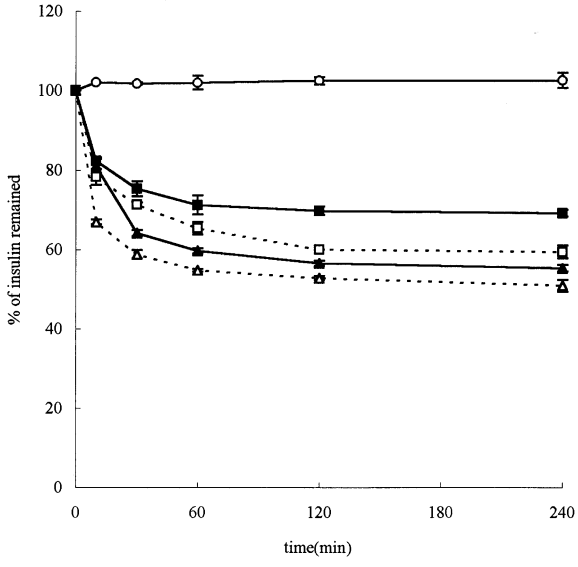

Fig. 2. Degradation of insulin in lung cytosol or subcellular pellets of normal or diabetic rat: (○) Tris buffer, (△) lung cytosol of normal rat, (▲) lung cytosol of diabetic rat, (□) lung subcellular pellets of normal rat, (■) lung subcellular pellets of diabetic rat. Each point represents the mean ± S.D. of three experiments.

cytosol or subcellular pellets of normal rat lung were much smaller than that of diabetic rat lung (Table 1).

Bacitracin inhibits the activities of aminopeptidase (Raehs et al., 1988). As seen in Fig. 3, bacitracin significantly inhibited enzyme activity in lung cytosol at a concentration of 2.5 mM. In the presence of bacitracin, 95.4% of insulin still remained after 10-min incubation, while in the absence of bacitracin, only 65.3% of intact insulin remained. The $T_{60\%}$ value in the presence of bacitracin was greater than 240 min. Fig. 3 also reveals that sodium cholate can partially inhibit the degradation of insulin at a concentration of 2.5 mM ($T_{60\%} = 102$ min), although it was less effective than bacitracin. Table 2 summarizes the effect of bacitracin and sodium cholate on $T_{60\%}$ of insulin degradation in lung cytosol. The lung cytosolic enzyme activity had optimum pH of 7.4 (Fig. 4). Cytosolic insulin degradation was much slower at pH 3.6 and 4.5.

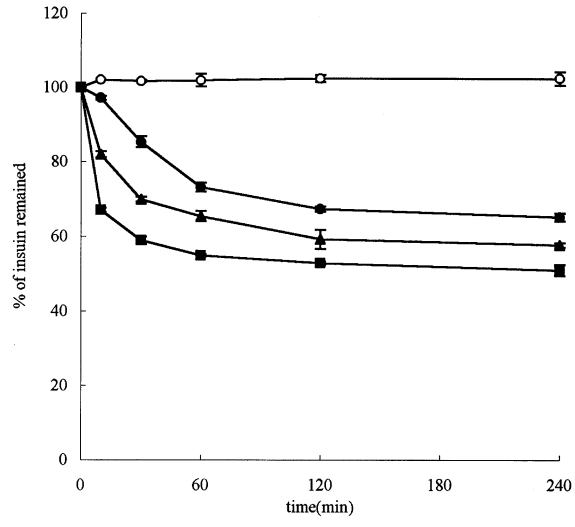

Fig. 3. Inhibition of insulin degradation by bacitracin and sodium cholate in the lung cytosol of normal rat: (○ Tris buffer, (■) lung cytosol, (●) 2.5 mM bacitracin, (▲) 2.5 mM sodium cholate. Each point represents the mean ± S.D. of three experiments.

Table 2
Effect of protease inhibitors on $T_{60\%}$ values of cytosolic insulin degradation in normal rat lung[a]

	$T_{60\%}$ (min)
Lung cytosol	27 ± 2.5
+2.5 mM bacitracin	>240*
+2.5 mM sodium cholate	102 ± 5.2*

[a] Results are expressed as the mean ± S.D. of three experiments.
* $P<0.01$ versus lung cytosol.

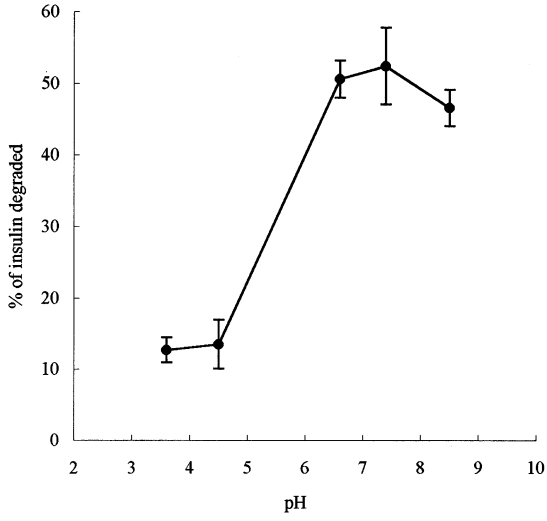

Fig. 4. The pH dependence of enzyme activity in the lung cytosol of normal rat. Each point represents the mean ± S.D. of three experiments.

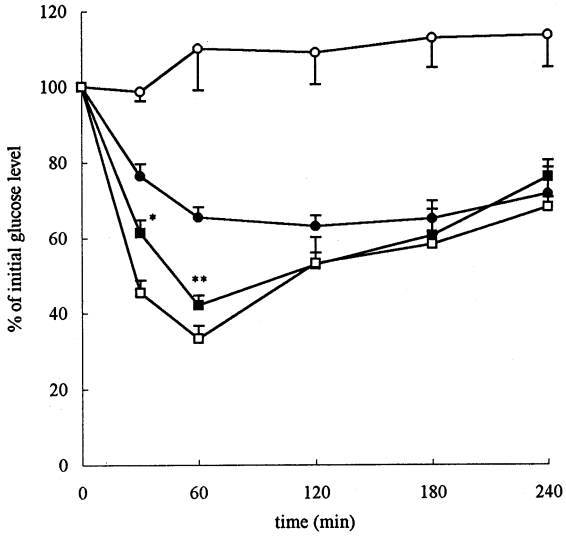

Fig. 5. Hypoglycaemic effect after pulmonary delivery of insulin solution (1.0 U/kg; pH 7.0) with bacitracin (20 mM) to normal rats: (○) control, (●) 1.0 U/kg insulin solution (pH 7.0), (■) 1.0 U/kg insulin solution (pH 7.0) with 20 mM bacitracin, (□) 0.5 U/kg insulin solution (pH 7.0) by subcutaneous injection. * $P < 0.05$ and ** $P < 0.01$ versus 1.0 U/kg insulin solution (pH 7.0) by intratracheal administration. Results are mean ± S.E., $n = 5–6$.

Blood glucose concentration–time profiles after subcutaneous administration (0.5 U/kg; pH 7.0) and intratracheal instillation (1.0 U/kg) of insulin solution (pH 7.0) with or without 20 mM bacitracin are shown in Fig. 5. In the absence of proteolytic inhibitors, a noticeable reduction in blood glucose level was observed at a dose of 0.5 U/kg, which had a Fr of 26.0% (Table 3). Bacitracin significantly enhanced the hypoglycaemic effect of pulmonary administered insulin. The Fr increased from 26.0 to 45.6% (Table 3). This result is in accord with the observations in lung cytosolic degradation experiments.

The maximum reduction in blood glucose level was approximately 40% 2 h after intratracheal administration of 0.5 U/kg (pH 7.0) insulin solution with 20 mM sodium cholate (Fig. 6). The Fr increased to 69.0% in the presence of sodium cholate, compared with 45.6% in the presence of bacitracin (Table 3).

The hypoglycaemic effects of insulin solutions in pH 3.0 and 7.0 conditions were also investigated. At pH 3.0, insulin had a more pronounced hypoglycaemic effect (Fig. 6), and its Fr reached 53.8%, which was onefold greater than the value obtained with pH 7.0 solution.

4. Discussion

In the present study, it was observed that a substantial amount of insulin was degraded in lung cytosol and subcellular pellets in normal and diabetic rats, suggesting the presence of some proteolytic enzymes responsible for insulin degradation in the lung. Recently, Hsu and Bai (1998) investigated the presence of insulin degrading enzyme in cultured type II alveolar cells and proved that insulin degrading enzyme is responsible for most insulin degradation in alveolar epithelial cell cytosol, even though other enzymes might play a small part. It has been reported that insulin degrading enzyme is also present in some organelles such as peroxisomes (Kuo et al., 1994) and microsomes (Liu et al., 1992).

The rapid and complete degradation of insulin by proteolytic enzymes in the gastrointestinal tract precludes effective absorption of insulin by oral delivery (Yamamoto et al., 1994a). Our

Table 3
Relative pharmacological bioavailability of insulin after intratracheal administration of solution under various conditions[a]

Condition	AAC (% min)		Relative pharmacological bioavailability (Fr %)
	Mean	±S.E.	
Insulin (0.5 U/kg) (s.c.)	181.7	6.6	100.0
Insulin (0.5 U/kg; pH 7.0) (i.t.)	47.3	5.2	26.0
+Sodium cholate (20 mM)	125.4	30.7	69.0
Insulin (0.5 U/kg; pH 3.0) (i.t.)	97.8	12.2	53.8
Insulin (1.0 U/kg; pH 7.0) (i.t.)	123.5	8.5	34.0
+Bacitracin (20 mM)	165.8	16.0	45.6

[a] AAC, Area above the blood glucose curve; s.c., subcutaneous administration; i.t., intratracheal administration.

present experiment demonstrates that degradation of insulin in intestine epithelial cytosol is much more significant than in lung cytosol (Fig. 1), suggesting larger amounts of proteases are present in the intestine. In our in vivo experiment, a low dose of insulin (0.5 U/kg) produced noticeable hypoglycaemic effect and its Fr (26.0%) was greater than the values previously determined via the oral route (Ziv et al., 1987; Lowe and Temple, 1994) These in vitro and in vivo results suggest that the lung may be a more effective site for the absorption of insulin than the gastrointestinal tract.

We found that the $T_{60\%}$ values of insulin in lung cytosol or subcellular pellets of diabetic rat were much greater than that of normal rat (Table 1), indicating that proteolytic enzyme activities in diabetic rat lung are lower than in normal rat lung. This finding suggests that the deficiency of insulin might induce a decrease of enzyme activities in the lung, although the mechanism is not clear.

The present data showed that bacitracin and sodium cholate improve the pulmonary absorption of insulin by inhibiting the degradation of insulin in lung cytosol. Our results are in agreement with an experiment by Yamamoto et al. (1994b), showing that 20 mM bacitracin can significantly enhance the pulmonary absorption of insulin (Fig. 5). It was reported that bile salts enhance permeability by removing the epithelial cells (Hersey and Jackson, 1987) or by interacting with cell membranes to form a reverse micelle to increase the permeation (Gordon et al., 1985). We found that sodium cholate partially inhibits the degradation of insulin in lung. Yamamoto et al. (1994a) reported that sodium glycocholate effectively reduces the degradation of insulin in homogenates of the small or large intestinal mucosae. In our in vivo experiments, a significant hypoglycaemic effect was observed by intratra-

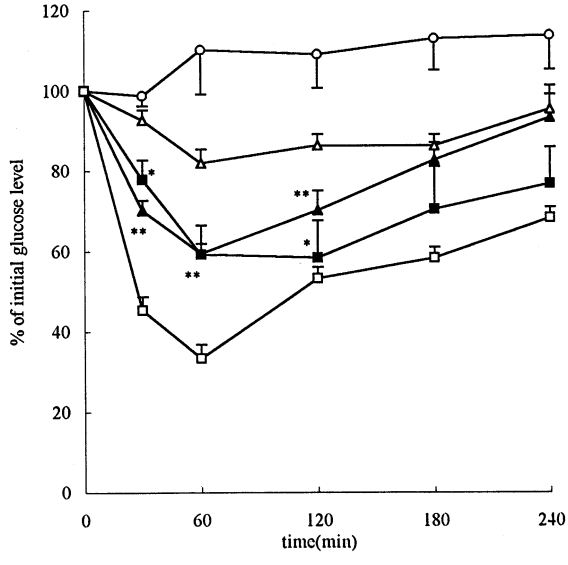

Fig. 6. Hypoglycaemic effect after pulmonary delivery of insulin solution (0.5 U/kg) with 20 mM sodium cholate in pH 7.0 or 3.0 conditions: (○) control, (△) 0.5 U/kg insulin solution (pH 7.0), (▲) 0.5 U/kg insulin (pH 3.0), (■) 0.5 U/kg insulin solution (pH 7.0) with 20 mM sodium cholate, (□) 0.5 U/kg insulin solution (pH 7.0) by subcutaneous injection. * $P < 0.05$ and ** $P < 0.01$ versus 0.5 U/kg insulin solution (pH 7.0) by intratracheal administration. Results are mean ± S.E., $n = 5–6$.

cheal administration of 0.5 U/kg insulin with 20 mM sodium cholate (Fig. 6). However, bacitracin, which strongly inhibited insulin degrading activity in vitro (Fig. 5), did not exhibit a greater Fr than sodium cholate in vivo (Table 3). These results imply that sodium cholate is more effective as an enhancer rather than as a protease inhibitor.

Fig. 4 shows cytosolic proteolytic enzymes have a maximum activity at pH 7.4. The glucose concentration–time profile following the intratracheal administration of insulin (0.5 U/kg) at pH 3.0 causes a rapid decrease in the blood glucose level with a Fr value twofold greater than that of pH 7.0 solution. (Fig. 6 and Table 3). This result may be due to lung tissue damage by the acid solution and increasing the permeability of insulin (Okumura et al., 1992). Alternatively, the acidity may have inhibited enzyme activities in acid conditions, as shown in our in vitro observations.

In conclusion, our in vitro and in vivo results suggest that proteolytic enzymes limit absorption of insulin in the lung. The enzyme activity should be minimized in order to improve the bioavailability of insulin via pulmonary delivery.

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