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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),