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Characterization of human insulin microcrystals and their absorption enhancement by protease inhibitors in rat lungs

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Abstract

Pulmonary route appears to be an attractive alternative as a non-invasive systemic delivery for peptide and protein drugs. An appropriate formulation, however, is important for increasing their bioavailability in lung. In this study, the human insulin microcrystals were produced. The particle size analysis and scanning electron microscopy (SEM) showed that the microcrystals were uniform and had a monodispersed size distribution (mean diameter = 0.95 μm) for pulmonary delivery. The physicochemical properties of the microcrystals developed were similar to those of the commercial crystalline powder in powder X-ray diffraction (XRD) and differential scanning calorimetry (DSC) analyses. The percentage of high molecular weight proteins (%HMWP), the percentage of other insulin related compounds (%OIRC) and the percentage of A-21 desamido insulin (%D) of the microcrystals were very low. In addition, the cytotoxicity of microcrystals developed and protease inhibitors (aprotinin, bacitracin and soybean-trypsin inhibitor) was investigated, and the enhancement of insulin absorption in the presence of these protease inhibitors at various concentrations was studied. The cell viability of A549 was over 80% at various concentrations of aprotinin and soybean-trypsin inhibitor, except for bacitracin (below 60%). The percent of decrease in blood glucose (D%) was 42.68 ± 1.62% after intratracheal instillation of insulin microcrystals (5 U/kg). An enhancement of hypoglycemic effect with protease inhibitors was also found. Soybean-trypsin inhibitor (48.86 ± 3.24% at 10 mg/ml; 55.78 ± 0.71% at 5 mg/ml; 51.49 ± 5.27% at 1 mg/ml) and aprotinin (52.57 ± 8.78% at 10 mg/ml; 51.97 ± 1.98% at 5 mg/ml; 56.90 ± 3.42% at 1 mg/ml) were effective for absorption enhancement. These findings suggest that the use of insulin microcrystals and protease inhibitors would be useful to improve the hypoglycemic effect in pulmonary route.

Keywords: Insulin; Microcrystal; Protease inhibitors; Cytotoxicity; Instillation; Pulmonary delivery

1. Introduction

Insulin is one of the leading therapeutic proteins, and many insulin analogs have been developed (Brunner et al., 2000; Vajo and Duckworth, 2000). Injection, however, is the only delivery method for insulin as well as other protein therapeutics. To overcome the drawbacks of injection (*e.g.*, pain, irritation, itching, redness, stinging, swelling, atrophy of subcutaneous fat tissue, and poor patient compliance), oral, transdermal, buccal, ocular, and nasal routes of administration have been investigated (Smith et al., 1992; Cullander and Guy, 1992; Ho et al., 1992; Harris et al., 1992; Edman and Bjork, 1992). These alternative approaches, however, have the major limitations that are variable bioavailability (Shen et al., 1992; Swenson and Curatolo, 1992) and the safety concerns of the enhancers used in the formulations.

The respiratory tract has been a target delivery site because of its convenience and perceived advantages. The respiratory tract has several unique features that can facilitate systemic delivery. Unlike the nasal cavity (approximately 180 cm^2), an adult's lung offers a large surface area for drug absorption (approximately 100 m^2). In addition, good vascularization and the ultra-thinness of the alveolar epithelium (approximately 0.1–0.5 μm) can facilitate rapid drug absorption. Moreover, respiratory delivery would avoid the first-pass effect of the gastrointestinal tract, and the lung has relatively low metabolic enzyme activity (Agu et al., 2001).

The combination of insulin analogs and pulmonary administration was not effective on the optimal control of blood glucose because of the short-acting property of insulin analogs and the need of formulation for pulmonary delivery (Cefalu et al., 1998; Skyler et al., 1998). Therefore, investigators have studied

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methods of prolonging insulin absorption in the lung, such as microencapsulation using a biodegradable polymer (Choi et al., 2004), but problems remain, *e.g.*, the accumulation of the biodegradable polymer in the lung (Patton et al., 1999) and the loss of insulin activity during the preparation of microspheres (Rosa et al., 2000). Currently, crystallization is a major technological process for drug formulation in pharmaceutical industries and, in addition, plays an important roll in enhancing the stability and drug release properties of the final dosage forms (Shekunov and York, 2000).

Enzymatic degradation limits pulmonary absorption of peptides and proteins. A strategy to improve pulmonary absorption of proteins via the lung may include co-administration with protease inhibitors. This approach has been shown to improve the bioavailabilities and pharmacodynamic response of biotherapeutic agents, including insulin. Aprotinin is a broad-based serine proteinase inhibitor isolated from bovine lung and has the potential to inhibit protease enzymes with serine residues as their active site (Bernkop-Schnürch, 1998). Bacitracin has been employed as an important investigational tool in the laboratory where it is used as an inhibitor of several diverse enzymatic reactions. These include enhancement of insulin activity in a variety of cell types, and the inhibition of a variety of enzymes including insulinase, enkephalinase and protein disulfide isomerase (PDI) on cell surfaces (Rogelj et al., 2000). Soybean-trypsin inhibitor (STI) reduces the activities of trypsin and chymotrypsin (Yamamoto et al., 1994).

The previous studies reported the preparation method of bovine insulin microcrystals for pulmonary delivery (Kwon and Kim, 2004; Lee et al., 2006a), and the bioavailability enhancement and long-acting property of microcrystals using intrapulmonary inhalation (Kwon et al., 2004; Lee et al., 2006b). In addition, the effects of various protease inhibitors have been investigated for pulmonary absorption enhancement of insulin. As reported, the pulmonary absorption of insulin dry powder (Todo et al., 2001) and insulin solution (Shen et al., 1999; Yamamoto et al., 1994, 1996) was enhanced by the co-administration of protease inhibitors effectively. However, there were no experimental data which showed pulmonary absorption enhancement of insulin microcrystals co-administered with protease inhibitors.

Therefore, in this study, human insulin microcrystals for pulmonary delivery were produced by the previous preparation method (Kwon and Kim, 2004) with brief modification and were characterized in the size and crystalinity. The cytotoxicity of various protease inhibitors and the adjuvant effect of protease inhibitors on the pulmonary absorption of insulin microcrystals were examined.

2. Materials and methods

2.1. Materials

Crystalline recombinant human insulin was purchased from Serological Corporation (Norcross, GA, USA). Aprotinin, bacitracin, soybean-trypsin inhibitor (STI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemical substances are of analytical grade.

2.2. Preparation of insulin microcrystals

Insulin microcrystals were prepared using the seed zone method of Kwon and Kim (2004) with brief modification. Crystalline insulin powder and zinc sulfate were dissolved in 0.1N acetic acid (pH 2.0). The pH of solution (1 mg/ml insulin and 0.2 mg/ml zinc sulfate) was increased slowly up to about pH 10.5 by adding 10N and 1N NaOH solutions. When the aqueous suspension became clear, the pH of solution was adjusted to pH 6.0 by adding 5N HCl solution immediately. The solution was stirred for 15–30 min at room temperature. Then the suspension of microcrystals was stored at 4 °C. The human insulin microcrystals were recovered by drying at room temperature.

2.2.1. Morphology of insulin microcrystals

Before fixation, the insulin microcrystal suspension was centrifuged and the supernatant was discarded. The initial fixation was with 2.5% glutaraldehyde for over-night at 4 °C and the insulin microcrystals were rinsed with cold distilled water (two times, 10 min). The microcrystals were fixed with 1% osmium tetroxide for 1 h at room temperature finally, dehydrated in a graded series of ethanol, and substituted with hexamethyldisilazane. After drying at room temperature absolutely, the microcrystals were sputter-coated with gold palladium before examination in the scanning electron microscope (SEM, Hitachi S-4700, Japan).

2.2.2. Particle size analysis

The insulin microcrystals and commercial crystalline powder were dispersed in acetic acid (0.1N, pH 6) at the concentration of 1 mg/ml. About 15 ml of sample was stirred and sonicated for seconds simultaneously in the particle size analyzer (CILAS 1640, France). Size distribution was measured by laser diffraction. The particle size was expressed as a volume mean diameter.

2.3. Analysis of physicochemical properties

2.3.1. X-ray diffractometry (XRD)

Human insulin microcrystals produced were examined using X-ray diffraction to determine the crystalinity. The sample was transferred into a 1 mm glass capillary and the capillary tubes were sealed with wax. Diffraction data were collected using a Rigaku R-AXIS IV^{++} image plate detector (Rigaku International Corporation, Shibuya-Ku, Japan) with a 50 kV, 100 mA rotating copper anode and focusing mirrors, using an oscillation of 240° and 30 min exposure time at a 10 cm material-to-detector distance.

2.3.2. Differential scanning calorimetry (DSC)

DSC measurements provide qualitative and quantitative information as a function of time and temperature regarding transitions in materials that involve endothermic or exothermic processes or changes in heat capacity. Differential scanning calorimeter (Suiko Instrument, DSC 6100, Chiba, Japan),

equipped with a refrigerated cooling accessory using liquid nitrogen, was operated at a heating rate of 5 °C/min, from 40 to 200 °C. Temperature was calibrated with indium. An empty pan was used as a reference.

2.4. Analysis of product related impurities during the process

The HPLC system used was a Waters Alliance® system (Waters Corporation, MA, USA) equipped with a quaternary pump, a thermostated column compartment, an autosampler, a Waters M2996 PhotoDiode Array Detector and data acquisition software, Empower Chromatography Manager. Deionized water prepared with a MilliQ apparatus (Millipore Corporation, MA, USA) was used. All solvents were filtered with 0.45 °C filters.

2.4.1. Size exclusion HPLC (SE-HPLC)

In order to measure the high molecular weight proteins (HMWP), a filtered and degassed mixture of arginine solution, acetonitrile, and glacial acetic acid (65:20:15) was used as a mobile phase. The column temperature was maintained at 40 °C and the flow rate was about 0.5 ml/min. Detection was performed at 276 nm. One hundred microliter of sample was applied. The percentage of HMWP (%HMWP) was calculated by the equation:

$$\%\mathrm{HMWP} = \frac{\Sigma R_\mathrm{H}}{\Sigma R_\mathrm{H} + R_\mathrm{M}} \times 100$$

where ΣR_H is the sum of the responses for all peaks having retention times less than that of insulin monomer, and R_M is the peak response of the insulin monomer (USP XXVI, 2002).

2.4.2. Reversed-phase HPLC (RP-HPLC)

A reversed-phase C-18 column (VyDAC, 4.6 × 250 mm, The separation Group, CA, USA) was used. The solvent was prepared by dissolving 28.4 g of anhydrous sodium sulfate in 1 l of water and adding 2.7 ml of phosphoric acid into this solution. The acidity of the solvent was adjusted to pH 2.3 with ethanolamine. A filtered and degassed mixture of the solvent and acetonitrile was used as a solution A (82:18) and solution B (50:50). The column temperature was maintained at 40 °C, and the flow rate was 1 ml/min. The elution peaks were detected at 214 nm.

Twenty microliters of the sample were applied to the column and the chromatography was performed as follows; initially, isocratic elution for about 36 min with a mobile phase consisting of a mixture of 78% solution A and 22% solution B. For gradient elution phase of 25 min, mobile phase was changed into a mixture of 36% solution A and 64% solution B. Following the gradient elution phase, the system was maintained isocratic elution for 6 min and returned to the initial conditions of 78% solution A and 22% solution B (USP XXVI, 2002). The percentage of insulin, %I, was calculated by the equation:

$$\%\mathrm{I} = \frac{R_\mathrm{I}}{R_\mathrm{S}} \times 100$$

where R_I is the peak response for insulin, and R_S is the sum of the responses for all of the peaks. The percentage of A-21 desamido insulin (%D) was calculated by the equation:

$$\%\mathrm{D} = \frac{R_\mathrm{D}}{R_\mathrm{S}} \times 100$$

where R_D is the peak response for A-21 desamido insulin, and R_S is the sum of the responses for all of the peaks. The percentage of other insulin related compounds (%OIRC) was calculated by the equation:

$$\%\mathrm{OIRC} = 100 - (\%\mathrm{I} + \%\mathrm{D})$$

where %I is the percentage of insulin, and %D is the percentage of A-21 desamido insulin.

2.5. Cytotoxicity

2.5.1. Culture of A549 cells

A549, a human lung cancer cell line, was purchased from KCLB (Korean Cell Line Bank, K10135). Cells were incubated in RPMI 1640 medium containing 10% FBS, 1% penicillin–streptomycin at 37 °C, 5% CO_2.

2.5.2. MTT assay

Cytotoxicity was determined using the MTT assay as an indicator of the viability of the cells. Cells were seeded at 1.5×10^4 cells/200 μl into 96-well culture plates and grown for 24 h. Then the test medium containing insulin microcrystals, commercial crystalline powder and protease inhibitors was added at the indicated concentrations. After completion of 4 h incubation, the test medium was eliminated, 200 μl of 0.5 mg/ml MTT stock in phosphate-buffered saline was added to each well, and the plate was incubated for 4 h. The solution was then discarded, 200 μl of 0.05N HCl in isopropanol was added to each well. Absorbance was measured at a wavelength of 570 nm and reference wavelength of 630 nm was used for the correction of a background noise.

2.6. Animal experiment

Male Sprague–Dawley rats (Taconic Anmed, Rockville, MD, USA) weighing 200–300 g were kept under a 12 h day-night rhythm at 20 °C and a relative humidity of 50% for 7 days. Animals were fasted for 12 h prior to an experiment but allowed free access to water.

Insulin microcrystal suspensions with or without protease inhibitors were administered by using a tracheal instillation according to the methods of Enna and Schanker (1972) and Brain et al. (1976) with brief modification. The animals were anesthetized by an intraperitoneal injection of 60 mg/kg ketamine and 20 mg/kg sodium pentobarbital. 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 and the animals were placed on a slanted board (30° from the vertical) hanging from their upper incisors. Then 100 μl of insulin microcrystals suspension was injected into the lung through a needle of a calibrated 250 μl microsyringe (1725LT

250 μl SYR, Hamilton Co., USA). For the injection, the needle was stabbed between the fifth and sixth tracheal rings. With the syringe positioned, the insulin microcrystals suspension was injected over a time of 1–2 s. The incision in the skin was sutured with a sterile 6/0 silk suture and the surgical incisions are swabbed with betadine solution. Then the animals were allowed to recover in clean, separate cages where they remained during the experimental period and were given water *ad libitum*. The blood was sampled from the tail vein of rat.

2.7. Data analysis

To estimate the pulmonary absorption of insulin, the blood glucose concentration was measured with LIFESCAN SureStep^{TM} (Johnson & Johnson Company, Milpitas, CA, USA) at the indicated time intervals. A decrease in the blood glucose concentration (D%) was calculated by a modified method of Hirai et al. (1981) from the following equation:

$$D\% = \left[\frac{1 - AUC_{0\rightarrow 24\,h}}{(100\% \times 24\,h)}\right] \times 100$$

where $AUC_{0\rightarrow 24\,h}$ is the area under the curve for 24 h.

Results were expressed as the mean ± S.D. Statistical significance was determined using the Student's unpaired *t*-test.

3. Results and discussion

3.1. Characterization of insulin microcrystals

3.1.1. Size distribution and morphology of insulin microcrystals

The size distribution and shape of the commercial human insulin crystalline powder and the microcrystals are compared in Fig. 1. The commercial crystalline powder was a large particle with a mean diameter of 10.97 μm and had a broad size distribution (Fig. 1A). By contrast, microcrystals produced in this study were small (mean diameter of 0.95 μm) and had a narrow, monodispersed size distribution (Fig. 1B). The shape of each material was observed using SEM. The commercial crystalline powder was very crude (Fig. 1A), but the microcrystals were monoclinic forms (Fig. 1B). Therefore, the microcrystals may be optimal to control the drug dosage with a narrow, monodispersed size distribution and to obtain an effective pulmonary delivery (1–5 μm) with a small size.

3.1.2. Physicochemical properties of insulin microcrystals

The crystalline structure of insulin microcrystals was measured using XRD. The XRD peaks and ring patterns of the commercial crystalline powder and the microcrystals are shown in Fig. 2. The commercial crystalline powder showed many weak diffraction peaks. The peak of the microcrystals was very similar to that of the commercial crystalline powder, although the intensity of the peaks for the commercial crystalline powder was a little higher than that of the microcrystals (Fig. 2A). The commercial crystalline powder diffraction data yielded a pattern of well-formed concentric rings of variable intensity (Fig. 2B). As shown in Fig. 2C, the rings of microcrystals were similar to those of the commercial crystalline powder at low resolution, but the microcystals had broader ring pattern than the commercial crystalline powder at intermediate resolution. It is known that the diffraction peak and ring pattern are affected by the crystal size and crystallinity of the particles (Gibson, 2001). In the case of the microcrystals, it is likely that the size of microcrystals may affect the reduction of diffraction intensity and the broadening of rings because the insulin microcrystals (0.95 μm) are about 11 times smaller than the commercial crystalline powder (10.97 μm) in the average diameter.

Representative DSC thermogrms of commercial crystalline powder and microcrystals were illustrated in Fig. 3. Both of the commercial crystalline powder and the microcrystals represent

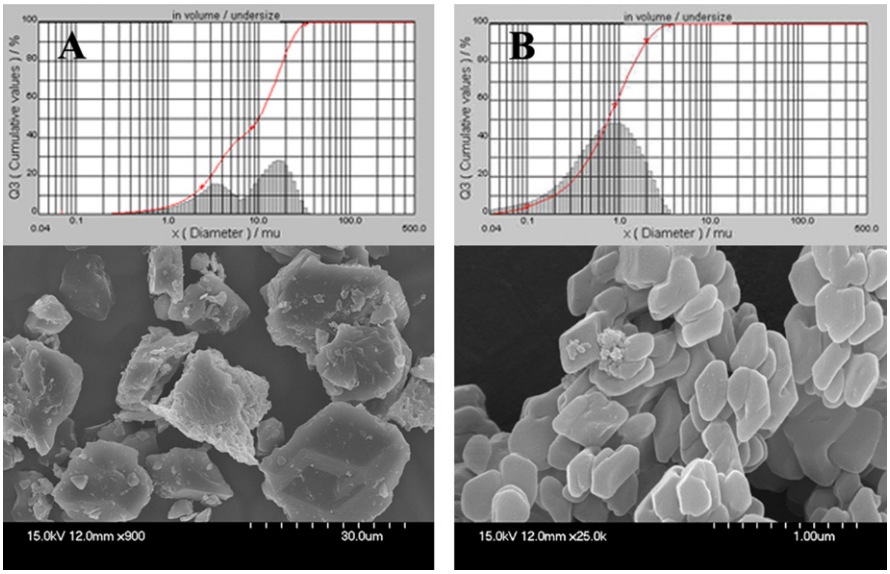

Fig. 1. Size distributions measured by laser diffraction and shapes observed by scanning electron microscope (SEM) of human insulin crystalline powder and microcrystals. (A) Human insulin crystalline powder (mean diameter = 10.97 μm, ×900); (B) microcrystals (mean diameter = 0.95 μm, ×25,000).

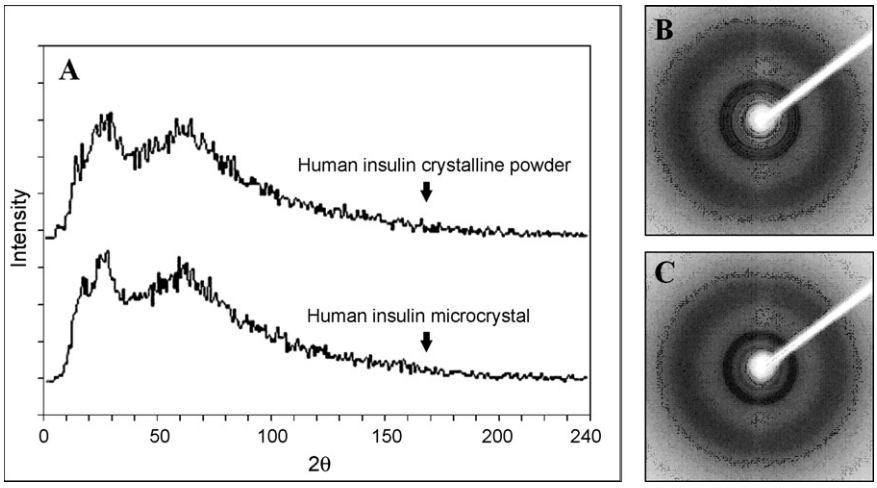

Fig. 2. Crystallinity of human insulin crystalline powder and microcrystals measured by X-ray diffractometry (XRD). XRD intensity peaks (A) and ring patterns of commercial crystalline powder (B) and microcrystals (C) were compared.

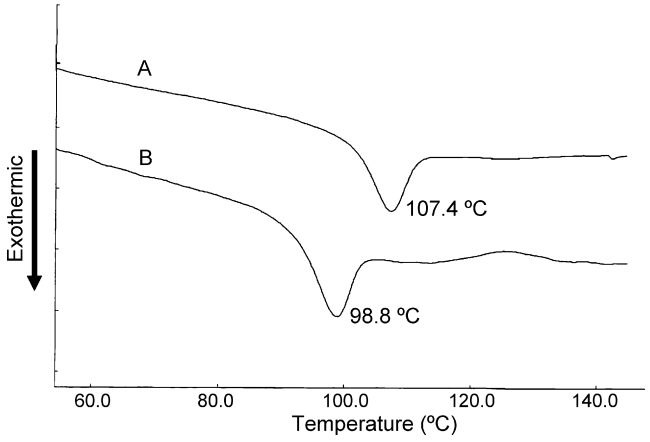

Fig. 3. Thermogram of human insulin crystalline powder and microcrystals measured by differential scanning calorimetry (DSC). The thermograms of human insulin crystalline powder (A) and microcrystals (B) were compared. Indicated value is melting temperature (T_m).

a certain endothermic peak at 107.4 and 98.8 °C, respectively. These endothermic melting peaks confirm the presence of crystallinity in both samples. The melting temperature (T_m) of crystalline powder was 8.6 °C higher than that of microcrystals. This result shows that the microcrystals have a little lower crystallinity compared with the crystalline powder used as a raw material. The previous study suggested that the different particle size between the same materials could promote the transformation of peak (Gibson, 2001). Therefore, it is likely that the small size of microcrystals causes the melting temperature to decrease. In addition, the conditions of particle such as humidity and composition can affect the change of thermogram (Shekunov and York, 2000).

3.1.3. Process related impurities of insulin microcrystals

The process related impurities of insulin produced during the microcrystallization process was investigated by the analytical method of US Pharmacopoeia (USP XXVI, 2002). In the case of HMWP analysis using SE-HPLC, the percentage of insulin monomer (%I) (99.83 ± 0.00%) of the microcrystals produced was almost same as that of the insulin powder (99.69 ± 0.01%) used as a raw material (Table 1). The %HMWP of the microcrystals was 0.17 ± 0.00% which is below the US Pharmacopoeia allowable level of not more than 1.0% (USP XXVI, 2002). Desamido insulin analysis using RP-HPLC indicated that the level of A-21 desamido insulin (%D) of the microcrystals (0.66 ± 0.12%) was almost the same level of the insulin crystalline powder (0.73 ± 0.17%). This value is also very low compared to the US Pharmacopoeia guideline for human insulin (not more than 2.0%). The percentage of OIRC (%OIRC) of the microcrystals (0.17 ± 0.01%) was a little higher than the insulin powder (0.16% ± 0.02%), but it was not significant enough for consideration. The contents of %HMWP,

Table 1
Impurity analysis of human insulin microcrystals

	SE-HPLC		RP-HPLC		
	%I[a]	%HMWP[b]	%I	%D[c]	%OIRC[d]
Insulin powder (raw material)	99.69 ± 0.01	0.31 ± 0.01	99.11 ± 0.17	0.73 ± 0.17	0.16 ± 0.02
Microcrystal	99.83 ± 0.00	0.17 ± 0.00	99.17 ± 0.12	0.66 ± 0.12	0.17 ± 0.01

Each value represents the mean ± S.D. ($n = 3$).
[a] %I: the percentage of insulin.
[b] %HMWP: the percentage of high molecular weight proteins.
[c] %D: the percentage of A-21 desamido insulin.
[d] %OIRC: the percentage of other insulin related compounds.

%D, and %OIRC results demonstrated that there was almost no degradation of inslin during the microcrystallization process.

3.2. Cytotoxicity

The cytotoxic effects of the commercial crystalline powder, microcrystals and protease inhibitors on the A549 cells were studied by the MTT assay. We used A549 cell line for toxicity study because it has been widely used in various kinds of studies, and because it has the structural and biochemical characteristics of human type II cells (Schins et al., 2002). The results of the MTT assay, as a measure of metabolic competence of the cells following 4 h treatment with commercial crystalline powder, microcrystals and protease inhibitors, are shown in Fig. 4. Following 4 h treatment with commercial crystalline powder and microcrystals, the cell viability of A549 was over 90%. This result shows that the microcrystals have no toxic factors which may be produced by the microcrystallization process compared with commercial crystalline powder. In the case of protease inhibitors, the cytotoxicity of aprotinin and soybean-trypsin inhibitor was also very low. Significant cytotoxicity, however, was observed at all concentrations of bacitracin tested.

3.3. Animal experiment

Before the experiment, we evaluated the relationship between the dose of insulin microcrystals administered intrapulmonarily and the decrease in the blood glucose concentration (D%), and there was a linear relationship between these two parameters over the range of 1–5 U/kg (data not shown). This suggests that the D% values can be used an index of insulin absorption from the lung. For this experiment, the dose of 5 U/kg was selected. The time course of glucose concentrations in blood after intrapulmonary administration of insulin microcrystals (5 U/kg) with or without various protease inhibitors is shown in Fig. 5 (soybean-tripsin inhibitor) and Fig. 6 (aprotinin), and the values of pharmacodynamic parameters are arranged in Table 2.

In the published studies, the insulin solution (5 U/kg) delivered in rat lungs showed the decrease in blood glucose concentration (D%) of 30% (Yamamoto et al., 1994) and the period of time maintained less than 70% of initial blood glucose was held (Time) for 7 h (Zhang et al., 2001). The 70% level of initial glucose is the threshold of optimal hypoglycemic effect and the 'Time' is the parameter of a long-acting property.

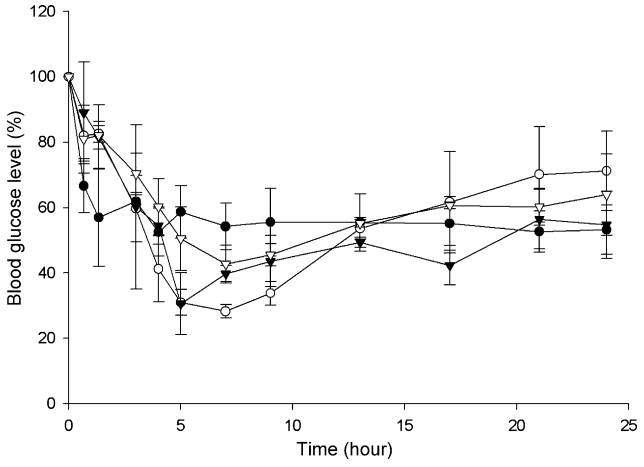

Fig. 5. Hypoglycemic effect after pulmonary delivery of insulin microcrystals (5 U/kg) with soybean-trypsin inhibitor (STI) in pH 6 conditions. (●) 5 U/kg insulin microcrystals; (◯) with 1 mg/ml STI; (▼) with 5 mg/ml STI; (▽) with 10 mg/ml STI. Each value represents the mean ± S.D. (n = 3–6).

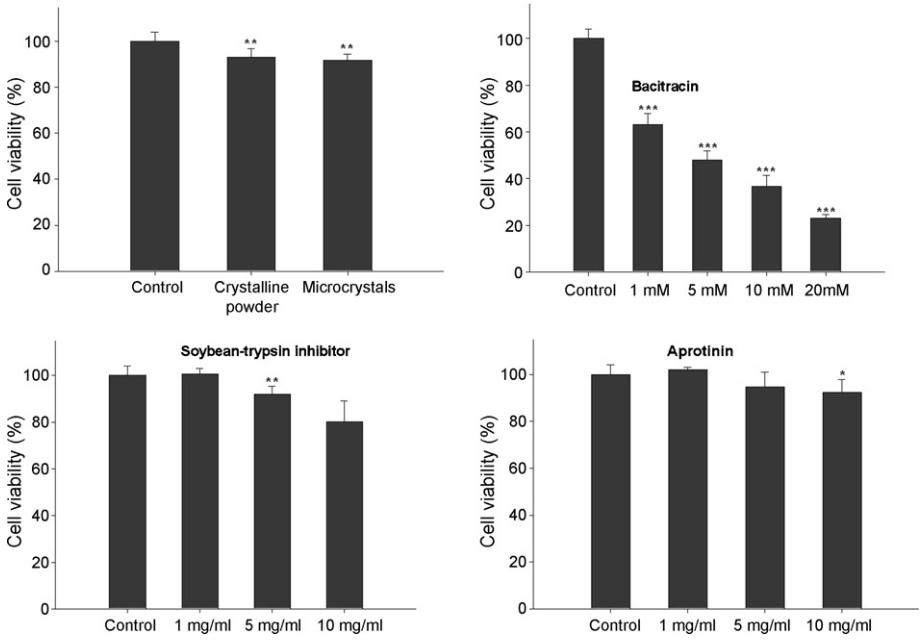

Fig. 4. Cytotoxicity of human insulin crystalline powder, microcrystals and protease inhibitors in A549 cells. The A549 cells were incubated with human insulin crystalline powder (1 mg/ml) or microcrystals (1 mg/ml), or a given concentration of protease inhibitors. After 4 h of incubation, the viable cells were measured by using the MTT assay. The viability of the cells for each treatment and concentration was expressed as a percentage of control values. Each value represents the mean ± S.D. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control.

Table 2
Effect of protease inhibitors on the pulmonary absorption of insulin microcrystals

	Concentration	D%	%MBGC[a]	T%MBGC[b] (h)	Time[c] (h)
Insulin 5 U/kg	–	42.68 ± 1.62	52.46 ± 7.29	4	0.6–24
+Soybean-trypsin inhibitor (mg/ml)	1	51.49 ± 5.27*	28.25 ± 2.05***	7	3–17
	5	55.78 ± 0.71 *	30.59 ± 9.47**	5	3–24
	10	48.86 ± 3.24	42.66 ± 5.78*	7	4–24
+Aprotinin (mg/ml)	1	56.90 ± 3.42***	44.06 ± 4.10*	7	0.6–24
	5	51.77 ± 1.98**	48.07 ± 5.36	5	1.3–24
	10	52.57 ± 8.78	35.84 ± 4.46	1.3	0.6–24

Each value represents the mean ± S.D. (n = 3–6). Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001
[a] %MBGC: the percent of minimum blood glucose concentration.
[b] T%MBGC: the time required to attain %MBGC.
[c] Time: the time during which less than 70% of blood glucose is held.

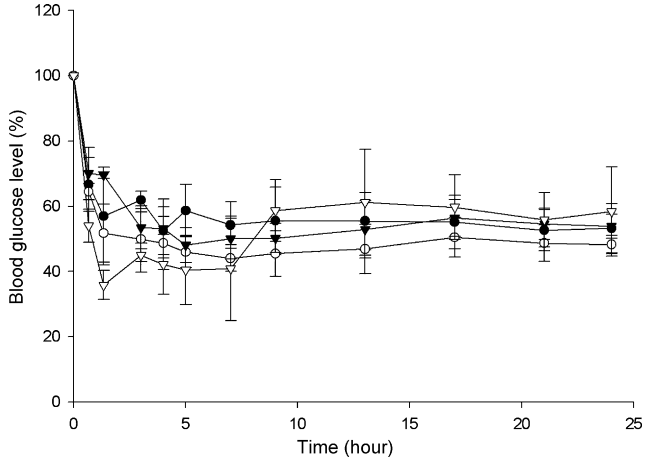

Fig. 6. Hypoglycemic effect after pulmonary delivery of insulin microcrystals (5 U/kg) with aprotinin in pH 6 conditions. (●) 5 U/kg insulin microcrystals; (◯) with 1 mg/ml aprotinin; (▼) with 5 mg/ml aprotinin; (▽) with 10 mg/ml aprotinin. Each value represents the mean ± S.D. (n = 3–6).

In this study, insulin microcrystals had more effective pharmacodynamic property (D% = 42.68 ± 1.62% and Time = 23.4 h) (Table 2, Fig. 7). In the presence of soybean-trypsin inhibitor and aprotinin, the pharmacodynamic property of insulin microcrystals in lungs was improved. The decrease in blood glucose level (D%) and the percent of minimum blood glucose concentration (%MBGC) were 42.7 and 52.5% in the absence of soybean-trypsin inhibitor and aprotinin but in their presence reached 48.9–56.9 and 28.3–48.1%, respectively (Table 2).

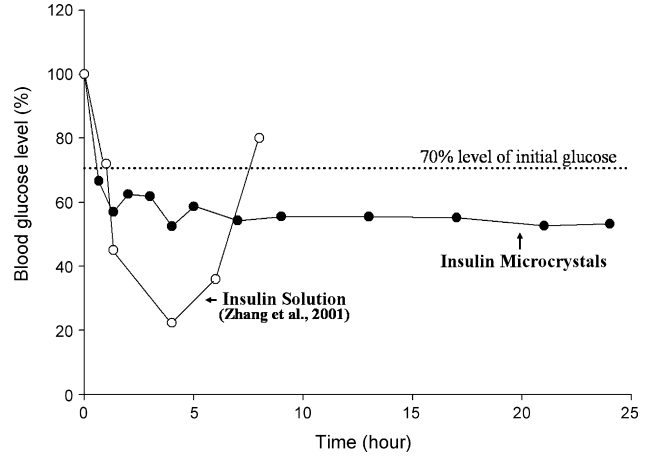

Fig. 7. Blood glucose level administered with insulin microcrystals and solution as a function of time. Insulin microcrystals have a long-acting effect (Time = about 24 h) compared with insulin solution (Time = about 7 h).

The blood glucose level showed a tendency to increase after 14 h by co-administration of soybean-trypsin inhibitor as compared with insulin microcrystals (Fig. 5). The enzyme inhibitors of pancreatic proteases such as soybean-trypsin inhibitor have a toxic potential caused by the inhibition of these enzymes themselves. It is demonstrated that the inhibition of protease stimulates the secretion of proteases by a feed-back regulation and this feed-back regulation rapidly leads to both hypertrophy and hyperplasia of the pancreas (Bernkop-Schnürch, 1998): the excessive secretion of protease and pancreatic disorder affect on the activity of insulin delivered and original insulin-releasing ability of pancreas. As shown in result, therefore, the prolonged hypoglycemic effect by insulin microcrystals was not maintained to 24 h, and it is considered that aprotinin is proper to absorption and long-acting profiles of insulin microcrystals.

The time–glucose concentration profile data of bacitracin is not shown. Because, in *in vivo* study, the intratracheal instillation of bacitracin caused the death of all experimental animals by hypoglycemia in 2 h or difficulty in breathing. It may be considered that high cytotoxicity of bacitracin revealed in MTT assay inhibited the lung function or enhanced an excessive insulin absorption by damage of lung epithelial layer.

4. Conclusion

From the above-mentioned results, the human insulin microcrystals prepared had optimal uniformity, morphological and physicochemical properties for pulmonary delivery. Furthermore, the generation of product-related impurities during the microcrystallization process such as desamido insulins or aggregated products and the cytotoxicity were not significant. In the intratracheal instillation, insulin microcrystals significantly reduced the blood glucose level and maintained the hypoglycemic effect compared with other dosage forms. In addition, protease inhibitors improved the absorption profiles of insulin microcrystals effectively. The side effects of protease inhibitors, however, should be considered significantly. In conclusion, these

results show that the co-administration of insulin microcrystals and appropriate protease inhibitors is a suitable therapeutic form of pulmonary delivery.

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