Tracheal Barrier and the Permeability of Hydrophilic Drugs and Dipeptides

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The permeability of model hydrophilic compounds with different molecular weights and model dipeptides were examined to characterize the tracheal epithelial barrier in *in vitro* experiments using excised rabbit trachea. 6-Carboxyfluorescein (6-CF; 376 Da) and fluorescein isothiocyanate (FITC)-dextran (FDs) with varying molecular weights (4 to 70 kDa) were used as model hydrophilic and macromolecular compounds, and glycyl-D-phenylalanine (Gly-D-Phe) and glycyl-L-phenylalanine (Gly-L-Phe) were used as model dipeptides in this experiment. The apparent permeability coefficients (P_{app}) of 6-CF and FDs with Mw 376 Da to 70 kDa ranged from 2.35×10^{-7} to 4.05×10^{-10} cm/s and exhibited a good inverse correlation with their molecular weights. The tracheal permeability of 6-CF, FD-4 (4 kDa) and FD-10 (10 kDa) were increased over three fold by 10 mM glycocholate, which is an absorption enhancer. The P_{app} of Gly-D-Phe was 1.03×10^{-6} cm/s and there was no metabolism during tracheal permeation. Gly-L-Phe was immediately degraded in the mucosal fluid and its intact form was not detected in serosal fluid during the 150 min experimental period. These results suggest that absorption of some peptide drugs *via* the respiratory tract may contribute to their systemic delivery following pulmonary administration by intratracheal insufflation and instillation.

Key words pulmonary delivery; tracheal permeability; permeability barrier; tracheal epithelium; dipeptide; peptide

Peptide and protein drug delivery *via* the pulmonary route has recently received much attention since the lung, with a large surface area for absorption and extensive vascularization, is thought to be a good site for absorption of this class of compounds.^{1,2)} There are several reports concerning pulmonary delivery of peptide and protein drugs, such as calcitonin, insulin, human growth hormone and rhG-CSF.³⁻⁶⁾ These studies of the pulmonary absorption of peptide drugs were performed *in vivo* using whole lung preparations following intratracheal instillation and aerosol inhalation. The tracheo-bronchial deposition of an inhaled formulation entrained in particles (5–40 μ m) was significantly greater than alveolar deposition.^{7,8)} Systemic delivery of drugs *via* the pulmonary route will be influenced by the physicochemical characteristics and regional deposition pattern of drug and its formulation. Therefore, it is important to understand the permeability characteristics and overall metabolism of the drug in different regions of the respiratory tract, which may have different morphological and physiological characteristics. Then, we focused on the tracheas, as they are more accessible to *in vitro* experimental manipulation than the bronchi and bronchioles.

In the present study, the permeability of model hydrophilic compounds with different molecular weights and model dipeptides were examined to characterize the structural and enzymatic barriers presented by the trachea in a series of *in vitro* experiments using excised rabbit trachea. 6-Carboxyfluorescein (6-CF; Mw 376 Da) and fluorescein isothiocyanate (FITC)-dextran (FDs) with varying molecular weights from 4 to 70 kDa were used as model hydrophilic compounds, and glycyl-D-phenylalanine (Gly-D-Phe) and glycyl-L-phenylalanine (Gly-L-Phe) were used as model dipeptides in this experiment. Furthermore, the effects of sodium glycocholate, as a permeation enhancer, and bestatin as a proteolytic enzyme inhibitor, on the permeation of these compounds through excised rabbit trachea were examined. Therefore, the

goal of this study is to estimate the contribution of tracheal absorption to the systemic delivery, following intratracheal administration of peptide and protein drugs by intratracheal insufflation and instillation.

MATERIALS AND METHODS

Materials 6-CF, FDs, Gly-D-Phe, Gly-L-Phe, L-Phe, sodium glycocholate, and bestatin were commercially obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Average molecular weights of FDs were 4.3 kDa (FD-40), 9.3 kDa (FD-10), 38.5 kDa (FD-40) and 71.2 kDa (FD-70). All other chemicals were of the highest purity available commercially.

Permeation Tests Permeation tests were carried out using noneverted sacs (2 cm segment) of excised rabbit (2.0–2.5 kg) trachea at 37 °C as previously described.⁹⁾ Drug solution, with or without permeation enhancer, and proteolytic enzyme inhibitor in pH 7.4 HEPES buffered solution (0.2 ml) was infused into the sac (mucosal side) which was then placed in serosal medium, 7 ml HEPES buffer solution (pH 7.4) which was bubbled with 95% O₂–5% CO₂. Samples (0.2 ml) were removed from the serosal fluid at predetermined times for 150 min and 0.2 ml, of portions fresh fluid was added to the cell to maintain the original volume. In separate experiments, samples (10 μ l) were removed from the mucosal fluid at a predetermined time to calculate the degradation rate of dipeptides in the tracheal mucosal fluid. The concentrations of 6-CF and FDs were determined by spectrofluorometry. The degradation of FDs in the final sample (150 min) of serosal fluid was checked by HPLC using a gel permeation column. Gly-D-Phe and Gly-L-Phe were determined by HPLC as previously described.¹⁰⁾ The spontaneous transepithelial electric resistance (TEER) of the excised rabbit trachea was measured by a modification of the method of Guo *et al.*¹¹⁾ TEER was approximately 300 $\Omega \cdot \text{cm}^2$ at 0 and

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Fig. 1. Correlation between the P_{app} and Molecular Weight of CF and FDs through Rabbit Tracheas
Each point represents the mean $\pm$ S.E. of 4 experiments.

150 min. Therefore, the excised rabbit trachea remained viable over the 150 min period of the experiments.

Data Analysis The cumulative amount of compounds and their metabolites in the serosal fluid was plotted as a function of time. The steady-state fluxes (J ; pmol/cm²/s) and apparent permeability coefficients (P_{app} ; cm/s) of these compounds were estimated from the linear slope of the plot using Eqs. 1 and 2, respectively. $J = (dQ/dt)/A$ (Eq. 1) and $P_{app} = J/C_0 = (dQ/dt)/A/C_0$ (Eq. 2), where dQ/dt is the solute transfer rate (mol/s), C_0 is the initial concentration of compound on the mucosal side and A is the surface area of the membrane exposed to the compound.

All data are expressed as means $\pm$ S.E. Statistical analysis was performed using Mann-Whitney's u-test or the unpaired Student's t -test. The level of significance was taken as $p < 0.05$ and $p < 0.01$.

RESULTS

Tracheal Permeability of Hydrophilic Compounds

The characteristics of tracheal epithelial permeability of CF and FDs with various molecular weights were examined using excised rabbit tracheas. The cumulative appearance in the serosal fluid of CF and FDs was linear over the 150 min period of the experiments. No measurable lag-time was observed, and no measurable metabolites were present in the serosal fluid. The cumulative appearance of hydrophilic solutes in the serosal fluid decreased as the molecular weight increased. The P_{app} of CF (376 Da), FD-4 (4.3 kDa), FD-10 (9.3 kDa), FD-40 (38.5 kDa) and FD-70 (71.2 kDa) through rabbit tracheas were $(2.35 \pm 0.38) \times 10^{-7}$ cm/s, $(2.22 \pm 0.33) \times 10^{-8}$ cm/s, $(8.61 \pm 2.85) \times 10^{-9}$ cm/s, $(7.25 \pm 1.08) \times 10^{-10}$ cm/s and $(4.05 \pm 1.07) \times 10^{-10}$ cm/s, respectively. In Fig. 1, the logarithm of P_{app} for CF and FDs was plotted against the logarithm of their molecular weight. The P_{app} of these compounds exhibited a good inverse correlation with the molecular weight ($R=0.993$).

Figure 2 shows the effects of sodium glycocholate (1 and 10 mM) on the permeability of CF, FD-4 and FD-10 through rabbit tracheas. One millimole glycocholate did not significantly change the P_{app} of 6-CF, FD-4 and FD-10, while

Fig. 2. Effects of Sodium Glycocholate (1 and 10 mM) on the Permeation of CF and FDs through Rabbit Tracheas

Each point represents the mean $\pm$ S.E. of 4 experiments. * $p < 0.05$, ** $p < 0.01$ compared with P_{app} without glycocholate.

10 mM glycocholate significantly increased the P_{app} of these compounds.

Tracheal Permeability of Dipeptides The characteristics of the tracheal epithelial permeability of Gly-D-Phe and Gly-L-Phe were examined using excised rabbit tracheas. Figure 3 shows the P_{app} of Gly-D-Phe and the effects of sodium glycocholate (1 and 10 mM) on the permeability of Gly-D-Phe (5 and 10 mM) through rabbit tracheas. The cumulative appearance of Gly-D-Phe (5 and 10 mM) in the serosal fluid was linear over the 150 min period of the experiment (data not shown). No measurable lag-time was observed, and no measurable metabolites were present in the serosal fluid. The P_{app} for Gly-D-Phe (5 and 10 mM) was $(1.03 \pm 0.05) \times 10^{-6}$ cm/s and $(0.77 \pm 0.10) \times 10^{-6}$ cm/s, respectively, and there was no

Fig. 3. P_{app} of Gly-D-Phe and the Effect of Sodium Glycocholate (1 and 10 mM) on the Permeation of Gly-D-Phe through Rabbit Tracheas
Each point represents the mean $\pm$ S.E. of 4 experiments. ** $p < 0.01$ compared with P_{app} without glycocholate.

Fig. 4. Time-Courses of the Permeated Amount of Gly-L-Phe and Its Metabolite, L-Phe, Following Administration of Gly-L-Phe (5 and 10 mM) to Rabbit Tracheas

Permeated amount of Gly-L-Phe (○) and L-Phe (●) following administration of 5 mM Gly-L-Phe. Permeated amount of Gly-L-Phe (□) and L-Phe (■) following administration of 10 mM Gly-L-Phe. Each point represents the mean $\pm$ S.E. of 4 experiments.

Fig. 5. Effects of Sodium Glycocholate (10 mM) on the Time-Courses of the Permeated Amount of Gly-L-Phe and Its Metabolite, L-Phe Following Administration of Gly-L-Phe (5 mM) through Rabbit Tracheas

Permeated amount of Gly-L-Phe (○) and L-Phe (●) following administration of 5 mM Gly-L-Phe. Permeated amount of Gly-L-Phe (□) and L-Phe (■) following administration of 5 mM Gly-L-Phe with 10 mM glycocholate. Each point represents the mean $\pm$ S.E. of 4 experiments.

concentration-dependence. Ten millimoles glycocholate significantly increased the P_{app} of Gly-D-Phe (5 mM).

Figure 4 shows the time-courses of permeated amounts of Gly-L-Phe and its metabolite, L-Phe, following administration of Gly-L-Phe (5 and 10 mM) to rabbit tracheas. No intact Gly-L-Phe was detected in the serosal fluid for the 150 min duration of the experiment. However, the cumulative appearances of L-Phe in the serosal fluid were linear over the 150 min period of the experiment. The unidirectional flux (pmol/cm²/s) of L-Phe on following administration of 5 and 10 mM Gly-L-Phe through rabbit tracheas was 3.87 ± 0.34 and 5.64 ± 0.28 , respectively. Gly-L-Phe (5 mM) in the mucosal fluid underwent degradation according to first-order kinetics. The calculated half-life ($t_{1/2}$) was about 13 min (data not shown).

Figures 5 and 6 show the effects of sodium glycocholate (10 mM) and bestatin (1 mM) on the time-course of the permeated amounts of Gly-L-Phe and its metabolite, L-Phe, following administration of Gly-L-Phe (5 mM) through rabbit tracheas, respectively. Table 1 shows the flux of Gly-L-Phe and its metabolite, L-Phe, following administrations of Gly-L-Phe (5 mM) through rabbit tracheas. When 10 mM glycocholate was added to the mucosal fluid, no intact Gly-L-Phe was detected in the serosal fluid over the 150 min period of the experiments. The cumulative appearance of L-Phe in the serosal fluid slightly increased with 10 mM glycocholate.

Fig. 6. Effect of Bestatin (1 mM) on the Time-Courses of the Permeated Amount of Gly-L-Phe and Its Metabolite, L-Phe, Following Administration of Gly-L-Phe (5 mM) to Rabbit Tracheas

Permeated amount of Gly-L-Phe (○) and L-Phe (●) following administration of 5 mM Gly-L-Phe. Permeated amount of Gly-L-Phe (□) and L-Phe (■) following administration of 5 mM Gly-L-Phe with 1 mM bestatin. Each point represents the mean $\pm$ S.E. of 4 experiments.

Table 1. Fluxes of Gly-L-Phe and L-Phe Following Administration of Gly-L-Phe to Rabbit Tracheas

	Gly-L-Phe flux (pmol/cm ² ·s)	L-Phe flux (pmol/cm ² ·s)
Gly-L-Phe (5 mM)	— ^{a)}	3.87±0.34
Gly-L-Phe (10 mM)	— ^{a)}	5.64±0.28*
Gly-L-Phe (5 mM)+glycocholate (10 mM)	— ^{a)}	4.65±0.70
Gly-L-Phe (5 mM)+bestatin (1 mM)	1.37±0.56	1.00±0.29*

^{a)} Not calculated because no permeation was observed. * Significant at $p < 0.05$ vs. L-Phe flux following administration of 5 mM Gly-L-Phe.

However, its flux was not a significantly different from that without glycocholate.

When bestatin (1 mM) was present in the mucosal fluid, intact Gly-L-Phe was detected in the serosal fluid. The cumulative appearance of intact Gly-L-Phe in the serosal fluid was linear for the 150 min duration of the experiments. The cumulative appearance of L-Phe in the serosal fluid was significantly reduced with 1 mM bestatin.

DISCUSSION

In this study, the permeability characteristics of the tracheal epithelial barrier were examined in a series *in vitro* experiments using excised rabbit tracheas. The P_{app} of 6-CF and FDs with 376 Da to 70 kDa, as model hydrophilic compounds, through rabbit tracheas ranged from 2.35×10^{-7} to 4.05×10^{-10} cm/s and exhibited a good inverse correlation with the molecular weight. These P_{app} values are consistent with findings in isolated rabbit tracheal epithelium with CF (376 Da), [¹⁴C] sucrose (342 Da), [³H] inulin (5.5 kDa) and FD-20 (20 kDa).^{12,13)} These results are also similar to the P_{app} for FD-4 and FD-10 (4 and 10 kDa) with respect to the permeability of air-interface cultured rabbit tracheal epithelial cell monolayers.¹⁴⁾ However, the P_{app} of FD-40 and FD-70 (40 and 70 kDa) was slightly lower than our results. These results show that the permeation of hydrophilic macromolecules is limited by their molecular size, 20 kDa. The electrical resistance values of these air-interface cultured rabbit tracheal epithelial cell monolayers was approximately *ca.* 1200 $\Omega \cdot \text{cm}^2$. Our electrical resistance for isolated rabbit tracheal epithelium was approximately 300 $\Omega \cdot \text{cm}^2$ (data not shown), which was consistent with the findings in isolated rabbit nasal, ileal and colonic epithelium.¹²⁾

Matsukawa *et al.* reported that the P_{app} ($\times 10^{-8}$ cm/s) for FDs across rat alveolar epithelial cell monolayers ranged from 1.35 for FD-4 (4 kDa) to 0.32 for FD-40 (40 kDa).¹⁵⁾ These P_{app} values are relatively similar to our own P_{app} data. Morita *et al.* reported that the cumulative percentages absorption after intratracheal administration of phenol red and FDs in rats was as follows; 37% for phenol red, 7.2% for FD-4, 2.0% for FD-10 and 0.37% for FD-70 up to 2 h.¹⁶⁾ Therefore, these results indicate that absorption of peptide drugs through tracheal epithelium may contribute to the systemic delivery following pulmonary administration of these drugs by intratracheal insufflation and intratracheal instillation.

One millimole glycocholate did not significantly change the tracheal permeation of 6-CF, FD-4 and FD-10. However, 10 mM glycocholate increased the tracheal permeation of these compounds over 3-fold. There are many reports of gly-

cocholate enhancing absorption through various mucosal tissues.¹⁷⁾ The mechanism of enhanced absorption of drugs by glycocholate is still not fully understood. It is suggested that glycocholate might loosen the tight junction of the tracheal epithelium and enhance paracellular permeability.

The permeation of model dipeptides, Gly-D-Phe and Gly-L-Phe, through isolated rabbit tracheal epithelium was determined. The lack of any concentration-dependence in the transport of Gly-D-Phe (222 Da) strongly suggests a simple diffusion mechanism is involved. The absence of any detectable D-Phe in the serosal fluid indicates that Gly-D-Phe is transported in intact form through the tracheal epithelium. Its P_{app} is about 10^{-6} cm/s, a value comparable with that of mannitol (a paracellular marker) of *ca.* 1.0×10^{-6} cm/s.¹⁹⁾ However, this P_{app} is 10 times greater than the P_{app} (*ca.* 1.6×10^{-7} cm/s) of Gly-D-Phe across rat alveolar epithelial cell monolayers.¹⁰⁾

No intact Gly-L-Phe was detected in the serosal fluid (receiver fluid) over the 150 min period of the experiment. Unlike Gly-D-Phe, which remained essentially intact during permeation through isolated rabbit tracheal epithelium, Gly-L-Phe was extensively metabolized by luminally secreted enzymes in the mucosal fluid.

Ten millimoles glycocholate increased the tracheal permeation of Gly-D-Phe about 3-fold. On the other hand, in the presence of 10 mM glycocholate in mucosal fluid, no intact Gly-L-Phe was detected in the serosal fluid and the concentration of metabolite (*i.e.* L-Phe formed from Gly-L-Phe) in serosal fluid was slightly increased over the 150 min period of the experiment, compared with that in the absence of glycocholate. This increase was not significant. Glycocholate is known to be not only an absorption enhancer, but an aminopeptidase inhibitor.¹⁷⁾ However, it was unclear why the effect of glycocholate (10 mM) on the tracheal permeation of Gly-L-Phe was a weak one. When 1 mM bestatin, a relatively specific inhibitor of leucin aminopeptidase, was added to the mucosal fluid, the concentration of L-Phe in the serosal fluid fell by about 80%. Gly-L-Phe is susceptible to metabolism by luminally secreted aminopeptidase, especially when Gly-L-Phe is present in the mucosal fluid. On the other hand, Yamashita *et al.*, reported that Gly-L-Phe was degraded during transport across primary cultured tracheal epithelial cell monolayers.²⁰⁾ Low levels of intact Gly-L-Phe in the serosal fluid (receiver fluid) were detected over the 150 min period of the transport experiments involving 10 mM Gly-L-Phe. This suggests that Gly-L-Phe, at 10 mM, might be transported mainly *via* paracellular diffusion and the L-Phe formed from Gly-L-Phe appears to be efficiently transported *via* the amino acid transporter for Gly-L-Phe across cultured tracheal epithelial cell monolayers. It is speculated from our results that Gly-L-Phe (at 5 and 10 mM) is immediately metabolized by aminopeptidase in mucosal fluid and then the L-Phe in mucosal fluid might be transported *via* paracellular diffusion or transcellular transport by the amino acid transporter. However, we were unable to characterize the transport mechanism of Gly-L-Phe through rabbit tracheal epithelium in our studies.

In summary, the P_{app} of 6-CF and FDs with molecular weights ranging from 376 Da to 70 kDa as model hydrophilic compounds through rabbit tracheas ranged from 2.35×10^{-7} to 4.05×10^{-10} cm/s and exhibited a good inverse correlation

with the molecular weight. The P_{app} of Gly-D-Phe was 1.03×10^{-6} cm/s and Gly-D-Phe was not metabolized during tracheal permeation. No intact Gly-L-Phe was detected in the serosal fluid over the 150 min period of the experiments. These results suggest that the absorption of some peptide drugs via the respiratory tract may contribute to the systemic delivery following pulmonary administration of these drugs by intratracheal insufflation and instillation.

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