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Original article

Modulation of insulin transport by D-glucose in alveolar epithelial cells

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ABSTRACT

D-glucose concentration in alveolar lining fluid is maintained much lower than that in the plasma, but is elevated by hyperglycemia. In the present study, we examined insulin uptake in A549 human alveolar epithelial cells; especially focusing on the effect of D-glucose on the uptake. Time- and temperature-dependence were observed in FITC-insulin uptake in A549 cells in the presence of D-glucose. Confocal laser scanning microscopic analysis showed the punctate localization of FITC-insulin in the cells. These results indicated that FITC-insulin was taken up by the cells through an endocytotic pathway. FITC-insulin uptake was increased 2–4 fold by extracellular D-glucose, but glucose analogs such as 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose did not show any enhancement effect. Moreover, FITC-albumin and FD-4 uptake were not affected by D-glucose. Intracellular ATP amount was not affected by D-glucose during the incubation. Phloretin, a facilitative glucose transporter inhibitor, significantly inhibited D-glucose uptake as well as D-glucose-stimulated FITC-insulin uptake. Taken together, D-glucose may regulate the insulin uptake after being taken up by A549 cells, through the mechanism that is not directly related to ATP production. The effect of D-glucose on insulin absorption was also examined in *in-vivo* pulmonary administration study in rats, and co-administration of D-glucose potentiated the hypoglycemic action of insulin. These findings suggest that endocytosis is involved in insulin uptake, and D-glucose specifically enhances the endocytosis of insulin in alveolar epithelial cells. D-glucose concentration in alveolar lining fluid may play an important role in the absorption of insulin from the lung.

1. Introduction

Frequent injections of insulin for the control of blood glucose concentration remain a significant problem for a large number of patients under insulin therapy. In order to overcome this problem, inhaled insulin has been widely investigated. It is important to understand the transport mechanisms of proteins and peptides in alveolar epithelial cells, for the development of efficient pulmonary delivery systems for protein and peptide drugs.

The alveolar surface is covered by a thin layer of fluid, which contains various ions and proteins including surfactant protein [1,2]. D-glucose also occurs in the alveolar lining fluid. It is reported that glucose concentration in alveolar lining fluid is usually maintained lower than that in the plasma in both human and animals [3,4]. For example, Saumon et al. [3] reported that D-glucose concentration in the epithelial lining fluid would be less than 5% of the plasma D-glucose concentration using fluid-filled isolated rat lung. More recently, Baker et al. [4] reported that D-glucose concentration in the fluid from lower respiratory tract was 12.5 times lower than that in the plasma, by measuring D-glucose concentration in the exhaled breath condensate in human.

On one hand, D-glucose concentration in alveolar lining fluid is reported to be elevated by hyperglycemia [4]. The lung is continually exposed to various pathogens and bacterium, and therefore low D-glucose concentration in alveolar lining fluid could contribute to pulmonary defense against infection. In fact, diabetic patients with hyperglycemia, whose alveolar D-glucose concentration is higher than normal subjects, are more likely to suffer from pneumonia [5,6].

Recently, several clinical studies have confirmed the efficacy and safety of inhaled insulin for the treatment of diabetes mellitus. The pharmacokinetics and pharmacodynamics of inhaled insulin were widely studied in healthy volunteers and in patients with type 1 and type 2 diabetes [7,8]. Several reports showed that bioavailability of inhaled insulin tended to be higher in diabetic patients than in healthy volunteers [9,10]. Based on these findings, we have hypothesized that the absorption of inhaled insulin from the lung

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is possibly regulated by D-glucose concentration in alveolar lining fluid.

The alveolar region of the lung is composed of type I and type II epithelial cells. We previously suggested that type II cells play an important role in the transport of proteins in alveoli, using primary cultured alveolar type II and type I-like epithelial cells [11,12]. In this study, we examined insulin uptake in A549 human alveolar epithelial cells, an *in-vitro* model of pulmonary epithelium having similar characteristics with alveolar type II epithelial cells [13], especially focusing on the effect of D-glucose on the uptake. Furthermore, the effect of co-administration of D-glucose with insulin was evaluated in rats under *in-vivo* conditions.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from MP Biomedicals (Solon, OH, USA). Trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Fluorescein isothiocyanate-labeled insulin from bovine pancreas (FITC-insulin), FITC-albumin from bovine serum, FITC-dextran (FD-4, 4 kDa), and phloretin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fructose, galactose, and D-glucose were purchased from Nacalai Tesque (Kyoto, Japan). 2-Deoxy-D-glucose (2DOG) was purchased from Kanto Chemical Co. (Tokyo, Japan), sodium azide (NaN_3) from Katayama Chem. (Tokyo, Japan), and 3-*O*-methyl-D-glucose (3OMG) from Wako Pure Chemical (Osaka, Japan). Hoechst 33342 solution as a fluorescent nucleus marker was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used for the experiments were of the highest purity commercially available.

2.2. Cell culture

A549 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were cultured as described previously [14,15], and were used for the experiments on the sixth day after seeding between passages 96 and 116.

2.3. Animals

Male Wistar rats (7 weeks old) weighing approximately 200 g were fasted overnight with free access to water, and then anaesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection before experiments. Experiments with animals were performed in accordance with the Guideline for the Committee on Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

2.4. Uptake of FITC-labeled compound in A549 cells

Uptake experiments were performed as described previously [14,15]. A549 cells grown on 12-well plates were used. After removal of the culture medium, each well was washed and preincubated with phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4) (PBS buffer) supplemented with 0-25 mM D-glucose at 37 °C or 4 °C for 10 min. Then, PBS buffer supplemented with each concentration of D-glucose containing FITC-insulin (100 μg/mL), FITC-albumin (50 μg/mL), or FD-4 (2000 μg/mL) was added to each well, and the cells were incubated at 37 °C or 4 °C for a specified period. In order to investigate the effect of other glucose analogs and other monosaccharides, A549 cells were preincubated with PBS buffer containing each concentration of 3OMG, 2DOG, fructose, or galactose at 37 °C or 4 °C for 10 min, and FITC-insulin uptake experiments were performed in the presence of each concentration of 3OMG, 2DOG, fructose, or galactose at 37 °C or 4 °C for 60 min.

For inhibition studies, A549 cells were preincubated with PBS buffer at 37 °C or 4 °C. Then, the cells were incubated with the PBS buffer containing FITC-insulin and each concentration of D-glucose with or without (control) a facilitative glucose transporter (GLUT) inhibitor, phloretin (200 μM), at 37 °C or 4 °C for 60 min.

At the end of the incubation, the uptake buffer was aspirated and the cells were washed with ice-cold PBS buffer. The scraped and collected cells were washed by centrifugation. After the supernatant was aspirated, the pellet was solubilized by 0.1% Triton X-100 in PBS buffer without $CaCl_2$ and $MgCl_2$, and centrifuged. The fluorescence of FITC-insulin in the supernatant was measured using a Hitachi fluorescence spectrophotometer F-2700 (Tokyo, Japan) at excitation and emission wavelengths of 500 and 520 nm, respectively. Protein contents in the supernatant were determined by the Lowry method with bovine serum albumin as the standard.

2.5. Confocal laser scanning microscopy

A549 cells were grown on 35-mm glass bottom culture dishes for 5 days. The cells were incubated with FITC-insulin (100 μg/mL) and Hoechst 33342 (10 μM) for 30 min at 37 °C. After washing the cells with ice-cold PBS buffer three times for 5 min each, florescence in the cells was visualized by confocal laser scanning microscopy (LSM5 Pascal, Carl ZEISS, Germany).

2.6. Detection of intracellular ATP amount

A549 cells grown on 96-well plates were washed two times with PBS buffer, and then incubated with various concentrations of D-glucose, 3OMG, 2DOG or fructose in the absence or presence of 100 μg/mL insulin for 60 min at 37 °C. At the end of the incubation, cells were washed three times with PBS buffer at room temperature followed by the incubation with the "Cell ATP Assay Reagent" comprising luciferin for 20 min. Luciferase activities were measured in GloMax™ 96 microplate luminometer at room temperature.

2.7. Pulmonary administration

Pulmonary administration was performed as reported previously [14,16]. After being anesthetized with pentobarbital, the trachea was exposed and incised transversely between the fourth and fifth tracheal rings. Cannulation (2.5 cm long polyethylene tubing, O.D. 3 mm) was made through the tracheal incision. Then, 75 μL of prepared drug solution containing 19.25 μg/rat (0.5 IU/rat) insulin with or without 330 μg/rat D-glucose was injected into the lung over a period of 1–2 s through the tracheal cannula using a 100 μL syringe. Because a depth of 2.5 cm below the incision is suitable, polyethylene tubing (PE-50) was attached to the needle of the syringe in order to insert the tubing at the precise position. After administration of the drug solution, blood samples were taken from the jugular vein for a specified period. Plasma was separated immediately by centrifugation at 10,000 rpm for 5 min, and glucose concentration in plasma was determined by using Glucose CII test Wako (Japan).

2.8. Statistical analysis

All data were expressed as the means ± standard error of mean (SE). Statistical analysis was performed by the Student's *t*-test or one-way ANOVA, followed by the Tukey-Kramer's test for multiple comparisons. The level of significance was set at $^{*}P < 0.05$ or $^{**}P < 0.01$.