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Research paper

Characterization of protein factor(s) in rat bronchoalveolar lavage fluid that enhance insulin transport via transcytosis across primary rat alveolar epithelial cell monolayers

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Abstract

The aim of this study was to characterize factor(s) in rat bronchoalveolar lavage fluid (BALF) that enhance(s) insulin transport across primary rat alveolar epithelial cell monolayers (RAECM) in primary culture. BALF was concentrated 7.5-fold using the Centricon device and the retentate was used to characterize the factor(s) involved in enhancing apical-to-basolateral transport of intact ^{125}I-insulin across various epithelial cell monolayers. These factor(s) enhanced transport of intact insulin across type II cell-like RAECM (3-fold increase) and type I cell-like RAECM (2-fold increase), but not across Caco-2 or MDCK cell monolayers. The insulin transport-enhancing factor(s) were temperature- and trypsin-sensitive. The mechanism of enhancement did not seem to involve paracellular transport or fluid-phase endocytosis, since fluxes of sodium fluorescein and FITC-dextran (70 kDa) were not affected by the factor(s) in the apical bathing fluid. BALF enhancement of intact ^{125}I-insulin transport was abolished at 4 °C and in the presence of monensin, suggesting involvement of transcellular pathways. Sephacryl S-200 purification of BALF retentate, followed by LC-MS/MS, indicated that the high molecular weight (>100 kDa) fractions (which show some homology to alpha-1-inhibitor III, murinoglobulin gamma 2, and pregnancy-zone protein) appear to facilitate transcellular transport of insulin across RAECM.

Keywords: Cell culture; Peptide delivery; Pulmonary; Insulin; Transcytosis

1. Introduction

Systemic delivery of peptides and proteins via the lung has been of interest over several decades, and clinical trials of drug molecules such as insulin have proven the potential and efficiency of inhalation as a method of systemic drug delivery [1]. The lung's large surface area offered by the thin alveolar epithelium and rich capillary blood supply are factors which have contributed to notable systemic bioavailability of peptides and proteins, while the mechanisms of absorption are unclear and need further investigation.

There are several barriers that a drug macromolecule faces as it traverses from the lung airspaces to the bloodstream, one of which is the epithelial lining fluid (ELF) [2]. ELF acts as a reservoir for lipid surfactants and is rich in proteins such as those produced by type II pneumocytes (e.g., surfactant proteins) and alveolar macrophages (e.g., cytokines/chemokines and growth factors), or those of plasma origin (e.g., immunoglobulin G (IgG), IgA, transferrin, macroglobulins and alpha 1-antitrypsin) [3,4]. It is thought that clearance by the mucociliary escalator is responsible for the maintenance of appropriate

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concentrations of these proteins in airway fluid, while secretion/reabsorption mechanisms may be responsible for homeostasis of these molecules in alveolar lining fluid [5]. Paracellular diffusion and transcytosis across the alveolar epithelium have both been proposed as mechanisms that contribute to reabsorption of proteins present in the alveolar milieu [5]. Although it appears that paracellular diffusion may be the predominant pathway in clearing proteins under pathological circumstances, receptor-mediated transcytosis has also been shown to contribute to the clearance of proteins such as IgG [6], albumin [7], human growth hormone [8], and surfactant protein A [9] in normal lungs.

The closest approximation of this ELF microenvironment is the bronchoalveolar lavage fluid (BALF), where a buffer solution (e.g., phosphate buffered saline) is instilled into the lungs and withdrawn. It has been reported that BALF yields an accurate reflection of ELF protein composition [10]. BALF has been utilized to determine some parameters of lung diseases [10]. In the context of drug delivery, some ELF components have been utilized as carriers that exploit the dynamic properties of the lung epithelium and thereby improve pulmonary delivery of ligand-conjugated peptides or proteins. For example, erythropoietin-Fc fusion protein studied *in vitro* [11] or *in vivo* [12] has shown enhanced absorption, presumably through the Fc receptor expressed in the pulmonary epithelial cells. Chemical conjugation of transferrin to granulocyte colony stimulating factor (GCSF) or horseradish peroxidase [13,14] has also led to increased transport of proteins via receptor-mediated endocytosis across alveolar epithelium *in vitro*. These examples indicate that endogenous protein factor(s), present in the ELF, may have potential application in protein drug delivery. However, the impact of ELF components on macromolecule drug absorption is still unclear. To our knowledge, the effect of proteins present in ELF on the absorption of inhaled insulin has not been studied to date. We have recently reported that (a) protein factor(s) in the conditioned medium of primary cultures of type II cell-like rat alveolar epithelial cell monolayers (RAECM) can increase the transport of insulin across cultured RAECM. The purpose of this study was to determine if factors in BALF similarly are involved in enhancement of transalveolar insulin absorption.

2. Materials and methods

2.1. Monolayer cultures of primary rat alveolar epithelial cells and MDCK and Caco-2 cell lines

Rat alveolar epithelial type II cells were isolated and purified as previously described [15]. Purified alveolar type II cells (>90% purity and >90% viability) were plated onto tissue culture-treated polycarbonate filters (12 mm Transwells, 0.4 μm pore size, Corning Costar, Cambridge, MA) at 10^6 cells/cm^2 and cultured for 6 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture medium consisted of minimal defined serum-free medium (MDSF, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12, Sigma Chemical, St. Louis, MO) supplemented with 10% (v/v) newborn bovine serum, 100 U/mL penicillin and 100 ng/mL streptomycin. Cells were fed on day 3 and every other day thereafter. These cells transdifferentiate into cells bearing type I cell-like morphology and phenotype, monolayers of which are designated as RAECM-I. In order to maintain the type II cell-like phenotype and morphology, keratinocyte growth factor (KGF, 10 ng/ml) was added to both apical and basolateral medium for feeding cells on day 3 and onward. Monolayers of these cells were designated as RAECM-II. Transepithelial electrical resistance (TEER) and potential difference (PD) of both RAECM-I and -II, when measured on day 6 using an epithelial volt-ohm meter (EVOM, World Precision Instruments, Sarasota, FL), are >2000 Ω cm^2 and >10 mV (apical side negative).

Madin-Darby canine kidney (MDCK) cells (2.2×10^4 cells/cm^2), a strain we originally obtained from Upjohn (Kalamzoo, MI), were cultured on Transwells using Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.5% (v/v) penicillin (10,000 U/mL)/streptomycin (10 mg/mL) solution (Sigma), 1% (v/v) L-glutamine (200 mM, GIBCO, Grand Island, NY), and 1% (v/v) non-essential amino acid solution (100 mM, Sigma). On day 3, MDCK cell monolayers exhibited a TEER of ~1000 Ω cm^2 and were replenished with the same culture medium but containing only 2.5% FBS. The concentration of FBS was reduced further to 1% from day 5 and onward until a final TEER of ~2500 Ω cm^2 was achieved (usually by 7–8 days in culture).

Human colon carcinoma (Caco-2) cells (4.2×10^4 cells/cm^2) obtained from ATCC (Manassas, VA) were cultured on Transwells using DMEM supplemented as described above for MEM with 10% FBS, which was used throughout the culture period. Caco-2 cells were fed every 3 days and confluence was reached within 7–8 days. The monolayers were maintained an additional 7–13 days when TEER of ~400 Ω cm^2 was obtained.

2.2. Preparation of bronchoalveolar lavage fluid (BALF)

BALF was collected from normal Sprague–Dawley rats by lavaging lungs of anaesthetized rats three times (10 mL per lavage) with warm (37 °C), phosphate-buffered Kreb's Ringer's phosphate solution (KRP, pH 7.4) comprised of 135 mM sodium chloride, 5 mM potassium chloride, 24 mM dibasic sodium phosphate septahydrate, 6 mM monobasic sodium phosphate monohydrate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 1 mg/mL glucose. The rat lung lavage fluid (~30 mL/rat and protein concentration ~0.1 mg/mL) was first centrifuged at 230*g* for 10 min at 4 °C to remove lung resident cells (e.g., macrophages) and then at 3685*g* for 15 min at 4 °C to remove cell debris. The combined BALF

supernatant was concentrated 7.5-fold using a Centricon device YM-10 (Millipore Corp, Bedford, MA), unless stated otherwise. The retentate from the 7.5× concentrate (designated as 7.5× BALF hereafter) contained approximately ~0.5–1 mg/mL total protein and was stored at 4 °C for use within 5 days.

2.3. Transport studies

RAECM-II were washed once with MDSF and incubated for 30 min at 37 °C. Apical medium (0.5 mL) was aspirated and replaced with an equal volume of 7.5× BALF or KRP containing ^{125}I-insulin. At 2 h post-dosing, basolateral fluid (1.5 mL) was collected and total radioactivity was measured using a Packard gamma counter. Basolateral samples were then treated with 15% trichloroacetic acid (TCA) on ice for 15 min, followed by centrifugation at 2235*g* for 15 min at 4 °C. After aspiration of supernatants, the radioactivity associated with pellets was measured again in the gamma counter. TCA is a simple analytical method which has been used to determine intact insulin-associated radioactivity [16]. In some experiments, apical-to-basolateral transport of ^{125}I-insulin across RAECM-II was compared with that across RAECM-I, MDCK, and Caco-2 monolayers using the same procedure described above except that MDCK and Caco-2 cell monolayers were washed once with serum-free MEM and DMEM, respectively, before transport experiments.

The influence of BALF on paracellular drug transport was determined using two paracellular markers: ^{3}H-mannitol (1 μCi/mL) and sodium fluorescein (1 mg/mL), which were dosed in 7.5× BALF or KRP for transport studies across RAECM-II at 37 °C. Apical-to-basolateral transport of ^{3}H-mannitol and sodium fluorescein was measured by sampling the basolateral receiver fluid at 2 h after apical dosing and their activities assayed using a liquid scintillation counter (Beckman LS1801) and a fluorometer (Hitachi F-200), respectively. Furthermore, to assess the effect of BALF factor(s) on fluid phase endocytosis, FITC-dextran 70 kDa (0.5 mg/mL) was apically dosed in a similar manner as mannitol transport experiments and the basolateral receiver fluid sampled at 2 h was assayed for fluorescence.

To determine temperature-dependency, apical-to-basolateral transport of ^{125}I-insulin dosed in 7.5× BALF or KRP was measured across RAECM-II at 37 and 4 °C after 1 h of apical dosing. Effects of an endocytosis inhibitor, monensin, were determined by first pre-incubating RAECM-II with serum-free MDSF containing monensin (60 μM) in both apical and basolateral compartments at 37 °C for 0.5 h. The apical medium was aspirated and replaced with ^{125}I-insulin dosed in 7.5× BALF or KRP with or without monensin (60 μM) and the apical-to-basolateral transport of ^{125}I-insulin was measured at 37 °C after 1 h of apical dosing as described above.

2.4. Characterization of the insulin transport-enhancing factor(s) in BALF

To determine the molecular weight range of "putative" factor(s) that retain the insulin transport-enhancing activity, rat BALF was concentrated 7.5-fold using Centricon devices with different molecular weight cutoffs (10, 30, and 50 kDa; or YM-10, -30 and -50). Radiolabeled insulin was added to each of the retentates and filtrates to assess their effects on insulin transport across RAECM-II, as described above. Two aliquots of the retentate obtained from the concentrated BALF (using YM-10) were treated either by heating at 80 °C for 15 min, followed by cooling to room temperature and centrifuging to remove precipitated material, or by digesting with trypsin (10 μM), incubating for 30 min at 37 °C and quenching trypsin activity with soybean trypsin inhibitor (20 μM) (Sigma). The effect of protein depletion by heat denaturation and precipitation, or protein degradation by trypsin digestion of BALF on the apical-to-basolateral transport of insulin across RAECM-II was determined using apical dosing with ^{125}I-insulin in these solutions similar to those described above.

2.5. Column chromatography of BALF

For purification purposes, a larger volume of rat bronchoalveolar lavage fluid (500 mL) was concentrated using a tangential flow filtration system (TFF) (Fisher Scientific, Pittsburg, PA) which was fixed directly to a Millipore Pellicon XL filter (50 kDa molecular weight cutoff). This procedure yielded a 20-fold concentrated BALF (or 20× BALF) with a higher protein concentration (2.5 mg/mL). The retentate obtained from the 20× BALF was loaded onto a Sephacryl S-200 column (1 cm × 40 cm) that was pre-equilibrated with PBS (pH 7.4), fractions (1 mL) collected, and their absorbance measured at 280 nm. Peak fractions from the Sephacryl S-200 chromatography were pooled and tested for their effects on insulin transport across RAECM-II, similarly to those described above.

2.6. LC-MS/MS and database search

Aliquots (35 μl) of sample fractions obtained from Sephacryl S-200 chromatography were analyzed using sodium-dodecyl-sulfate polyacrylamide gel (7.5%) electrophoresis (SDS–PAGE). Coomassie stained protein bands in the gel were excised and destained with 50% acetonitrile in 50 mM ammonium carbonate. In-gel tryptic digestion was carried out according to Gallaher et al. [17]. Briefly, samples were reduced with 10 mM dithiothreitol (in 50 mM ammonium carbonate) for 60 min at 56 °C and alkylated with 55 mM iodoacetamide (in 50 mM ammonium carbonate) for 45 min in the dark at room temperature. The samples were digested overnight at 37 °C using reductively methylated trypsin. Digestion products were extracted from the gel twice with a 5% formic acid/50% acetonitrile solution, followed by once extraction with

100% acetonitrile. The solvent was evaporated using an APD SpeedVac (ThermoSavant, Holbrook, NY) and samples were resuspended in 10 μL of 60% acetic acid, injected via autosampler (Surveyor, ThermoFinnigan, San Jose, CA) and subjected to reverse-phase liquid chromatography. Mass analysis was done using a LCQ Deca XP Plus ion trap mass spectrometer equipped with a nano-spray ion source (ThermoFinnigan). The column was equilibrated for 5 min at 1.5 μL/min with 95% solution A (0.1% formic acid in water) and 5% solution B (0.1% formic acid in acetonitrile) prior to sample injection. A linear gradient was initiated 5 min after sample injection, ramping to 35% solution A and 65% solution B after 50 min and 20% solution A and 80% solution B after 60 min. Mass spectra were acquired in the mass/charge (m/z) range of 400–1800. Protein identification was carried out with the MS/MS search software Mascot 1.9 (Matrix Science, London, UK) [18] and complementary analyses with TurboSequest as implemented in the Bioworks Browser 3.2, build 41 (ThermoFinnigan).

2.7. Statistical analyses

Data are presented as means ± SD. For comparisons of multiple ($\geqslant 3$) group means, one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests were performed using Prism v4.0 software (Graphpad, San Diego, CA). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Transport of insulin across RAECM-II and -I, MDCK and Caco-2 monolayers

The apical-to-basolateral transport of acid-precipitable ^{125}I-insulin (as determined by TCA precipitation) across RAECM-II when dosed in the retentate obtained from 7.5× BALF using Centricon YM-10 was increased by 4-fold as compared to that dosed in KRP (Fig. 1). Our data indicate that ~5% and ~10% of total transported ^{125}I-insulin dosed in 7.5× BALF and KRP, respectively, were acid-precipitable. Moreover, incubating ^{125}I-insulin in 7.5× BALF or KRP at 37 °C for 1 h in a water bath resulted in ~20% and 1% dissociation of total insulin, respectively (data not shown). This suggests that the lower percent of acid-precipitable ^{125}I-insulin observed when dosed in 7.5× BALF and transported across RAECM-II was partly a result of protease activity present in BALF but which was absent in KRP. All data regarding insulin transport hereafter represent the acid-precipitable ^{125}I-insulin which is most relevant to our study.

When transport of insulin across RAECM-I and -II was compared, 7.5× BALF enhanced transport of insulin across both monolayer types (2- and 3-fold, respectively), indicating that the putative factor(s) may be involved in enhancing insulin transport across both type I and type II cell monolayers (Fig. 1). To determine whether the transport-enhancing activity of BALF factor(s) was also efficacious in other cell monolayers, both MDCK and Caco-2 cell monolayers were used. As shown in Fig. 2, the insulin transport-enhancing effect of 7.5× BALF was absent when tested across both MDCK and Caco-2 monolayers. Furthermore, transport of insulin across RAECM-II when dosed in filtrate (in place of retentate) was not significantly different from transport observed with KRP. Since enhancement of insulin transport was most prominent across RAECM-II, we chose to focus on this cell monolayer for characterization and identification of the factor(s) using 7.5× BALF retentate.

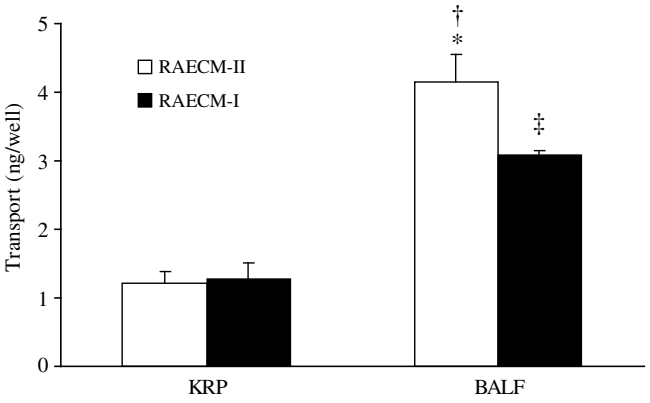

Fig. 1. Transport of insulin across RAECM-II and -I. Apical-to-basolateral transport of intact insulin, when dosed apically at 5 μg/mL in either KRP or 7.5× BALF across RAECM-II and -I. Data represent means ± SD ($n = 3$). *Significantly different ($p < 0.05$) compared to KRP across RAECM-II; ‡significantly different ($p < 0.05$) compared to KRP across RAECM-I; and †significantly different ($p < 0.05$) compared to BALF across RAECM-I.

3.2. Effects of putative factor(s) in BALF on enhancing transport via paracellular versus transcellular pathways

The effect of BALF on transport of paracellular markers (mannitol and sodium fluorescein) and fluid-phase endocy-

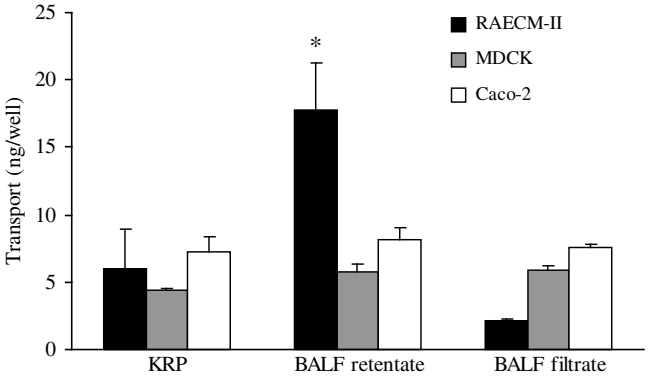

Fig. 2. Transport of insulin across RAECM-II and MDCK and Caco-2 monolayers. Apical-to-basolateral transport of intact insulin, when dosed apically at 5 μg/mL in either KRP or 7.5× BALF retentate or 7.5× BALF filtrate across RAECM-II and MDCK and Caco-2 cell monolayers. Data represent means ± SD ($n = 3$). *Significantly different ($p < 0.05$) from all others.

tosis marker (FITC-dextran, 70 kDa) across RAECM-II was also investigated (Table 1). Permeability of ^{3}H-mannitol and sodium fluorescein in either KRP or 7.5× BALF showed similar fluxes across RAECM-II, with no significant changes in TEER. The permeability of FITC-dextran across RAECM-II was similar in magnitude, irrespective of the dosing solution used (i.e., KRP or 7.5× BALF).

Effects of BALF factor(s) on transport across RAECM-II were assessed by dosing insulin apically in either KRP or 7.5× BALF at 37 or 4 °C. Fig. 3 shows that transport of insulin dosed in KRP decreased by ~40% at 4 °C compared to that at 37 °C. The 2.5-fold enhanced insulin transport observed with BALF at 37 °C was decreased to the same level of transport observed with KRP when temperature was lowered to 4 °C, suggesting involvement of a transcellular pathway such as transcytosis, in the presence of BALF factor(s). To further corroborate this observation, we used monensin, which is an endocytosis inhibitor. Monensin did not affect insulin transport observed with KRP (Fig. 3). However, in the presence of monensin, enhanced insulin transport observed with 7.5× BALF decreased by ~90% to a level similar to that of baseline (i.e., insulin transport observed with KRP in the absence of monensin). Given that monensin did not change TEER (2000 ± 230 ($n = 10$) and 2250 ± 150 ($n = 10$) $\Omega\,cm^2$ in the absence and presence of monensin, respectively), the inhibition is likely due to interference with an endocytosis-related pathway. These data indicate that BALF factor(s) enhance transport of insulin via a transcellular pathway, but do not affect paracellular or fluid phase endocytosis transport of solutes.

Table 1
Effect of 7.5× BALF on paracellular and fluid phase endocytosis transport across RAECM-II

Solute	Papp (cm/s)	
	KRP	BALF
Sodium fluorescein (1 mg/mL)	5.8 ± 0.9 ($\times 10^{-7}$)	5.0 ± 0.89 ($\times 10^{-7}$)
^{3}H-Mannitol (1 μCi/mL)	7.35 ± 1.2 ($\times 10^{-7}$)	7.93 ± 1.34 ($\times 10^{-7}$)
FITC-dextran 70 kDa (0.5 mg/mL)	3.2 ± 0.16 ($\times 10^{-9}$)	2.6 ± 0.65 ($\times 10^{-9}$)

Values are means ± SD, ($n = 4$).
TEER measured in $\Omega\,cm^2$ of RAECM-II did not change when either KRP or BALF was used as dosing solution (2000 ± 304 ($n = 24$) and 2150 ± 450 ($n = 24$), respectively).

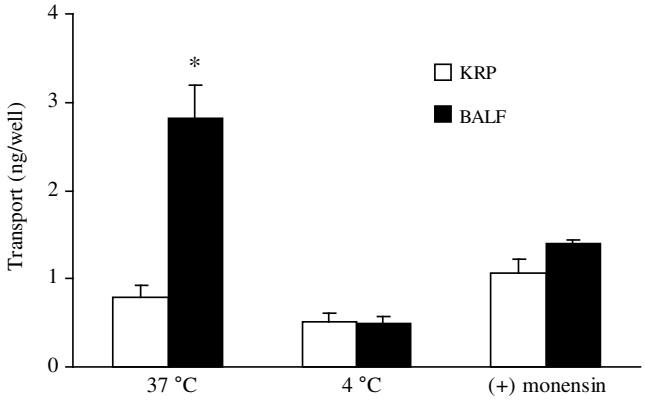

Fig. 3. Effects of temperature and monensin on enhanced insulin transport across RAECM-II. Apical-to-basolateral transport of intact insulin, when dosed apically at 5 μg/mL in KRP or 7.5× BALF across RAECM-II at 37 and 4 °C after 1 h and with or without 60 μM monensin at 37 °C after 1 h. Data represent means ± SD ($n = 4$). *Significantly different ($p < 0.05$) from all others.

3.3. Characterization of insulin transport-enhancing factor(s) in BALF

To determine the molecular weight range of the factor(s) in BALF involved in enhancing insulin transport across RAECM-II, the retentates obtained from the BALF concentration Centricon devices YM-10, -30, or -50 were used as dosing solutions. As shown in Table 2, insulin transport-enhancing effect was highest in the retentate obtained from the Centricon device YM-10. Transport was decreased to 56% of that with the YM-10 retentate when dosed in retentates obtained from Centricon devices YM-30 and -50 ($p < 0.05$). A 12% SDS–PAGE revealed that the same number of bands was present (data not shown) in all retentates, but that the intensity/level of proteins in retentates was greater in the retentate obtained from YM-10 ultrafiltration compared to that from YM-30 and YM-50.

In order to determine the nature of the factor(s) contributing to enhancement of insulin transport across RAECM-

Table 2
Effects of various 7.5× BALF retentates and KRP as dosing solutions on enhancing intact ^{125}I-insulin transport across RAECM-II

Dosing solution	% ^{125}I-insulin transport enhancement as compared to that observed with the BALF retentate from using 10 kDa cutoff[a]
BALF retentate from YM-10	100
BALF[b] retentate from YM-30	56 ± 8.32
BALF[c] retentate from YM-50	56 ± 6.31
KRP	20 ± 0.63
BALF[d] retentate treated at 80 °C for 15 min	4 ± 0.35
BALF[e] retentate treated with 10 μM trypsin and 20 μM soybean trypsin inhibitor	20 ± 2.08

Values are means ± SD, ($n = 3$).
[a] BALF was concentrated 7.5× in a Centricon device with 10 kDa cutoff and retentate was used as dosing solution for ^{125}I-insulin transport across RAECM-II.
[b] BALF was concentrated 7.5× in a Centricon device with 30 kDa cutoff and used as dosing solution for ^{125}I-insulin transport across RAECM-II.
[c] BALF was concentrated 7.5-fold in a Centricon device with 50 kDa cutoff and used as dosing solution for ^{125}I-insulin transport across RAECM-II.
[d] 7.5× BALF retentate from a Centricon device YM-10 was denatured at 80 °C for 15 min.
[e] 7.5× BALF retentate from a Centricon device YM-10 was digested with 10 μM trypsin and quenched with 20 μM soybean trypsin inhibitor.

II, insulin transport was examined in 7.5× BALF (obtained from the Centricon device YM-10) which was treated either by heat denaturing or by digesting with trypsin. Table 2 shows that enhancement of insulin transport across RAECM-II in BALF concentrates treated by heat precipitation or trypsin digestion of proteins was decreased to 4% and 20% of that observed with untreated 7.5× BALF, respectively ($p < 0.05$), suggesting that a protein or a number of proteins in BALF were involved.

3.4. Purification of BALF proteins

Concentrating BALF 20-fold using a TFF system yielded a larger amount of protein (~2.5 mg/mL) than the ultracentrifugation technique. The fractions obtained from 20× BALF using a Sephacryl S-200 column showed two prominent peaks: #22, which represented the fraction containing high molecular weight proteins with insulin transport-enhancing activity across RAECM-II, and #29 which consisted mainly of albumin, the major protein component of BALF (Fig. 4A and B). Fraction #22 was loaded onto a 7.5% SDS–PAGE, stained with Coomassie blue, and analyzed for size. Three prominent high molecular weight protein bands at molecular weight of ~200 kDa were found (Fig. 4C). In-gel digestion and LC-MS/MS of the three bands suggested that the proteins may be: alpha-1-inhibitor III, murinoglobulin gamma 2, and pregnancy-zone protein (Table 3), all of which belong to the macroglobulin family.

4. Discussion

The present study demonstrates that BALF obtained from rat lung lavage contains factor(s) which enhance(s) transport of insulin across RAECM. It was necessary, first, to concentrate BALF, which represents a diluted sample of rat epithelial lining fluid, by ~7.5-fold to obtain significant transport-enhancing activity. The lack of such enhancement in Caco-2 or MDCK cell monolayers indicates tissue specificity (i.e., for alveolar epithelium) of the factor(s) involved. Molecular sizing using a Centricon device suggested that the factor(s) is greater than 50 kDa. The insulin transport-enhancing effect of 7.5× BALF was decreased by 96% and 80% upon heat deactivation and tryptic-digestion, respectively, suggesting that the factor(s) involved represent protein(s).

Transport across the alveolar epithelial barrier may take place via paracellular or transcellular routes, depending on the size, shape, or charge of the transported molecule. For insulin (5.8 kDa), transport is thought to be predominantly paracellular as indicated by its permeability across RAECM [19] as well as its small diameter (~2.2 nm) relative to the estimated pore diameter (10–12 nm) of the alveolar epithelium [20]. Our results showed that transport of insulin when dosed in 7.5× BALF across RAECM-II was enhanced ~3-fold as compared to that dosed in KRP. Our observation that enhancement of insulin transport

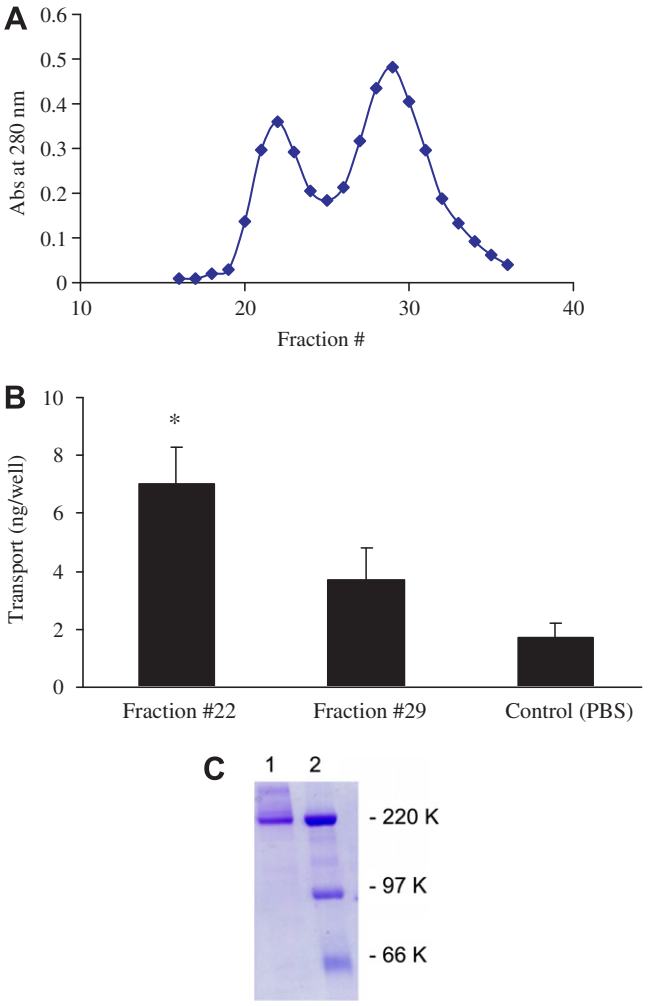

Fig. 4. Elution profile and SDS–PAGE of BALF proteins. BALF was concentrated 20-fold using TFF system, and purified by applying to a Sephacryl S-200 column (1 cm × 40 cm, PBS (pH 7.4)). (A) Absorbance profile (♦) at 280 nm of fractions (1 mL) from purified 20× BALF. (B) Apical-to-basolateral transport of intact insulin, when dosed apically at 10 μg/mL in fractions #22 and #29, and control (PBS) across RAECM-II. Data represent means ± SD ($n = 3$). *Significantly different ($p < 0.05$) from both the transport observed under control conditions (PBS) and that observed with dosing in fraction #29. *C:* BALF protein detected by 7.5% SDS–PAGE. Lane 1: fraction #22 of purified 20× BALF. Lane 2: rainbow molecular weight markers (myosin, 220,000; phosphorylase b, 97,000; bovine serum albumin 66,000).

dosed in 7.5× BALF across RAECM-II was not due to enhanced passive diffusion via paracellular routes is evidenced by the unchanged permeability of two paracellular markers, ^{3}H-mannitol and sodium fluorescein, when dosed in either 7.5× BALF or KRP. Moreover, 7.5× BALF did not affect TEER (>2000 ohm·cm^2). Fluid phase endocytosis did not seem to be the main route for enhanced insulin transport, as no significant difference in transcytosis of FITC-dextran (70 kDa) was noted with 7.5× BALF or KRP as the dosing solution. These findings provide evidence that a non-diffusional mechanism is responsible for enhancement of insulin transport across RAECM in the presence of BALF factor(s).

Table 3
Analysis and identification of proteins, obtained from SDS–PAGE gel bands of rat 20× BALF (fraction #22), using LC-MS/MS and Mascot Search Engine

Protein name	Database accession #	Mass	Score[a] ($p < 0.05$)	% Sequence coverage[b]
Alpha-1 inhibitor III precursor (Rattus norvegicus)	gi 34858400	164986	623	16
Murinoglobulin 2 (Rattus norvegicus)	gi 62647940	162752	677	14
Pregnancy-zone protein (Rattus norvegicus)	gi 21955142	168422	330	7

[a] Score is the summation of all observed unique peptides, which is based on individual peptide scores (ion scores) >36 and that are indicative of identity and extensive homology.
[b] The % sequence coverage is determined by the percentage of amino acids matched to the identified protein based on observation of tryptic peptides with high confidence match ($p < 0.05$) as per Mascot scoring.

To investigate whether enhancing effects of BALF factor(s) involved cell energy-requiring process(es), insulin transport was studied at 37 °C and 4 °C. Lowering temperature from 37 to 4 °C reduced insulin transport by ~37% when dosed in KRP. This is comparable to the decrease (~40%) in flux of small molecular weight dextran (4 kDa) as a result of lowering temperature from 37 to 4 °C and is consistent with a predominant diffusional pathway [20]. Transport of insulin observed with 7.5× BALF at 37 °C was reduced to baseline transport similar to that observed for insulin with KRP at 4 °C, which implies that an additional pathway besides paracellular transport and one that involves an energy-dependent mechanism such as transcytosis may be involved [21]. Monensin, an ionophore reported to interfere with Na^+/H^+ exchange in endosomes, has been used in various studies to determine the involvement of clathrin-mediated receptor endocytosis of certain ligands [9,22]. By altering the acidic pH of the endosome environment, monensin disrupts dissociation of ligand-receptor complex and prevents recycling of the receptor to the plasma membrane, thereby inhibiting endocytosis [5]. Addition of monensin to 7.5× BALF completely abolished the observed enhanced insulin transport across RAE-CM-II and reduced it to that observed with KRP alone. Taken together, these findings suggest involvement of a transcellular process as the mechanism for the enhancement of intact insulin transport by BALF factor(s).

SDS–PAGE analysis of the BALF concentrate depicts a large number of proteins in epithelial lining fluid of the lungs. In fact, proteomic data from human or rat BALF show an array of more than 50 proteins ranging from plasma proteins to proteins produced by cells in the lung [3,23]. The BALF factor(s) contributing to the insulin transport-enhancing activity were in the high molecular weight fraction when partially purified using the S-200 gel chromatography. After in-gel digestion of the high molecular weight protein bands and analysis using LC-MS/MS, alpha-1-inhibitor III precursor, murinoglobulin gamma 2, and pregnancy-zone protein were identified by the Mascot Search Engine. These plasma proteins of relatively large molecular weights have been recently identified in BALF of normal rats [24].

Among the three proteins identified using LC-MS/MS, alpha-1-inhibitor III (α_1I_3) and murinoglobulin gamma 2, which may be identical [25], and pregnancy-zone protein, all belong to the macroglobulin family [26]. In fact, α_1I_3 (most abundant in the analyzed fraction) is a homologue of alpha-2 macroglobulin (α_2M; mwt 700 kDa), which is found in a number of species including human [26]. As proteinase inhibitors, they share a common mechanism whereby a protease-susceptible region (known as the "bait" region) lures the endopeptidase, which in turn cleaves that region of the macroglobulin [27]. The cleaving of the bait region exposes β-cysteinyl-γ-glutamyl thiol esters which attack lysine residues present in the proteinase, resulting in a conformational change that in turn leads to the inactivation of the proteinase [28]. Only this activated form of α_1I_3 or α_2M-proteinase complex binds with high affinity [29] to its cognate receptor: the low density lipoprotein receptor-related protein/α_2M receptor (LRP/α_2MR). Various studies have shown that, in addition to its proteinase inhibitory activity, α_2M exhibits nonproteolytic ligand binding capacity [30]. The ligands for α_2M include growth factors [31,32], antigens [33], cytokines [34] and insulin [35], among others. This binding (mainly covalent bonding due to nucleophilic attack) occurs only when protein ligand is in the presence of both α_2M and its associated proteinase. On the one hand, it is well established that both α_1I_3 and α_2M share a common proteinase inhibitory mechanism; but on the other hand, simultaneous presence of insulin is a prerequisite for its incorporation during the proteinase-induced conformational change of α_2M [35]. Although it is possible that α_1I_3 proteinase may have already been formed in BALF, this does not completely eliminate the presence of free α_1I_3 partly because it exists in a state of equilibrium and partly due to the reversibility of the thiol ester bond under certain conditions [36]. The mechanism(s) involved in mediating the interaction between insulin and α_1I_3 with subsequent transcytosis of insulin across the alveolar epithelium will need further investigation. Recent results in our lab have shown that enhanced insulin transcytosis mediated by fraction #22 of S-200 purified BALF was inhibited by 70% in the presence of activated human α_2M (200 nM) and not by albumin when used as control (unpublished results), suggesting the involvement of receptors related to α_2M-receptor in the transport process.

In summary, alveolar epithelial lining fluid contains a large number of proteins whose effects on drug delivery have not been fully explored. Our present findings indicate that BALF contains a protein factor or factors which

enhance(s) transport of insulin across RAECM. We have previously reported a similar observation of enhanced insulin transport mediated by protein factor(s) present in conditioned medium obtained from apical fluid of primary cultured RAECM-II [37]. Observations from both studies highlight the importance of the alveolar epithelium in maintaining lung homeostasis through various mechanisms, including endocytosis [21], and may further explain the high bioavailability of several peptide and protein drugs *in vivo*.

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