Receptor-Mediated Peptide Delivery in Pulmonary Epithelial Monolayers

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The present study investigated the feasibility of utilizing receptor-mediated endocytosis as a means to enhance peptide delivery to the pulmonary epithelium. The strategy employs a molecular conjugate consisting of a cognate moiety, transferrin (TF), covalently-linked to a model polypeptide, horseradish peroxidase (HRP), via a reversible disulfide linkage. A cultured alveolar epithelial monolayer system was used to simulate the conditions of the pulmonary epithelium and to allow accurate quantitation of intra- and transcellular peroxidase transport. The alveolar cells were isolated from rat lungs by enzymatic digestion and grown on microporous tissue culture-treated polycarbonate filters. A significant increase in the uptake of HRP by the cell monolayer was observed upon its conjugation with TF. The effect was found to be concentration-dependent, being more pronounced at low concentrations, i.e., 3.9- and 1.2-fold increase over unconjugated HRP controls at the concentration levels of 0.05 and 1.50 U/ml respectively. Effective peroxidase uptake was shown to require the TF cognate moiety for the cell surface receptor. Specific internalization of the conjugate by the TF endocytic pathway was verified by competition for the TF receptor. Conjugate internalization was not followed by a proportional increase in transcytosis, i.e., at 0.05 U/ml conjugate level, a 1.7-fold increase in transcytosis was observed as compared to 3.9-fold for endocytosis. Effective enhancement of transcytosis was achieved by treating the monolayers with brefeldin A (BFA), a compound known to affect intracellular transport of TF receptor complexes. At 1.6 μ/ml concentration level, BFA promoted a >20-fold increase in the rate of transcytosis of the conjugate in both the apical-to-basal and basal-to-apical directions. This effect was not associated with membrane leakage since BFA-treated monolayers maintained tight barrier to transport of the paracellular permeability solute ^{14}C mannitol. In addition, BFA had no significant effect on the transport of free HRP. Instead, the effect of BFA on conjugate transport was mediated by TF receptors since excess free TF competitively inhibited transcytosis of the conjugate. Thus, our results are consistent with the TF receptor-mediated transport of the conjugate and its enhancement through the intracellular rerouting of the conjugate by BFA. The findings in this study may potentially be relevant to the design of drug delivery systems that can enhance intra- or transcellular uptake of therapeutic peptides in the pulmonary epithelium.

KEY WORDS: pulmonary absorption; receptor-mediated endocytosis; transcytosis; alveolar epithelium.

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INTRODUCTION

Drug delivery through the pulmonary route provides an attractive means for systemic application of peptides and proteins due to its large absorptive surface area, extensive microvasculature, and relatively low enzymatic activities in the alveoli. Bioavailability studies by numerous investigators (1–3) indicate that peptide delivery through the pulmonary route exhibits greater systemic bioavailability than most other alternate routes. Consistent with these studies, our recent permeability studies (4) also indicate that the epithelial membranes of the lung are more permeable to solute flux than those from other epithelia, i.e., respiratory > vaginal > rectal > corneal > buccal > skin. Despite its great therapeutic potential, the pulmonary route is still considerably less effective in delivering peptides and proteins than the injectable route, i.e., its systemic bioavailability is typically 10–20% (5). This relative inefficiency has been largely attributed to the protective permeability barrier, which limits drug absorption across the pulmonary wall. Thus, numerous efforts have been made to enhance pulmonary peptide absorption, most notably through the use of penetration enhancers. However, the practical use of these enhancers faces an uphill regulatory battle because of possible irritation and other safety concerns.

As an alternate strategy to circumvent this problem, the present study investigates the feasibility of delivering peptides via the natural receptor-mediated endocytosis pathway. To accomplish peptide delivery, this study utilizes a molecular conjugate consisting of TF, a cognate moiety for a cell surface receptor, covalently linked to HRP, a model polypeptide to be transported. TF receptor was previously reported to exist on the surface of respiratory epithelial cells (6). The recycling efficiency of the TF receptor is high (>98%) with a recycling half time of 10–20 minutes (7), making it an effective candidate for use in such a delivery system. When recognized by the receptor, the conjugate is internalized by the efficient receptor-mediated pathway, co-transporting the peptide. Because this method accomplishes peptide delivery by capitalizing on normal physiologic cellular pathway, it is potentially non-toxic, allowing the potential to administer peptides on a repetitive or continuous basis.

To facilitate transport studies and to allow accurate quantitation of the endocytosed and transcytosed peptide, the present study utilizes a cultured monolayer system of alveolar epithelial cells. The alveolar monolayer system is used also because it represents the major transport barrier of the lung *in vivo*. The results obtained in this study indicate that peptide delivery to the alveolar epithelium can be effectively enhanced *in vitro* by receptor-mediated endocytosis of the TF conjugate.

EXPERIMENTAL

Preparation of Transferrin-Peroxidase Conjugate

HRP (EC 1.11.1.7, Sigma) was conjugated to TF (iron-free, Sigma) through a reversible disulfide linkage with the aid of the bifunctional cross-linking agent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Boehringer Mann-

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heim), according to the modified method of Carlsson *et al.* (8). HRP (0.05 μmol) was dissolved in 0.5 ml of phosphate-buffered saline (PBS) (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4), pH 7.4. An ethanol solution of (50 μl) containing 10 mM SPDP was added to the stirred protein solution and the reaction proceeded for 30 min at room temperature. Excess of the reagent was removed by centrifugal filtration through the Durapore™ dialysis filter (CL5K, Millipore Corp.) at 5,000 g for 30 min. The modified protein was then collected and reconstituted in 0.5 ml sodium acetate buffer, pH 4.5. Modification of the TF was similarly performed except that the protein was reconstituted in PBS.

The HRP-2-pyridyl disulfide derivative obtained was then converted into a thiol derivative by specific reduction of the 2-pyridyl disulfide groups with dithiothreitol (10 μmol) for 30 min, afterwhich it was filtered (to remove excess dithiothreitol) and reconstituted in 0.5 ml PBS. The modified HRP was then mixed with the TF-2-pyridyl disulfide derivative and, after a 18 hr incubation period at 4°C, the reaction mixture was again centrifugally filtered through the Durapore™ CL100K dialysis membrane to remove unreacted proteins. The disulfide HRP-TF conjugate was then collected, iron saturated, and stored at 4°C in HEPES-buffered medium for further studies. The conjugate prepared under these condition retained approximately 70% of the initial peroxidase activity and yielded approximately 200 peroxidase units/mg conjugate. Peroxidase activity was determined spectrometrically using 2,2′-azino-bis(3-ethylbenzolinesulfonic acid) (ATBS, Sigma) as an enzyme substrate (9). One unit of peroxidase is the amount that oxidizes 1 μmol of ATBS per minute.

Isolation and Culture of Alveolar Epithelial Cells

Isolation and culture of alveolar cells were conducted according to the method previously described (10,11). Male, pathogen-free, Sprague-Dawley rats (100–150 g; Hilltop Lab, Scottdale, PA) were anesthetized with pentobarbital sodium (150 mg/kg body wt) and the lungs were removed. They were perfused with 0.9% NaCl to remove blood cells, and free alveolar macrophages were removed by tracheal lavage with PBS. The lungs were then excised and filled with PBS containing elastase (40 U/ml, type I, US Biochemical) and DNase (0.006%, Sigma) and incubated at 37°C for 20 min to free lung cells. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% fetal bovine serum and 0.006% DNase (to help prevent cell clumping). The crude extract was sequentially filtered through 160 and 45 μm screens, centrifuged, and the resulting cell pellet was spun on a sterile Percoll density gradient. The second cell band from the surface was collected, washed twice, and resuspended in 1:1 F_{12} and Eagle's modified minimum essential medium plus 10% fetal bovine serum, 100 U/ml penicillin, 10 μg/ml gentamicin, and 0.1 μg/ml dexamethasone. The cell suspension yielded 15–20 × 10^6 cells/rat with viability greater than 95% as determined by the Coulter counter (model Z_B, Coulter Instrument) and trypan blue exclusion respectively. The suspended cells were plated onto 0.4-μm pore, 1.2-cm^2 tissue culture-treated Nuclepore filters (Transwell, Costar, Cambridge, MA) at 1.5 × 10^6 cells/cm^2 in 12-well plates. The cells on filters were maintained in a humidified 5% CO_2 incubator at 37°C and the nutrient medium was changed every 48 hr after plating. Cell confluency was monitored by electrical resistance measurements using the Millicell ERS testing device (Millipore, Bedford, MA). A typical peak resistance value of the resulting monolayers (normally obtained after 5 days) was ≈1.3 kΩ.cm^2. Detailed characterization of the monolayers obtained by this procedure was previously described (10).

Transport Studies

After 5 days in culture, the monolayers on filters were incubated with HEPES-buffered medium (136 mM NaCl, 2.2 mM Na_2HPO_4, 5.3 mM KCl, 10 mM HEPES, 5.6 mM glucose, 1.0 mM $CaCl_2$, pH 7.4) at 37°C, 5% CO_2. After an incubation period of 1 hr, either HRP on HRP-TF conjugate was added to the donor (apical) compartment to give the final concentration of 0.05 U/ml. In some experiments in which the effect of concentration was studied, 0.05–1.50 U/ml of HRP or HRP-TF conjugate were used. To test potential membrane leakage, ^{14}C mannitol (1 μCi/ml) was also added in some experiments. At various time intervals, the receptor (basolateral) medium was removed and assayed for peroxidase activity or ^{14}C mannitol radioactivity. The former was used to indicate the amount of peroxidase transcytosed and the latter indicate the paracellular permeability or the tight junction integrity of the monolayers. Paracellular permeability was calculated from the equation; Papp = Jss/Cd = $(dm/dt.A)(1/Cd)$, where Papp is the apparent permeability, Jss is the steady state flux, Cd is the donor radioactive concentration, M is the amount transferred, and A is the exposed surface area.

To determine the amount of peroxidase endocytosed by the cells, the monolayers were removed, vigorously washed with cold HEPES buffer containing 1 mg/ml TF, and then lysed with 0.1% Triton X-100. The peroxidase activity in the cell extracts (1 ml/monolayer) was then measured and used to indicate endocytosis. In experiments designed to evaluate conjugate uptake via the TF receptor-mediated pathway, conjugate alone (0.05 U/ml) or in combination with either excess TF or albumin (0.01–0.1 mg/ml) was used. The effect of BFA on TF-mediated transcytosis was investigated using BFA (Sigma, 1.6 μg/ml) added to either the donor or receptor compartment.

RESULTS AND DISCUSSION

Intra- and Transcellular Transport of HRP and HRP-TF Conjugate

Figure 1A and 1B show the time-dependent intracellular uptake (endocytosis) and transepithelial transport (transcytosis) of HRP and HRP-TF conjugate in the alveolar epithelial monolayers. The monolayers treated with the conjugate exhibited greater peroxidase activities both in the cell extracts and in the receptor medium than those from free HRP-treated monolayers. Control studies using untreated monolayers showed minimal endogenous peroxidase activity and this had no interfering effect on the assay. These results indicate that the HRP-conjugate, despite its larger molecular

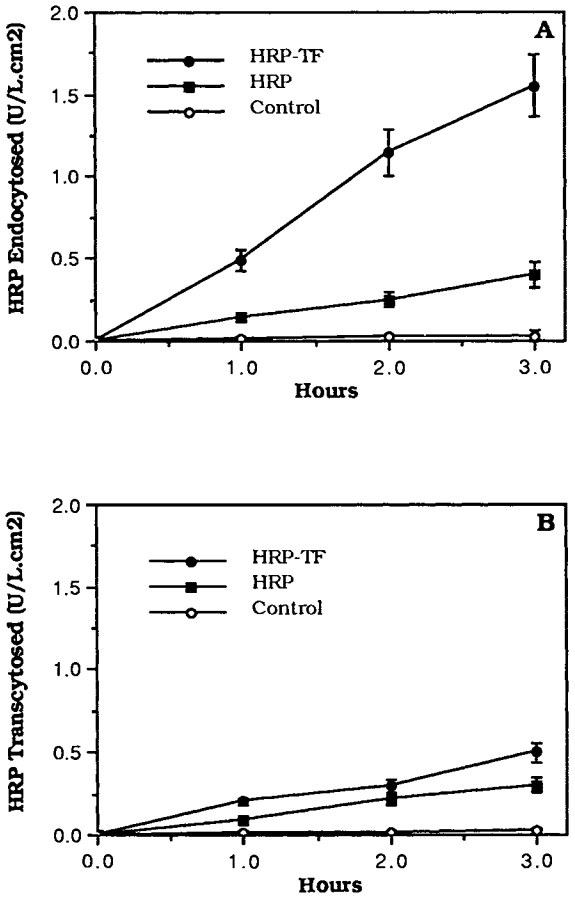

Fig. 1. Endocytosis (A) and transcytosis (B) of HRP and HRP-TF conjugate in alveolar epithelial monolayers. Monolayers were exposed for various times at 37°C to HRP or its conjugate (0.5 U/ml), added to the donor medium. At the indicated times, the monolayers and receptor media were removed and analysed for peroxidase activities as described in the Experimental section. Control indicates untreated monolayers. The values represent mean ± SE for four measurements obtained from different cell preparations.

size, facilitates cellular uptake of HRP to a greater extent than free HRP. This promoting effect was more pronounced in the cell extracts as compared to that in the receptor medium, i.e., a 3.9- and 1.7-fold increase over the HRP controls after a 3-hr incubation period respectively. This suggests that the internalized peroxidase from the conjugate accumulated in the intracellular compartment and did not traverse the basolateral membrane at a proportional rate. This limited transcellular transport was not believed to be due to the resistance to cleavage of the conjugate (to yield free HRP), the condition which was shown to be important in transcytosis of HRP from its conjugate in other epithelia (12). Supporting this notion is the evidence that the HRP recovered from the medium after conjugate treatment was smaller in size than the intact conjugate, i.e., >94% of the total HRP activity was recovered in the filtrate after medium filtration through the Durapore™ CL100K filter (the same filter that was used to retain the intact conjugate during its preparation). Intracellular cleavage of disulfide bonds was also reported in several disulfide conjugate systems such as drug-macromolecular carrier conjugates (13–14), tyramine-polylysine conjugates, and HRP-polylysine conjugates (12), in a variety of cell systems. In the present study, the observed low transcellular transport of HRP following conjugate treatment was consistent with the concept that the internalized TF and its receptor are localized in the endocytic vacuoles and are recycled back to the plasma membrane instead of being transcytosed (16). Using a similar disulfide linkage, Willingham *et al.* (17) were be able to demonstrate that in KB cells the HRP-TF conjugate was specifically bound to the surface TF receptor and was endocytosed and recycled back out of the cell with the same kinetics as native TF.

Because HRP is expected to be poorly absorbed across the monolayers (due to its molecular size and hydrophilicity) and because our experimental data indicate significant transepithelial transport of free HRP, there is a possibility that this may have been due to membrane leakage. To test this possibility, flux of the paracellular permeability marker ^{14}C mannitol was simultaneously monitored (Fig. 2). The results showed that the monolayers' permeability to mannitol was maintained, i.e., Papp = $3.2 \pm 0.3 \times 10^{-7}$ cm/sec as compared that observed *in vivo* of $\approx 3.4 \times 10^{-7}$ cm/sec (18), and this value was unaffected by the treatment with either free HRP or its conjugate. In addition, an induction of epithelial leakage by the calcium chelator EGTA (0.1 mM), the compound known to promote tight junction opening in alveolar epithelium (10), was shown to cause an almost 10-fold increase in the monolayers' permeability to mannitol (Fig. 2). Thus, these results indicated that the monolayers maintained their integrity and that the observed transepithelial transport of HRP following HRP or HRP-TF treatment was not simply due to membrane leakage. This conclusion was also supported by the observed higher flux of HRP-TF conjugate as compared to that of free HRP.

In the absence of evidence of paracellular HRP transport, it is logical to assume that this macromolecule may penetrate the membrane through a specialized transport mechanism. Indeed, previous studies have indicated that the cells of the alveolar epithelium were capable of transporting macromolecules via vesicular transport mechanisms (19–

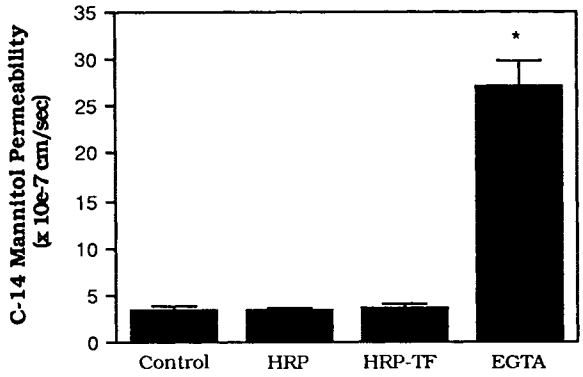

Fig. 2. Alveolar epithelial permeability to ^{14}C mannitol in the absence (control) or presence of 0.05 U/ml HRP, or 0.05 U/ml HRP-TF, or 0.1 mM EGTA. Experiments were conducted at 37°C in HEPES-buffered medium. The apparent permeability (mean ± SE, n = 4) was calculated from steady state flux of the radioactive tracer as described in the Experimental section. *significant increase over control ($p < 0.01$).

21). In bullfrog alveolar epithelium, ^{14}C-labeled albumin was found to be transported in the alveolar to pleural direction to a much greater extent than that in the opposite direction, i.e., about an order of magnitude higher (19). Although the exact transport mechanism was not fully elucidated in this study, it was suggested that vesicular transport was responsible for such an increased uptake. In hamsters and guinea pigs, studies were also shown that the epithelial lining of the lung was capable of transporting HRP via fluid-phase transcytosis (20,21). Ultrastructural analyses of the alveolar epithelium indicated HRP-stained pinocytic vesicles and the absence of junctional HRP activity.

Transport Mechanisms of HRP and HRP-TF Conjugate

To further investigate the transport mechanisms of HRP and its conjugate, dose dependence studies were conducted (Figs. 3A & 3B). Increasing the concentration of HRP in the donor medium (0.05–1.50 U/ml) caused a proportional increase in the rate of endocytosis and transcytosis of HRP. On the other hand, the rate of endocytosis following conjugate treatment appeared to be relatively less pronounced at increasing concentrations, i.e., a 3.9- and 1.2-fold increase over HRP controls at 0.05 and 1.5 U/ml respectively. The observations of the relatively constant and slower rate of HRP uptake suggested fluid-phase endocytosis as the likely mechanism for HRP uptake. In contrast, the more efficient and saturable process of the conjugate suggested its transport via the receptor-mediated process.

To further confirm that the transport of HRP-TF conjugate occurred through the TF receptor-mediated pathway, experiments were conducted in which an increasing amount of TF (0.01–0.1 mg/ml) was simultaneously added to the donor medium (to provide competitive inhibition for the receptor) (Fig. 4). Under this condition, a concentration-dependent inhibition of conjugate uptake was found, indicating that the conjugate must have entered the cells through the TF receptor-mediated pathway. This conclusion was further substantiated by the lack of specific inhibition of conjugate uptake in the presence of the non-specific competitor albumin (0.1 mg/ml) (Fig. 4). In addition, inhibition of the internalized conjugate was also observed if the experiments were conducted at 4°C, the conditions known to inhibit cellular internalization (results not shown). Taken together, our results showed that in alveolar epithelium the uptake of TF-HRP conjugate occurred via the TF receptor-mediated pathway.

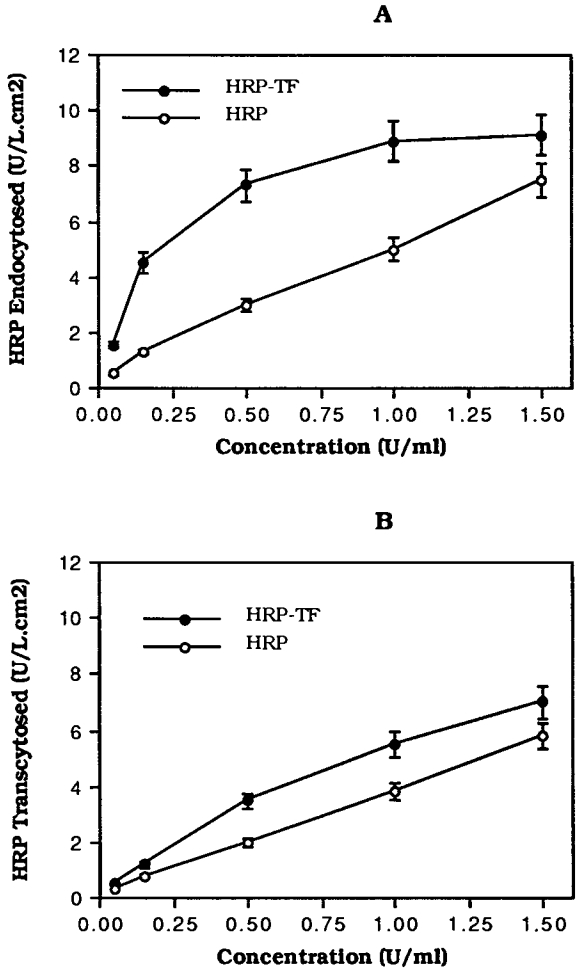

Fig. 3. Effects of concentration on intracellular (A) and transcellular (B) transport of HRP and HRP-TF conjugate. Monolayers were exposed to varying concentrations of HRP or HRP-TF conjugate (0.05–1.50 U/ml) and the appearance of peroxidase activities in the cell extracts and receptor medium were measured. The values (mean ± SE, n = 4) indicate the amount of peroxidase endocytosed or transcytosed after a 3-hr incubation period.

Effect of Brefeldin A on Transcytosis of HRP-TF Conjugate

The limitation to the use of TF-mediated uptake as a means of delivering drugs across epithelial cells is the recycling pathway through which both TF and its receptor undergo (22). However, recent findings suggest possible means of modulating the recycling pathway to achieve greater transport across the cells. BFA, a compound known to cause intracellular missorting of the TF receptor, has been shown to increase the transcytosis of TF or TF-HRP in a number of cell systems including the MDCK and Caco-2 cells (23–26). The enhancing effect of BFA on TF transport was found to be bidirectional, i.e., both from the basal-to-apical and api-

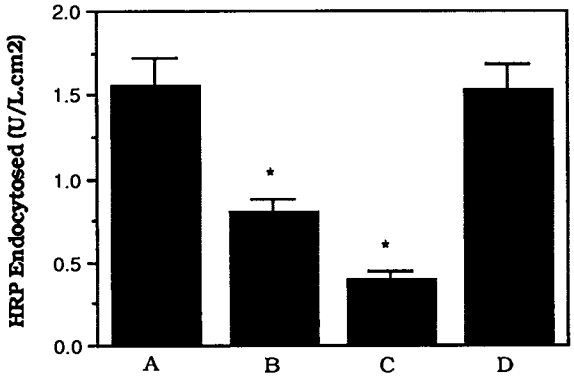

Fig. 4. Competitive inhibition of TF receptor-mediated endocytosis of HRP-TF conjugate in alveolar epithelial monolayers. Monolayers were exposed to 0.05 U/ml HRP-TF conjugate in the absence (A) or presence of 0.01 mg/ml TF (B) or 0.1 mg/ml TF (C) or 0.1 mg/ml albumin (D). The values (mean ± SE, n = 4) indicate the amount of peroxidase endocytosed after a 3-hr incubation period. *significant decrease over control ($p < 0.01$).

cal-to-basal directions. In alveolar epithelial monolayers, our studies similarly demonstrated that BFA markedly enhanced transcytosis of HRP-TF conjugate in both directions (Fig. 5). At a 0.05 U/ml conjugate level, BFA (1.6 μg/ml) caused a 21- and 14-fold increase in transcytosis of the conjugate in the apical-to-basal and basal-to-apical direction respectively. This enhancing effect was not observed with free HRP ($p < 0.05$) (Fig. 6), suggesting that BFA did not interfere with fluid-phase transport and that this effect was not due to BFA-induced membrane leakage. The latter conclusion was also supported by the observation that the monolayers' permeability to ^{14}C mannitol was maintained in the presence of BFA (results not shown). Instead, the effect of BFA on TF-HRP transcytosis was mediated through the TF receptor since excess free TF (0.1 mg/ml) completely inhibited the BFA-induced effect (Fig. 6). These results are consistent with the known effect of BFA in interfering with TF intracellular trafficking which results in enhanced TF transcytosis. In other epithelia, BFA was suggested to alter the traffic of TF and its receptor by missorting the internalized complex within an endosome-*trans*-Golgi network and diverting it from the normal recycling pathway to the transcytotic pathway (23).

The exploitation of receptor-mediated endocytotic and transcytotic processes is likely to become useful for the delivery of therapeutic macromolecules. Furthermore, its practical use will depend on a thorough understanding of the intricate cellular transport and processing mechanisms involved. However, pharmacological modification of these cellular processes to achieve enhanced therapeutic uptake of drugs remains to be further explored.

CONCLUSIONS

Advances in biotechnology are providing proteins and peptides of therapeutic importance. If significant endocytosis and transcytosis of these molecules occur in the pulmonary epithelium, the large surface area could serve as a useful route of administration, i.e., by inhalation of these drugs which are orally ineffective. In the present study, we demonstrated the feasibility of utilizing TF receptor-mediated endocytosis as a means to enhance peptide uptake in alveolar epithelial monolayers. The monolayers treated with HRP-TF conjugate exhibited intracellular peroxidase activity significantly greater than that from HRP-treated monolayers. This enhanced uptake was due to conjugate internalization via the TF receptor-mediated pathway since competitive inhibition of this receptor by excess TF abolished such transport. Unlike endocytosis, transcytosis of HRP was minimally enhanced upon conjugation with TF, suggesting recycling process of the TF conjugate to the cell surfaces. Coadministering the conjugate with BFA, however, was found to greatly promote transcytosis of the conjugate, presumably by inhibiting its recycling process and diverting it to the transcytosis pathway.

The findings in this study may potentially be useful to the development of drug delivery systems designed to improve intra- or transcellular uptake of peptides and proteins. For examples, enhanced intracellular uptake of peptides would be applicable to the delivery of antioxidant enzymes to the lung epithelium for its protection against oxidative injury. On the other hand, enhanced transepithelial transport would benefit systemic peptide delivery via non-injectable routes. The feasibility of utilizing receptor-mediated processes for *in vivo* peptide delivery remains to be established. Potential problems associated with peptide instability in endocytic vacuoles as well as the potential side effect of pharmacologic modulators such as BFA need to be further investigated. In addition, the uneven distribution of TF receptor on the alveolar epithelial cells has not been delineated. Localization of TF receptor on the basal cell layer would dictate a systemic route of administration of the conjugate to target these cells. On the other hand, localization of TF receptor at the apical pole of lumenal alveolar cells would permit pulmonary delivery of the conjugate. In this regard, TF level in the epithelial lining fluid of the lung is enriched

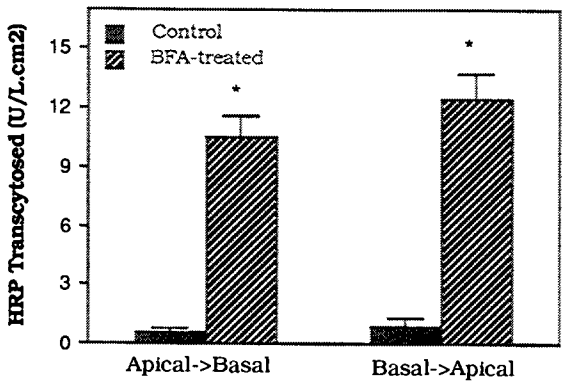

Fig. 5. Effects of BFA on transcytosis of HRP-TF conjugate in the apical-to-basal and basal-to-apical direction. Monolayers were exposed to HRP-TF conjugate (0.05 U/ml) in the absence (control) or presence of BFA (1.6 μg/ml), added to the apical or basal bathing medium. The medium collected from the opposite side to that of conjugate administration was then analysed for peroxidase activity. The values (mean ± SE, n = 4) indicate that amount of peroxidase transcytosed after a 3-hr incubation period. *significant increase over untreated controls ($p < 0.01$).

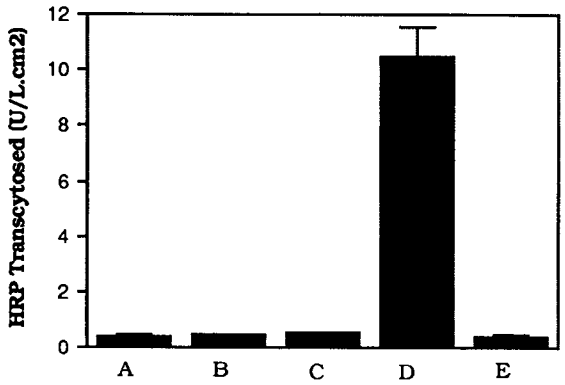

Fig. 6. Effects of BFA on TF receptor-mediated transcytosis of HRP and HRP-TF conjugate. Monolayers were treated either with HRP (0.05 U/ml) in the absence (A) or presence (B) of BFA (1.6 μg/ml), or with HRP-TF (0.05 U/ml) in the absence (C) or presence of BFA (1.6 μg/ml) (D) or BFA (1.6 μg/ml) + TF (0.1 mg/ml) (E). All testing agents were added to the apical medium and peroxidase activities in the basal medium were monitored. The values (mean ± SE, n = 4) indicate the amount of peroxidase transcytosed after a 3-hr incubation period.

relative to serum (27). Such localization of the TF in lung lumen suggests co-localization of its corresponding surface receptor, thus potentially allowing the delivery of TF conjugate by the pulmonary route.

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