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Enhancement Effect of Poly(amino acid)s on Insulin Uptake in Alveolar Epithelial Cells

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Summary: In this study, we elucidated the effect of poly(amino acid)s such as poly-L-ornithine (PLO) on FITC-insulin uptake in cultured alveolar type II epithelial cells, RLE-6TN. FITC-insulin uptake by RLE-6TN cells as well as its cell surface binding was markedly increased by PLO without cytotoxicity. The uptake of FITC-insulin in the presence of PLO was shown to be mediated by endocytosis, but in contrast to the uptake in the absence of PLO, the contribution of macropinocytosis emerged. Colocalization of FITC-insulin and LysoTracker Red was observed by confocal laser scanning microscopy both in the absence and presence of PLO, indicating that FITC-insulin was partly targeted to lysosomes in the cells and degraded. The half-life of the intracellular degradation of FITC-insulin was, however, prolonged by the presence of PLO. PLO also stimulated the uptake of other FITC-labeled compounds. Among them, the enhancement effects of PLO on FITC-albumin and FITC-insulin uptake were prominent. The effect of PLO on insulin absorption was also examined in in-vivo pulmonary administration in rats, and co-administration of PLO enhanced the hypoglycemic action of insulin. These findings suggest that co-administration of poly(amino acid)s such as PLO is a useful strategy for enhancing insulin uptake by alveolar epithelial cells and subsequent absorption from the lung.

Keywords: alveolar epithelial cells; RLE-6TN; insulin; poly-L-ornithine; endocytosis; macropinocytosis

Introduction

The majority of protein and peptide drugs were developed as injection formulations, and have a variety of problems, especially for patients, in terms of safety, pain, and needle phobia. Instead, the lung has attracted a great deal of interest as an alternative administration route for protein and peptide drugs due to its large surface area (approximately 100 m²), extremely thin epithelial barrier and extensive blood supply. The inhalation pulmonary delivery system, which may enable the systemic absorption of protein and peptide drugs due to its large surface area, has been extensively studied. In particular, clinical research on inhaled insulin has confirmed the efficacy and safety of inhaled insulin in the treatment of diabetes mellitus. In addition, the bioavailability of inhaled insulin via the lung is greater compared to other non-invasive routes such as the gut and nose. However, information concerning the handling of insulin in alveolar epithelial cells is still lacking.

On one hand, the bioavailability of inhaled insulin is still not very high (about 10–15%), and therefore it is important to search for absorption-enhancing strategies. There are many kinds of so-called absorption enhancers having different modes of action. So far, various studies have been done to improve the absorption of proteins and peptides, especially insulin. The methods employed include incubation with a tight junction modulator which leads to an increase in paracellular transport, prevention of the degradation by co-administration with a peptidase/protease inhibitor, and conjugation of peptide to transferrin to deliver the conjugate via the transferrin receptor-mediated endocytosis pathway. However, these techniques have not been used in clinical pharmacotherapy yet. Recently, several researchers have used cationic peptides for enhancing

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the uptake of high-molecular weight compounds into the cells.\textsuperscript{16-18} Many mechanisms have been proposed concerning their membrane-permeation enhancing activities, but structural differences of cationic peptides often cause considerable differences in their internalization mechanisms and cellular localization. In addition, when the cationic peptide was conjugated with a high-molecular weight compound, the decrease in the bioactivity of the conjugate was often observed. Poly-L-ornithine (PLO) and poly-L-lysine (PLL) are cationic poly(amino acid)s having positive charges at physiological pH. In addition, covalent binding is not needed to enhance the plasmid DNA delivery into the cells.\textsuperscript{19} However, it has not been examined whether or not these poly(amino acid)s are useful to enhance protein/peptide transport into the alveolar epithelial cells.

Clearance mechanisms of various peptides and proteins from the alveolar space have been widely studied, and among the mechanisms proposed, transcytosis seems to be the primary mechanism of protein/peptide absorption across the alveolar epithelial cells.\textsuperscript{20,21} In addition, we recently reported that insulin was taken up through endocytosis in RLE-6TN, which is a well characterized model of alveolar type II epithelial cells,\textsuperscript{22} and an insulin receptor might be involved partly in insulin endocytosis in the cells.\textsuperscript{23}

The alveolar region of the lung is composed of two major types of epithelial cells. One is type I cells, which have a squamous morphology, and the other is type II cells, which are cuboidal epithelial cells.\textsuperscript{20} We previously suggested that type II cells play an important role in the transport of proteins in alveoli.\textsuperscript{24} Therefore, we have been using cultured alveolar type II epithelial cells to characterize the endocytosis function.

In this study, we investigated the effects of poly(amino acid)s such as PLO on insulin endocytosis, and the mechanism(s) of enhancement effect on insulin uptake in RLE-6TN cells. Furthermore, the effect of PLO on insulin absorption from the lung was examined in rats under in vivo conditions.

Materials and Methods

Materials: Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F-12), trypsin-EDTA, and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Fluorescein isothiocyanate-labeled insulin from bovine pancreas (FITC-insulin), FITC-albumin from bovine serum, FITC-IgG from human serum, FITC-transferrin from bovine, poly-L-ornithine (PLO, average MW = 23,500), poly-L-lysine (PLL, average MW = 26,900), phenylarsine oxide (PAO), indomethacin (IND), nystatin (NYS), 5-(N-ethyl-N-isopropyl) amiloride (EIPA), cytochalasin D (Cyto D) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,4-Dinitrophenol (DNP) and sodium deoxycholate (SD) were purchased from Nacalai Tesque (Kyoto, Japan). 2-Deoxy-o-glucose (2DOG) was purchased from Kanto Chemical Co. (Tokyo, Japan) and sodium azide (NaN\textsubscript{3}) from Katayama Chem. (Tokyo, Japan). LDH-Cytoxic Test Wako and Glucose C2 Test Wako were purchased from Wako Pure Chemical (Osaka, Japan). LysoTracker\textsuperscript{R} Red DND-99 (LysoTracker Red), a fluorescent lysosomal marker, was purchased from Molecular Probes (Eugene, OR, USA). Hoechst 33342 solution, 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.

Cell culture: RLE-6TN cells were obtained from the American Type Culture Collection (ATCC no. CRL-2300; Manassas, VA, USA) and were cultured as described previously.\textsuperscript{24,25} The cells were used for the experiments on the seventh day after seeding between passages 50 and 64.

Animals: Male Wistar rats (seven weeks old) weighing approximately 250 g were fasted overnight with free access to water, then anesthetized 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.

Uptake of FITC-labeled compound by RLE-6TN cells: Uptake experiments were performed as described previously.\textsuperscript{24,25} Briefly, RLE-6TN cells grown on 35-mm culture dishes were used. After removal of the culture medium, each dish was washed and preincubated with phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, and 0.5 mM MgCl\textsubscript{2}, pH 7.4) (PBS buffer) supplemented with 5 mM D-glucose (PBS-G buffer) at 37°C for 10 min. Then, PBS-G buffer containing FITC-insulin (20 µg/ml), FITC-albumin (20 µg/ml), FITC-IgG (200 µg/ml), FITC-transferrin (400 µg/ml), or FITC-dextran (4 kDa; 2,000 µg/ml) was added to each dish in the presence or absence (control) of various concentrations of PLO and PLL, and the cells were incubated at 37°C or 4°C for a specified period.

For inhibition studies, RLE-6TN cells were preincubated with PBS or PBS-G buffer at 37°C or 4°C with or without the inhibitors as follows: 1 mM DNP and 10 mM Na\textsubscript{3}I plus 5 mM 2DOG for 10 min in PBS buffer and 300 µM IND for 10 min in PBS-G buffer, 50 µM NYS and 5 µM PAO for 10 min, 50 µM EIPA and 2 µM PMA for 30 min in PBS-G buffer containing 0.5% DMSO. The same vehicles were used for each control experiment. Then, the cells were incubated with 1 ml of the buffer containing FITC-insulin with or without (control) the inhibitors at 37°C or 4°C for a specified period in the presence of PLO (10 µg/ml). PAO was used only in the preincubation buffer and was not added.
to the uptake buffer.

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 CaCl₂ and MgCl₂, and centrifuged. The fluorescence of FITC-insulin in the supernatant was measured using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at excitation and emission wavelengths of 500 and 520 nm, respectively. Protein content in the supernatant was determined by the Lowry method with bovine serum albumin as the standard.

Confocal laser scanning microscopy: RLE-6TN cells were grown on 35-mm glass bottom culture dishes for 5 days. The cells were incubated with FITC-insulin (20 µg/ml), LysoTracker Red (75 nM) and Hoechst 33342 (10 µM) in the presence or absence of PLO (5 µg/ml) for 30 min at 37°C as described above, and after washing the cells with ice-cold PBS buffer three times for 5 min each, fluorescence in the cells was visualized by confocal laser scanning microscopy (LSM5 Pascal, Carl ZEISS, Germany).

Pulse-chase analysis of intact FITC-insulin in RLE-6TN cells: To evaluate the intactness of FITC-insulin in RLE-6TN cells, the cells were incubated with FITC-insulin in the presence of PLO (10 µg/ml) for 60 min, and after washing, the cells were further incubated with PBS-G buffer without FITC-insulin for 30, 60, or 90 min. These cell samples were solubilized in a loading buffer consisting of 2% SDS, 50 mM Tris-HCl, and 10% glycerol. Then, the sample was subjected to SDS-PAGE with 15% polyacrylamide gel. After SDS-PAGE, the fluorescence intensity of the gel was analyzed by fluoroimage analyzer FLA-2000 (Fuji Photo Film, Tokyo, Japan). The elimination half-life of intact FITC-insulin in the cells was calculated from the semilogarithmic plot using the equation for first-order elimination kinetics: log X = log X(0) − 0.3t/t½, where X is the amount (fluorescence intensity) of intact FITC-insulin, X(0) is the initial amount of FITC-insulin, t is the incubation time, and t½ is the elimination half-life of FITC-insulin.

Cytotoxic assay: After removal of cell culture medium and preincubation with PBS-G buffer for 10 min, RLE-6TN cells were incubated with PBS-G buffer or serum-free culture medium (negative control), PLO (10, 50 µg/ml), or sodium deoxycholate (SD; 10 mM) for 1 h, 24 h, or 1 h/day for 3 days at 37°C. At the end of each treatment, lactate dehydrogenase (LDH) activity released into the incubation buffer (culture medium) was measured with LDH-Cytotoxic Test Wako (Japan). Total LDH activity (the activity in the incubation buffer and the cells) was measured after solubilizing the cells with Tween 20 (0.2%) for 1 h at 37°C. Cell injury rate (%) was calculated by dividing the LDH activity in the incubation buffer by the total LDH activity.

Pulmonary administration: Pulmonary administration was performed as reported previously.26 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 III/rat) insulin with or without 9.625 µg/rat PLO (insulin:PLO = 2:1) 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 C2 test Wako (Japan).

Statistical analysis: Data are expressed as means ± S.E. Statistical analysis was performed by Student’s t-test or one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. The level of significance was set at *p < 0.05 or **p < 0.01.

Results

Effect of various concentrations of poly(amino acids) on FITC-insulin uptake: The effects of two positively charged poly(amino acids), PLO and PLL, on FITC-insulin uptake by RLE-6TN cells were examined. Figure 1 shows the effects of various concentrations of PLO and PLL on the uptake. The uptake of FITC-insulin was increased by PLO and PLL, and maximum effects were observed at 10 µg/ml (PLO) and 7.5 µg/ml (PLL). At higher concentrations, the stimulation effects of these poly(amino acids) decreased, and at 50 µg/ml, no significant stimulation of FITC-insulin uptake was observed.

Characteristics of FITC-insulin uptake in the presence of PLO: To compare FITC-insulin uptake in the presence or absence of PLO, time- and temperature-dependence of FITC-insulin uptake was examined (Fig. 2). FITC-insulin uptake at 37°C was much higher than at 4°C, and the uptake increased with time for up to 60 min regardless of the presence of PLO. The uptake of FITC-insulin both at 37°C (cell association) and at 4°C (cell surface binding) were higher in the presence of PLO than in the absence of PLO. In the following inhibition experiments, the amount of FITC-insulin taken up into the cells (specific uptake) was estimated by subtracting the cell surface binding at 4°C from the total cell association at 37°C.

Relationship between cell surface binding and uptake of FITC-insulin in the presence of PLO: The uptake of FITC-insulin at 37°C and at 4°C was examined in the presence of various concentrations of PLO. As shown in Figure 3A, specific uptake of FITC-insulin (37°C – 4°C) and surface binding (4°C) increased as the concentration of PLO increased up to 10 µg/ml. In addition, there was a good correlation (R² = 0.89) between FITC-insulin binding...
and specific uptake (expressed as the ratio normalized with the value in the absence of PLO) estimated in the presence of various concentrations of PLO (Fig. 3B). Similar data were obtained in the case of PLL (data not shown).

**Endocytic pathway of FITC-insulin uptake in the presence of PLO:** The effects of various endocytosis inhibitors on FITC-insulin uptake in the presence of PLO were examined. As shown in Figure 4, in the presence of PLO, treatment of RLE-6TN cells with NaN₃ plus 2-DOG and DNP, metabolic inhibitors, inhibited the specific uptake of FITC-insulin. PAO, a clathrin-mediated endocytosis inhibitor, also inhibited the specific uptake of FITC-insulin. NYS and IND, caveolae mediated endocytosis inhibitors, did not show inhibitory effects. These characteristics of FITC-insulin uptake in RLE-6TN cells were essentially similar to those observed in the absence of PLO.²³ On the other hand, EIPA, a macropinocytosis inhibitor, inhibited FITC-insulin specific uptake in the presence of PLO, while it showed no inhibitory effect in the absence of PLO.²³ In addition, treatment of PMA, which is known to activate macropinocytosis, increased FITC-insulin specific uptake by about 75% (Fig. 4). These results indicate that FITC-insulin is taken up by macropinocytosis, at least in part, in the presence of PLO.

**Intracellular localization of FITC-insulin taken up by RLE-6TN cells:** Intracellular localization of FITC-insulin was examined by confocal laser scanning microscopy. When RLE-6TN cells were incubated with FITC-insulin at 37°C, punctate localization of fluorescence was observed both in the absence and presence of PLO (Figs. 5A...
On the other hand, when the cells were incubated with FITC-insulin at 4°C, intracellular localization of FITC-insulin was not observed (data not shown). Figures 5B and 5E showed the fluorescence of LysoTracker Red, a lysosomal marker, simultaneously added to the uptake buffer. As shown in Figures 5C and 5F, colocalization of FITC-insulin and LysoTracker Red was observed both in the absence and presence of PLO, indicating that part of FITC-insulin taken up by the cells was targeted to lysosomes.

Degradation/elimination of intracellular intact FITC-insulin in RLE-6TN cells: The intactness of FITC-insulin in the cells was evaluated by a fluorolmage analyzer after SDS-PAGE. Figure 6A shows the result of pulse-chase analysis, and FITC-insulin taken up by RLE-6TN cells gradually degraded/were eliminated over time in the presence of PLO. Using semilogarithmic plots (Fig. 6B), the half-life of FITC-insulin in the cells in the presence of PLO was calculated to be 143 min. We previously reported that the half-life of FITC-insulin in the cells in the absence of PLO was 77 min. Therefore, the half-life of FITC-insulin in the presence of PLO was about two times as long as that in the absence of PLO.

Effect of PLO on the uptake of various FITC-labeled compounds by RLE-6TN cells: We further investigated the effect of PLO on the uptake of FITC-albumin, FITC-IgG, FITC-transferrin, and FITC-dextran by RLE-6TN cells (Fig. 7). Like FITC-insulin uptake, the uptake of FITC-albumin was dramatically stimulated by PLO. Uptake of other FITC-labeled compounds was also stimulated by PLO, but the enhancement effects were quite small. Therefore, the enhancement effect of PLO would depend on the ligand.

Cytotoxicity of PLO in RLE-6TN cells: In order to investigate the cytotoxicity of PLO, LDH leakage into the incubation buffer was measured. RLE-6TN cells were treated with PLO (10, 50 µg/ml) or SD (10 mM) for 1 h (Fig. 8A), 24 h (Fig. 8B), or 1 h/day for 3 days (Fig. 8C) at 37°C. PLO at the concentration of 10 µg/ml, which showed maximum effect on FITC-insulin uptake (Fig. 1A), showed very low or no cytotoxicity under these treatment conditions. On the other hand, PLO at the concentration of 50 µg/ml (the highest concentration examined in Fig. 1A) showed 8 to 21% cell injury rates, but was still much lower than those induced by 10 mM SD. Because most of the cells were detached even after 1 h incubation with SD, the cytotoxicity of SD after repeated treatment could not be estimated.

In vivo pulmonary administration of insulin: The effect of co-administration of PLO on insulin absorption from the lung was examined in rats in vivo. A solution containing insulin with or without PLO was administered into the lung, and the plasma glucose concentration was measured over time. The weight ratio of insulin:PLO for the administration was set at 2:1, since this ratio enhanced the uptake of insulin most effectively under in vitro conditions (Fig. 1). Plasma glucose concentration decreased after pulmonary administration of insulin alone (73.0% of the
initial value at 1.5 h. Co-administration of PLO with insulin further decreased plasma glucose concentration compared with insulin alone (50.5% of the initial value after 1.5 h) (Fig. 9). The hypoglycemic effect was calculated by using the trapezoidal method, and the calculated area above the time-concentration curve (AAC) was 53.8% h (insulin alone) and 102.8% h (with PLO). The enhancement ratio was 1.9-fold.

Discussion

In the present study, we examined the enhancement effect of PLO and PLL on FITC-insulin uptake in RLE-6TN cells.
PLO and PLL, which have positive charges at physiological pH, markedly stimulated FITC-insulin uptake; maximum enhancement ratios were 5.7-fold and 4.0-fold in the presence of 10 µg/ml PLO and 7.5 µg/ml PLL (Fig. 1). Interestingly, 30 and 50 µg/ml poly(olinoo acid)s suppressed the FITC-insulin uptake compared with 10 µg/ml poly(olinoo acid)s. Thus, there was an optimum FITC-insulin:poly(olinoo acid)s ratio for the enhancement of FITC-insulin uptake. The precise reason why such an optimum ratio was observed was not clear, but when the concentration of PLO increased, the number of FITC-insulin molecule(s) bound to one PLO molecule would decrease. Therefore, if there is an upper limit (or saturation) for the rate of FITC-insulin-PLO complex uptake by the cells, then the uptake of FITC-insulin may decrease.

The stimulation effect of PLO on FITC-insulin uptake was somewhat stronger than that of PLL. PLO and PLL have comparable particle sizes and zeta potentials, and their monomers lysine and ornithine differ only by an additional –CH₂– in the side-chain of lysine. It was suggested that this additional –CH₂– in the side-chain of lysine affects the conformation of these poly(olinoo acid)s and their interaction with plasmid DNA. Therefore, though the precise mechanism is not known, the difference in FITC-insulin uptake enhancement activity between these two poly(olinoo acid)s may be due to their conformational differences.

Temperature dependence was observed in FITC-insulin uptake in the presence of PLO (Fig. 2). The cell surface binding of FITC-insulin observed at 4°C increased as the concentration of PLO increased (Fig. 3A), and a good correlation (R² = 0.89) was observed between FITC-insulin binding and specific uptake in the presence of various concentrations of PLO (Fig. 3B). Therefore, the increase in FITC-insulin binding would be involved in the increase in the specific uptake by PLO.

Equilibrium dialysis showed that FITC-insulin could bind to PLO (data not shown), most likely due to the electrical interaction. Assuming that the FITC-insulin-PLO complex has a net positive charge, the complex would bind to negatively charged cell surfaces containing proteoglycan and sialic acid. Several reports indicated that cationic peptides bound to heparan sulfate proteoglycan (HSPG). In this study, the increased FITC-insulin binding at 4°C in the presence of PLO may be due to the interaction of the positively charged FITC-insulin-PLO complex with proteoglycans such as HSPG or chondroitin sulfate proteoglycan.

There have been several reports that examined the internalization mechanisms of arginine-rich cationic peptides including TAT peptide, penetratin and octaarginine. Among various mechanisms, macropinocytosis is reported to play a major role in endocytic uptake of cationic peptides and their complexes with other molecules. In addition, it was reported that histidylated poly-lysine and plasmid DNA complexes were taken up by HepG2 cells via clathrin-mediated endocytosis and macropinocytosis. In this study, we investigated the uptake mechanisms of FITC-insulin using various endocytosis inhibitors.

NYS and IND were used as inhibitors of caveolae-mediated endocytosis, FITC-insulin uptake by RLE-6TN cells was not affected by these inhibitors (Fig. 4). Therefore, caveolae-mediated endocytosis would not be involved in FITC-insulin uptake in the presence of PLO. On the other hand, FITC-insulin uptake in the presence of PLO was inhibited by PAO, which is a clathrin-mediated endocytosis inhibitor. Therefore, FITC-insulin uptake in the presence of PLO was suggested to be mediated in part by clathrin-mediated endocytosis, like the uptake in the absence of PLO. Furthermore, EIPA, which inhibits Na⁺/H⁺ exchanger in the plasma membrane and inhibits macropinocytosis, inhibited FITC-insulin uptake in the presence of PLO, but not in the absence of PLO. In addition, the protein kinase C activator PMA stimulated FITC-insulin uptake in the presence of PLO (Fig. 4). PMA treatment has been known to stimulate macropinocytosis by activating Ras-related GTP-binding protein, Rac. It has been suggested that oligoarginine and TAT peptide would bind to cell surface HSPG, and activate Rac 1 and macropinocytosis. Taken together, in the presence of PLO, macropinocytosis would be involved in the uptake of FITC-insulin/PLO complex and/or FITC-insulin in RLE-6TN cells.

The intracellular localization of FITC-insulin after being taken up by RLE-6TN cells was evaluated by confocal laser scanning microscopy. Using LysoTracker Red as a lysosomal marker, it was suggested that part of the FITC-insulin was localized in lysosomes either in the absence or presence of PLO (Fig. 5). A fluorescence analysis after SDS-PAGE showed that part of the FITC-insulin in the cells was gradually degraded over time, though the rest remained intact (Fig. 6). As the localization of FITC-insulin in lysosomes was observed by confocal laser scanning microscopy, FITC-insulin was possibly degraded in the lysosomes. On the other hand, the half-life of FITC-insulin in the presence of PLO was about twice as long as that in the absence of PLO. This finding may indicate that FITC-insulin/PLO complex is more resistant to degradation enzymes in lysosomes. Another possibility is the difference in the intracellular localization of FITC-insulin in the absence and presence of PLO. As discussed above, macropinocytosis was suggested to be involved in FITC-insulin uptake in the presence, but not in the absence, of PLO. It is reported that clathrin-coated endosomes eventually fused with lysosomes, while macropinosomes were not targeted for lysosomal degradation. Therefore, the difference in the uptake mechanism and intracellular localization of FITC-insulin may result in the difference in the degradation rate in the absence and presence of PLO.

It was suggested that a cell penetrating peptide such as oligoarginine had a ligand-specific enhancement effect on the intestinal absorption of macromolecules. Morishita et al. reported that an oligoarginine such as L-R6 potentially
stimulated the intestinal absorption of insulin when co-administered, while it did not increase the absorption of other molecules such as interferon-β and dextran. This may be due to the differences in the electrical charges of the molecules; insulin, interferon-β, and dextran are negatively charged, positively charged, and non-charged molecules, respectively. In the present study, we examined the effect of PLO on the uptake of various macromolecules, and found that PLO markedly stimulated FITC-insulin and FITC-albumin uptake, while the enhancement effects on FITC-transferrin, FITC-IgG, and FD-4 uptake were very weak (Fig. 7). FITC-albumin, insulin, and transferrin are negatively charged molecules, FITC-IgG is a positively charged molecule, and FD-4 is a non-charged molecule. Therefore, the extent of the enhancement effect of PLO on the uptake of a macromolecule could not be simply explained by the charged property of the macromolecule.

Application of an absorption enhancer often causes the decrease of TEER and severe plasma membrane damage, which leads to LDH leakage. As shown in Figure 8, however, 10 μg/ml PLO showed very low or no cytotoxicity under various treatment conditions, including repeated treatment. Even at the higher dose of PLO (50 μg/ml), LDH leakage was much lower than those induced by 10 mM sodium deoxycholate, which was often used as a permeation enhancer. These results suggested that PLO enhanced FITC-insulin uptake without cytotoxicity.

Using an in-vivo pulmonary administration method, the effect of co-administration of PLO with insulin was examined in rats. The plasma glucose concentrations were significantly lower when PLO was co-administered with insulin, compared with those after the administration of insulin alone (50.5% versus 73.0% of the initial value at 1.5 h) (Fig. 9). These results indicated that the pulmonary absorption of insulin was enhanced by PLO under in vivo conditions.

Seki et al. examined the effects of sperminated polymers on the pulmonary absorption of insulin in vivo, and showed that co-administration of sperminated pullulan (SP-H) with insulin decreased plasma glucose concentration to 70.5% of baseline level. They suggested that the enhanced absorption of insulin from the lung would be due to the increased paracellular flux induced by sperminated polymers, though the cytotoxicity of sperminated polymers was not known. Patel et al. examined the effect of conjugation with a cationic cell-penetrating peptide on the pulmonary absorption of insulin, and showed that blood glucose level was drastically reduced (about 70% of control at 2 h and about 20% at 5 h) after intratracheal instillation of INS-cr9 (insulin conjugated with cationic cr9 peptide). The permeation enhancing effect in vitro was not observed when unconjugated cr9 was co-administered with insulin. In contrast to the effect of sperminated pullulan described above, they suggested that adsorptive transcytosis appeared to be in part responsible for INS-cr9 absorption. In addition, some reports indicated that cationic poly(AMINO acid)s and their conjugates did not affect transendothelial electrical resistance in corneal endothelium or paracellular flux of 14C-sucrose across MDCK monolayers. Thus, though the precise mechanism underlying the increased insulin absorption from the lungs by PLO is not clear at this moment, co-administration of PLO may enhance the transcellular transport (transcytosis) of insulin after being taken up by endocytosis in alveolar epithelial cells. Unfortunately, the tight junction of RLE-6TN cells was too leaky to evaluate the transcytosis of FITC-insulin quantitatively, for example, by using the cells grown on Transwell filters. These points should be clarified further in future, but PLO co-administration may be useful as a simple (no conjugation is necessary), effective, and fairly safe strategy to enhance pulmonary absorption of insulin.

In conclusion, cationic poly(AMINO acid)s such as PLO stimulated FITC-insulin uptake, having the optimum concentration for the stimulation without undesirable toxic effect. In the presence of PLO, FITC-insulin was taken up via macropinocytosis. In addition to clathrin-mediated endocytosis. Under in vivo conditions, the plasma glucose concentrations were significantly lower when PLO was co-administered with insulin, indicating that the pulmonary absorption of insulin was enhanced by PLO. These results would provide useful information to develop strategies for enhancing insulin uptake by alveolar epithelial cells and subsequent absorption from the lung.

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