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Transport of proteins and peptides across human cultured alveolar A549 cell monolayer

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Abstract

An in vitro cultured monolayer system of alveolar epithelial cells was used as a model to investigate the transport pathway of the peptides and proteins, salmon calcitonin (sCT), insulin (INS), recombinant hirudin (rHAV2), and recombinant human growth hormone (rhGH), in pulmonary epithelium. Human lung adenocarcinoma A549 cells formed continuous monolayers when grown on the polycarbonate filters of Transwell plates. The transport of the peptides and proteins having MW of 3400–22,000 Da was studied under different conditions. The results showed that the apparent permeability coefficients (P_{app}) of these macromolecules across A549 cell monolayers ranged from 2×10^{-6} to 5×10^{-6} cm s^{-1} and exhibited a good inverse correlation with molecular weight. No concentration, direction, or temperature dependence was observed in the permeation of sCT, INS, and rHAV2. While the P_{app} of rhGH in the BA direction (2.25×10^{-6} cm s^{-1}) was less than that in the AB direction at both concentrations (3.20×10^{-6} and 3.29×10^{-6} cm s^{-1}). The P_{app} values of rhGH were concentration and temperature independent in the AB direction. These findings suggest that the hydrophilic peptides and proteins used in this study, sCT, INS, rHAV2, and rhGH, appear to cross the A549 cell monolayers via a paracellular pathway by a passive diffusion mechanism.

Keywords: Pulmonary absorption; Proteins and peptides; A549

1. Introduction

The pulmonary route has been proposed as one of the more promising routes for the systemic delivery of peptides and proteins (Wall, 1995; Robert, 1997; Hoover et al., 1992). Drugs, such as proteins and peptides, which are poorly absorbed through alternate routes, demonstrate an improved bioavailability when administrated through the lungs due to the large absorptive surface area, high rate and volume of blood supply, and highly permeable blood barrier in the alveoli (Patton et al., 1994; Stone et al., 1992; Zhou, 1994). However, peptide delivery through the pulmonary route is still considerably less efficient than through the injectable route due mainly to the protective nature of the pulmonary epithelium. The permeability barrier of the pulmonary airspaces is controlled largely by the epithelial cells, which are joined by tight junctions. Therefore, a detailed study of the barrier properties, including the transport processes and permeability characteristics are crucial for a better understanding of drug absorption in lungs. The emergence of epithelial cell culture techniques makes it possible for in vitro cell culture models to allow the dissection of permeation pathways and elucidation of drug transport across alveoli, and

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therefore, the investigation of in vivo pulmonary drug absorption processes at a molecular level.

Although it would be more desirable to utilize a monolayer system of alveolar type I cells because of their dominant role in the alveolar transport barrier, the lack of a suitable method for isolating and culturing type I cells has prevented the use of this terminally differentiated cell system. Therefore, the type I cells have been widely used to model the alveolar epithelium for drug transport studies because they can differentiate into type I cells in vivo and possess the in vivo morphological and biochemical characteristics of type I cells (Forbes et al., 1999; Dodoo et al., 2000; Kinnard et al., 1994). In addition, the use of cell lines derived from various lung tumors, such as human lung adenocarcinoma A549 cells with similar morphological and biochemical characteristics of epithelial type II cells, has advantages over primary culturing of alveolar epithelial cells because of the ease of cell culture and purity of cell types (Adjei and Carrigan, 1992; Shapiro et al., 1978). Therefore, A549 cells have been used as a model for the elucidation of peptide and protein absorption mechanism (Fujita et al., 1995; Kobayashi et al., 1995). In this study, we reported the in vitro permeability of four hydrophilic peptide and protein drugs with MW from 3400 to 22,000, namely, salmon calcitonin, insulin, recombinant hirudin, and recombinant human growth hormone, across A549 cell monolayers and elucidated the possible transpulmonary absorption mechanisms of these drugs in vivo.

2. Materials and methods

2.1. Materials

The following macromolecules and reagents were used: salmon calcitonin (sCT, MW 3400) from Hengtong Biotech. Co. Ltd., China; insulin (INS, MW 5700) from Xuzhou Biochemical Co. Ltd., China; recombinant hirudin (HAV2, MW 6900) from Peking University College of Life Science, China; recombinant human growth hormone (hGH, MW 22,000) from Jinsai Pharmaceutical Co. Ltd., China; F12 medium from GIBCO; fetal bovine serum (FBS) from TBD Biotechnology Development Center, China; trypsin (1:250) from Sigma; fluorescein isothiocyanate (FITC) from Sigma; ethylenediaminetetraacetic acid (EDTA), potassium chloride (KCl), magnesium sulfate ($MgSO_4 \cdot H_2O$), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$), and D-glucose from Beijing Beihua Fine Chemicals Co. Ltd., China.

2.2. Cell culture

The human lung adenocarcinoma A549 cell line provided by Cell Center in the Chinese Academy of Medical Science was cultured in F12 medium supplemented with 10% FBS and incubated at 37 °C in 5% CO_2–95% air. Medium was replaced every second day. After treating with 0.05% trypsin and 0.02% EDTA, the suspended cells were plated onto 3-μm pore, 1.13 cm^2 tissue-treated, polycarbonate filters (Transwell, Costar) at 0.8×10^6 to 1.0×10^6 cells/cm^2 in 12-well plates on day 0. The cells on filters were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C and F12 nutrient medium was changed every two days after plating. Cell confluency was monitored by transepithelial electrical resistance (TER) measurements using EVOM (World Precision Instrument). The TER value is an indication of the integrity of the alveolar epithelial cell monolayers (Adson et al., 1994). In our study, the TER of the A549 cell monolayers almost reached equilibrium on or after day 5. By day 7, the TER value had generally reached a maximum value of $384 \pm 11\ \Omega\ cm^2$ (mean ± S.E.; $n = 10$). After day 7, the TER value declined steadily. The cells became completely confluent on day 5 of cultivation and were used for transport experiments on day 7. This pattern is consistent with that reported by other researchers (Kobayashi et al., 1995).

2.3. Transport experiment

Transport experiments were carried out using a modified Eagle's balance solute solution consisting of KCl 0.4 g/l, $MgSO_4 \cdot H_2O$ 1.4 g/l, NaCl 6.8 g/l, $NaHCO_3$ 2.2 g/l, $NaH_2PO_4 \cdot H_2O$ 1.4 g/l, and D-glucose 1.0 g/l (transport medium). The cell monolayers were washed twice with transport medium and the apical (A) and basolateral (B) compartments of the filter cups filled with 0.5 and 1.5 ml of transport medium, respectively. The cells were equilibrated in a 5% CO_2–95% air atmosphere at 37 °C for 1 h. Stock solutions of the model peptides and proteins

were placed in either apical or basolateral reservoir at the desired final concentration and allowed to stand in a humidified atmosphere of 5% CO_2–95% air at either 37 or 4 °C. Samples of 100 μl were taken from the receptor chamber at 15, 30, 60, 90, 120, 150 and 180 min to estimate transport from the A to B side and the B to A side at 37 °C and from the A to B side at 4 °C. At each time point, an equal volume of fresh transport medium was placed in the reservoir. The TER across each monolayer was measured at the beginning and end of each experiment in order to verify that no significant changes in the integrity of the monolayers had occurred.

The apparent permeability coefficients (P_{app}, cm/s) for each macromolecular drug was calculated using the following equation (Morimoto et al., 1993):

$$P_{app} = \left(\frac{dQ}{dt}\right)\left(\frac{1}{AC_0}\right)$$

where dQ/dt is the solute transfer rate, A is the surface area of the membrane (1.13 cm^2; the normal surface area of the Transwell filter), and C_0 is the initial concentration of solute.

2.4. Assay

FITC labeled sCT and FITC labeled rHAV2 were assayed using a fluorescence spectrometer under the following conditions (Pohl et al., 1999): exciting wavelength: 490 nm, emission wavelength: 520 nm, and slit width: 10 nm. The others were assayed using commercially available kits: INS was assayed using ^{125}I-labeled CT radioimmunoassay kit (Isotope Co., China), and rhGH was assayed using ^{125}I-labeled GH radioimmunoassay kit (North Biotech. Institute, China). All assays were performed in duplicate.

3. Results and discussion

3.1. Apparent permeability coefficients for each drug

The time courses of macromolecular drug transport across A549 cell monolayers are depicted in Fig. 1. The cumulative appearance of all four drugs in the receiver fluid was linear at both high and low initial concentrations. The transfer rate of drugs at the higher initial concentration was significantly greater than that obtained at the lower concentration. The changes of temperature and transport direction slightly affect their transfer rate values. No measurable lag time was observed.

Table 1 summarizes the apparent permeability coefficients (P_{app}) values for the four drugs. The P_{app} (AB) values for the peptides and proteins at 37 °C ranged from 3×10^{-6} to 5×10^{-6} cm s^{-1}. There was no

Table 1
Apparent permeability coefficients (P_{app}) for peptides and proteins using A549 cell monolayers

Peptide (MW)	Direction	Concentration	P_{app} ($\times 10^{-6}$ cm s^{-1})	
			37 °C	4 °C
sCT (3400)	AB	30 μg ml^{-1}	4.83 ± 0.17	–
		3 μg ml^{-1}	4.54 ± 0.50	4.72 ± 0.88
	BA	3 μg ml^{-1}	4.97 ± 1.02	–
INS (5700)	AB	100 ng ml^{-1}	4.54 ± 0.50	–
		10 ng ml^{-1}	4.72 ± 0.56	4.91 ± 0.38
	BA	10 ng ml^{-1}	4.74 ± 0.73	–
rHAV2 (6900)	AB	10 μg ml^{-1}	4.43 ± 0.30	–
		1 μg ml^{-1}	4.78 ± 0.67	4.49 ± 0.45
	BA	1 μg ml^{-1}	4.38 ± 0.57	–
rhGH (22000)	AB	500 ng ml^{-1}	3.29 ± 0.58	–
		50 ng ml^{-1}	3.20 ± 0.30	3.57 ± 0.25
	BA	50 ng ml^{-1}	2.25 ± 0.35*	–

Each data represent the mean ± S.D. ($n = 3$).
* $P < 0.05$ vs. the lower concentration with AB direction at 37 °C.

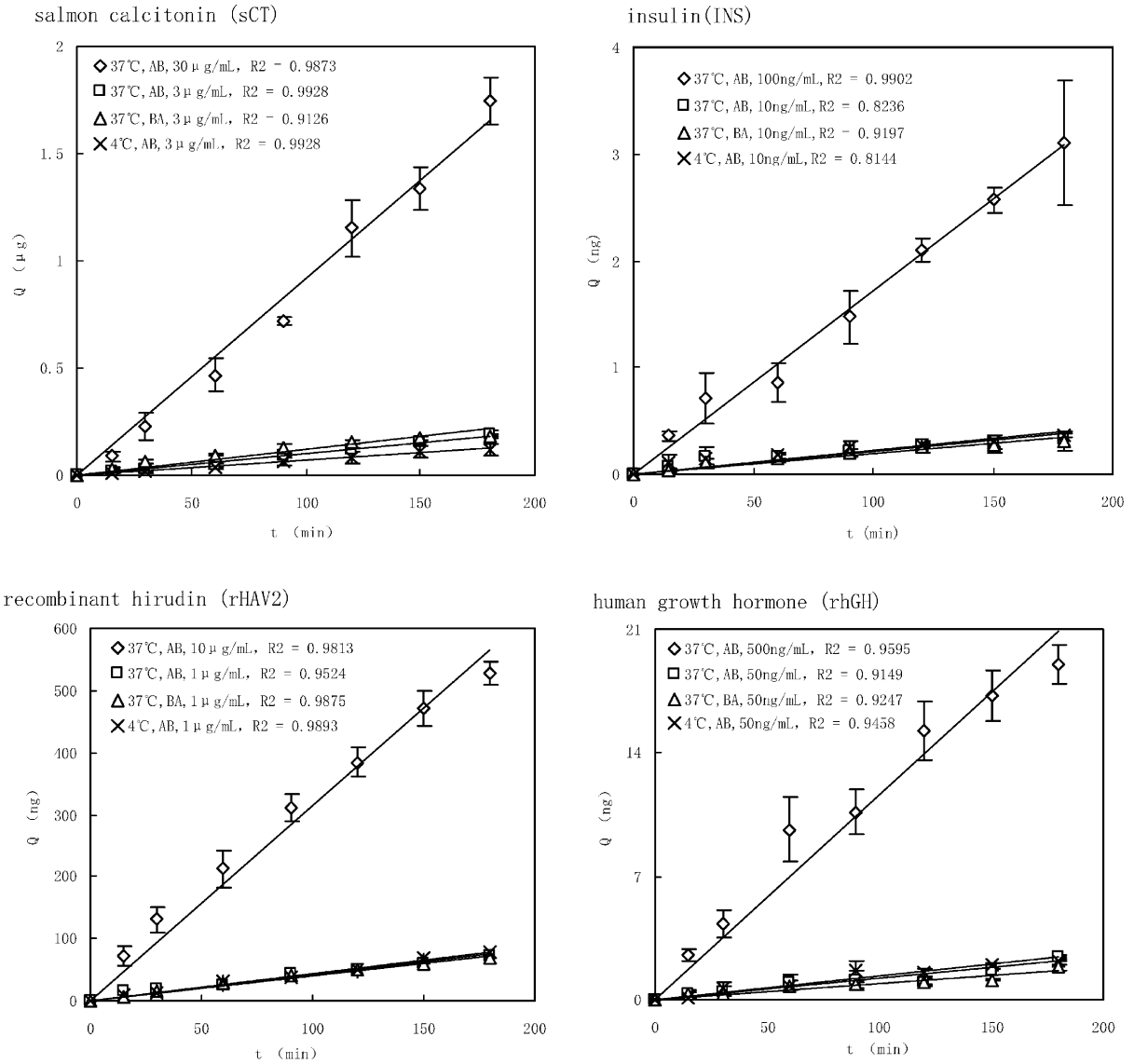

Fig. 1. Time course of accumulation of proteins and peptides in the receiver fluid under different conditions. Each data point represents the mean ± S.D. ($n = 3$).

obvious difference for the transport of sCT, INS, and rHAV2 when the concentration, direction or temperature was altered. The P_{app} of rhGH in the BA direction was $2.25 \pm 0.35 \times 10^{-6}$ cm s^{-1} and significantly less than that in AB direction at the lower concentration ($P < 0.05$). There was no concentration or temperature dependence of P_{app} in the AB direction for rhGH.

The P_{app} values obtained were far higher than those of similar molecular weight drugs obtained by other researchers using primary cultured alveolar type II cell monolayers (Forbes et al., 1999; Dodoo et al., 2000; Kinnard et al., 1994). The transepithelial resistance of their monolayer system was about 2000 Ω cm^2 and 5–6 times higher than that of our A549 cell system (Dodoo et al., 2000). The tight junctions of the A549

cells may be more leaky than those of normal alveolar type II cells, and the difference in the strength of the tight junctions in their system might have resulted in the different P_{app} values. It was reported that the permeability of thyrotropin-releasing hormone (TRH) declined to 1/5–1/6 of the initial value when the resistance was nearly doubled in a transepithelial transport experiment using cultured cell monolayers of Caco-2 (Thwaites et al., 1993).

3.2. Correlation between apparent permeability coefficient and molecular weight

Some researchers have studied in vivo pulmonary epithelial permeability and reported the existence of an inverse correlation between log molecular weight and log pulmonary clearance in which clearance changed dramatically over the molecular weight range of 1000–100,000 Da (Patton et al., 1994; Hastings et al., 1992). In this study, we found that the molecular weights of sCT, INS, rHAV2, and rhGH exhibited a good inverse correlation with P_{app} (AB) at 37 and 4 °C as shown in Fig. 2 ($R = 0.9415$). It is similar with the results reported by Patton and Hastings.

3.3. Permeation mechanism

As Table 1 shows, the apparent permeability coefficient value for each model drug changed slightly over a ten-fold change in donor concentration. There were no significant difference in P_{app} between cultures at 37 and 4 °C. No direction dependence and lag time were observed in the permeation of peptides and proteins except rhGH. Although the apical-to-basolateral transport of rhGH was significantly higher than that in the reverse direction, the P_{app} (AB)/P_{app} (BA) ratio was less than 1.5, indicating no obvious pathway of efflux in the process of the in vitro pulmonary rhGH absorption. However, the more detailed studies about direction selection should be performed in the future research. On the other hand, as shown in Fig. 2, the molecular weight of each of these four drugs exhibited a good inverse correlation with P_{app} over the range of 3400 to 22,000 Da. These findings strongly suggest that transport of the proteins and peptides used in this study, salmon calcitonin (sCT), insulin (INS), recombinant hirudin (rHAV2), and recombinant human growth hormone (rhGH), across A549 cell monolayers is via a simple passive diffusion mechanism. These hydrophilic drugs may penetrate the epithelial monolayer through some paracellular pathway, such as bijunctional or trijunctional aqueous channels at the intersection of two or three cells.

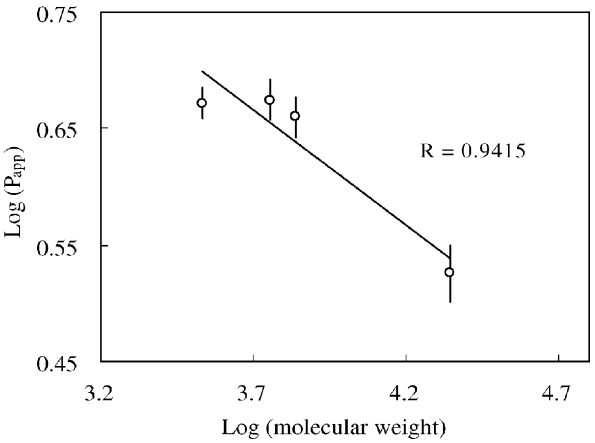

Fig. 2. Correlation between apparent permeability coefficient and molecular weight of peptides and proteins. Each point represents the mean of P_{app} values ± S.D. in the apical-to-basal direction.

Although the mechanisms of protein and peptide pulmonary absorption by the healthy lung are still poorly understood, it is widely accepted that the possible mechanisms might be transcytosis and paracellular absorption. For macromolecules > 40 kDa, transcytosis may be the dominant transport mechanism across pulmonary epithelia. For macromolecules < 40 kDa, paracellular and transcytotic mechanisms may both play roles in pulmonary epithelial transport (Hastings et al., 1994; Patton, 1996). In this study, we found that the peptides and proteins, salmon calcitonin, insulin, recombinant hirudin, and recombinant human growth hormone, which are all drugs potentially delivered by the pulmonary route in the future, cross A549 cell monolayers by a passive diffusion mechanism. This result indicates that these drugs may be absorbed through the lungs via a paracellular pathway in vivo.

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