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Research Paper

Enhancement of Insulin Transport Across Primary Rat Alveolar Epithelial Cell Monolayers by Endogenous Cellular Factor(s)

Rana Bahhady,[1] Kwang-Jin Kim,[2,3] Zea Borok,[2,3] Edward D. Crandall,[2,3] and Wei-Chiang Shen[1,4]

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Purpose. To characterize factor(s) contained in apical medium of primary cultured rat alveolar epithelial type II cell-like monolayers (RAECM-II) that enhance insulin absorption across alveolar epithelial cells.
Materials and Methods. Primary rat alveolar epithelial cell monolayers cultured on Transwells in the presence and absence of 10 ng/ml keratinocyte growth factor for 6 days were dosed from the apical compartment with radiolabeled insulin in: newborn bovine serum-containing medium (SM), conditioned medium from apical compartment of rat alveolar epithelial type I cell-like monolayers (RAECM-I) (CMI), or conditioned medium from apical compartment of RAECM-II (CMII). At the end of 2 h incubation, basolateral medium was collected and amounts of transported radiolabeled insulin were determined using a gamma counter. In order to determine the molecular size range of the enhancing factor(s), CMII was centrifuged in 50 kDa molecular weight cut-off Centricon tubes, and both retentate and filtrate were used as separate dosing solutions. Heat denaturation and ammonium sulphate precipitation were used to determine if the involved factor(s) represent proteins or other smaller soluble factors. Transalveolar transport rates of a paracellular marker, ^{14}C-mannitol, and fluid-phase marker, horseradish peroxidase, were determined in the presence and absence of the factors. Effects of temperature (4, 16 and 37°C) on radiolabeled insulin fluxes were also measured.
Results. Conditioned medium obtained from the apical compartment of RAECM-II, CMII, increased transport of insulin across the monolayers when compared to SM or CMI. The enhancing effect of CMII was retained in the precipitate following ammonium sulfate treatment and in the retentate after Centricon filtration. The enhancing effect of CMII was significantly decreased when heated at 80°C for 15 min. CMII did not affect the transport of ^{14}C-mannitol or HRP, while its effect on insulin transport was decreased by 87% when temperature was lowered to 4°C from 37°C.
Conclusions. Conditioned medium from type II cell-like monolayer cultures appears to contain protein factor(s) which seem to be involved in facilitating active transcellular transport of insulin across primary cultured RAECM-II.

KEY WORDS: alveolar epithelial cell monolayer; conditioned medium; insulin; transport.

INTRODUCTION

The lung has long been an attractive organ for non-invasive delivery of drugs due to its large surface area, thin epithelial barrier and extensive blood supply. Compared to oral, nasal and subcutaneous delivery of insulin in humans, pulmonary administration allows for a profile that closely simulates the physiological release of insulin following food ingestion (1,2), thereby emphasizing it as an alternative route to invasive delivery of proteins and peptide. Aerosol inhalation of peptides/proteins in most instances provides higher bioavailability relative to oral or nasal routes (3).

Cellular and whole lung studies of pulmonary insulin transport have indicated predominance of a paracellular pathway (4). Therefore, amphipathic agents such as bile salts (5–7), and polymers such as carbopol (8), among many other conventional tight junction modulators, have been utilized for paracellular enhancement of insulin and other macromolecule transport. Peptidase and protease inhibitors have also been studied for their effects on enhancement of pulmonary peptide/protein absorption and have been shown to improve the stability (9) and bioavailability (10,11) of insulin by inhibiting proteolytic enzymes present in the lung cytosol, although disruption of tight junctions in the lung epithelial barrier by some of these agents may also attribute to the enhanced bioavailability but which, on the downside, cause a potential threat of lung edema. More recent strategies for improving pulmonary delivery of peptide/protein have utilized the dynamic properties of the lung epithelium and endogenous proteins present in the epithelial microenviron-

[1] Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, California 90089-9121, USA.
[2] Department of Medicine, University of Southern California, Los Angeles, California 90089-9121, USA.
[3] Will Rogers Institute Pulmonary Research Center, University of Southern California, Los Angeles, California 90089-9121, USA.
[4] To whom correspondence should be addressed. (e-mail: weishen@usc.edu

ment. For example, erythropoietin-Fc fusion protein studied *in vitro* (12) or *in vivo* (13), has shown enhanced absorption presumably through the Fc receptor expressed by the pulmonary epithelial cells. Transferrin chemically conjugated to granulocyte colony stimulating factor (GCSF) or horseradish peroxidase (14,15), has also lead to increased transport of proteins across alveolar epithelium, *in vitro*, via receptor-mediated endocytosis. To date, no investigation of endogenous protein factor(s) for increasing insulin absorption in the lungs has been reported.

Primary culture of rat alveolar epithelial cell monolayers has been widely used as a reliable *in vitro* model for mechanistic studies of peptide and macromolecule drug delivery via the distal respiratory epithelial tract of the lung. This *in vitro* model resembles *in vivo* alveolar epithelium in morphology and phenotypic characteristics, and has been successfully used to study transport of various proteins and peptides (14,16,17). More recently, primary culture of human alveolar epithelial cells which was used to study transport of various proteins and peptides (18), showed that discrepancies in permeability do exist for some proteins when compared to permeability studies done in rat alveolar cells; however, a similar transport trend and magnitude was observed for most proteins including insulin. When plated on tissue culture-treated polycarbonate filters, the isolated rat type II pneumocytes begin to transdifferentiate by day three and onward into type I cell-like pneumocytes, acquiring thin cytoplasmic processes and losing their characteristic lamellar bodies and surfactant protein production. The presence of keratinocyte growth factor (KGF) in the culture medium has been shown to inhibit or reverse such transition (19), allowing the cultured cells to retain type II cell-like phenotype and morphological traits. Data from our laboratories and others regarding transport of small proteins such as insulin and GCSF across rat alveolar epithelial cell monolayers show low rates of transepithelial passive restricted diffusion (14,17). However, the presence of certain entities in the surrounding microenvirons of the alveolar epithelium has a direct effect on uptake of drugs. For example, surfactant lipids, which are endogenous in the lungs and secreted by type II pneumocytes, have been implicated in insulin absorption enhancement (20). In this study, primary cultured rat alveolar epithelial cell monolayers were used to investigate the effects of conditioned medium on transalveolar insulin absorption. We determined that rat alveolar epithelial type II cell-like monolayers express and secrete protein(s) that significantly increase the rate of absorption of insulin across the alveolar epithelial barrier.

MATERIALS AND METHODS

Isolation and Culture of Rat Alveolar Epithelial Cells

Primary rat alveolar epithelial cells were obtained and purified following the procedure described by Borok *et al.*. (19). The partially 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 Inc., Acton, MA, USA) at 1.0×10^6 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 a defined serum-free medium (MDSF, a 1:1 mixture of DMEM and Ham's F-12, Sigma Chemical, St. Louis, MO, USA) supplemented with 10% newborn bovine serum, 1.25 mg/ml bovine albumin, 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 and are designated as RAECM-I. In order to maintain the type II cell-like phenotype and morphology, keratinocyte growth factor (KGF, 10 ng/ml, Calbiochem, San Diego, CA, USA) was added to bathing medium from day 0 and to fresh culture medium for feeding cells on day 3 and onward. This latter group of cultured cells was designated as RAECM-II. Transepithelial resistance (TEER) and potential difference (PD) of these RAECM-I and -II, when measured on day 6 using an epithelial volt-ohm meter (EVOM, World Precision Instruments, Sarasota, FL, USA) is >2,000 Ω.cm^2 and >10 mV, respectively.

Transport of Insulin

Human insulin (Sigma) was iodinated using the chloramine T method (21). ^{125}I-insulin had a specific activity of 3×10^9 cpm/mg. RAECM-I and -II, used on day 6 for transport studies, were washed once with MDSF and incubated for 30 min at 37°C. Apical medium was aspirated and replaced with medium containing ^{125}I-insulin (4 μg/ml final solution i.e. 44×10^4 cpm/μg). At 2 h post-dosing, basolateral medium was collected and total radioactivity was measured using a Packard gamma counter. Samples were treated with 15% trichloroacetic acid (TCA) on ice for 15 min, followed by centrifugation in a microfuge (Beckman J-6B, 2,000 rpm, 4°C). Supernatant was aspirated and radioactivity associated with the pellet was measured in the gamma counter to determine intact insulin-associated radioactivity.

Conditioned Medium Collection and Characterization

Effect of Serum, KGF, CMII and CMI on Insulin Transport

Radiolabeled insulin was dissolved in defined serum-free medium supplemented with 10% newborn bovine serum (NBS) and abbreviated as SM unless mentioned otherwise, or SM supplemented further with KGF (10 ng/ml). Conditioned medium, CMII and CMI, from rat alveolar cell cultures with and without KGF, respectively, were collected on day 6, and used to dose radiolabeled insulin for comparison using RAECM-I and -II. In some experiments, 1% NBS was used in lieu of 10% NBS in KGF-containing culture medium (denoted as 1 and 10% SM, respectively) to examine if the extent of transport enhancing factor(s) in CMII would be affected.

Molecular Sizing

On day 6, conditioned medium was collected from the apical (or basolateral) bathing medium of either RAECM-I or RAECM-II and designated as aCMI (bCMI) or aCMII (bCMII), respectively. Of these various conditioned media, we focused on characterizing aCMII (hereafter designated as CMII for clarity), as others did not show much activity in enhancing insulin transport (see details below). CMII was

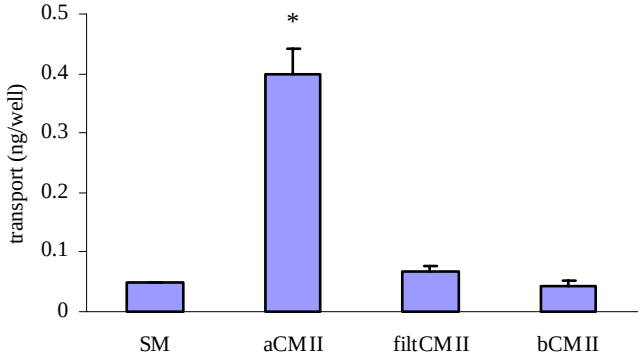

Fig. 1. Effects of various media on apical-to-basal transport of insulin across RAECM-II. Apical-to-basal transport of intact ^{125}I-insulin measured at apical donor concentration of 4 μg/ml across RAECM-II in culture medium containing 10% NBS (SM), conditioned apical medium from RAECM-II (aCMII), filtrate from type II apical conditioned medium (filtCMII) centrifuged with Centricon-50 at 2,000 g, at 4°C for 30 min, and conditioned basal medium from RAECM-II (bCMII). Data represent mean ± SD. * indicates $p<0.01$ from all others as determined by one-way analysis of variances ($n=4$).

concentrated in the Centriplus YM-50 (molecular weight cut off of 50 kDa, Millipore, Amicon, USA), where both retentate and filtrate were used to test the activity of CMII in modulation of radiolabeled insulin transport across RAECM-II by the method described above.

Characterization of Conditioned Medium

To test the heat lability of the putative transport enhancing factor(s), an aliquot of CMII was heated at 80°C for 15 min, cooled, centrifuged to remove coagulated proteins and used for transport studies of radiolabeled insulin. Supersaturated ammonium sulphate solution (4M), which renders salting out of protein(s), was added slowly to an aliquot of CMII on ice to a final concentration of 50%. After centrifugation, the supernatant was removed and the precipitate was re-suspended to original volume with water and dialyzed twice against phosphate-buffered saline (PBS). The resulting protein-rich CMII was used to dose radiolabeled insulin for transport studies, using PBS as vehicle control.

Transport of Paracellular vs Transcellular Markers

To determine whether CMII was affecting the paracellular pathway of drug transport, ^{14}C-mannitol (1 μCi/ml), a paracellular marker, was dosed in SM, CMI and CMII for transport studies across RAECM-II at 37°C for 2 h. The basolateral medium was then collected and measured for radioactivity using a liquid scintillation counter (Beckman LS1801). Furthermore, to assess CMII effect on fluid phase endocytosis, horseradish peroxidase (1 mg/ml) was dosed in a similar manner as mannitol transport experiments and the amount transported was measured using enzymatic assay (22). Transport of ^{125}I-insulin (4 μg/ml) dosed in SM or CMII was studied across RAECM-II at 37, 16 and 4°C for 1 h to determine the contribution of transcellular transport of insulin versus paracellular transport by assessing the temperature-dependency of insulin transport across the alveolar epithelial barrier. Finally, protease inhibitor cocktail (Sigma) added to the dosing vehicle was used to study the effect of CMII versus SM on ^{125}I-insulin degradation pathway.

Statistical Analyses

Data are presented as mean ± SD. Statistical analysis was performed using one-way analysis of variances between groups (≥ 3), followed by Tukey's post-hoc tests. The level of statistical significance was $p<0.05$ or lower.

RESULTS

Insulin Transport in CMII

A four-fold increase in the apical-to-basolateral transport of the intact ^{125}I-insulin across RAECM-II was observed in the presence of the conditioned medium which was collected from the apical compartment of RAECM-II (CMII), as compared to that in fresh serum-supplemented medium (SM) (Fig. 1). This increase of ^{125}I-insulin transport was not observed when CMII was replaced by the conditioned medium collected from the basolateral compartment of RAECM-II monolayers. As shown in Fig. 2, CMII-enhanced apical-to-basolateral transport of ^{125}I-insulin was significant only across RAECM-II ($p<0.05$), but not RAECM-I ($p>0.05$). On the other hand, CMII did not affect the basolateral-to-apical transport of ^{125}I-insulin across

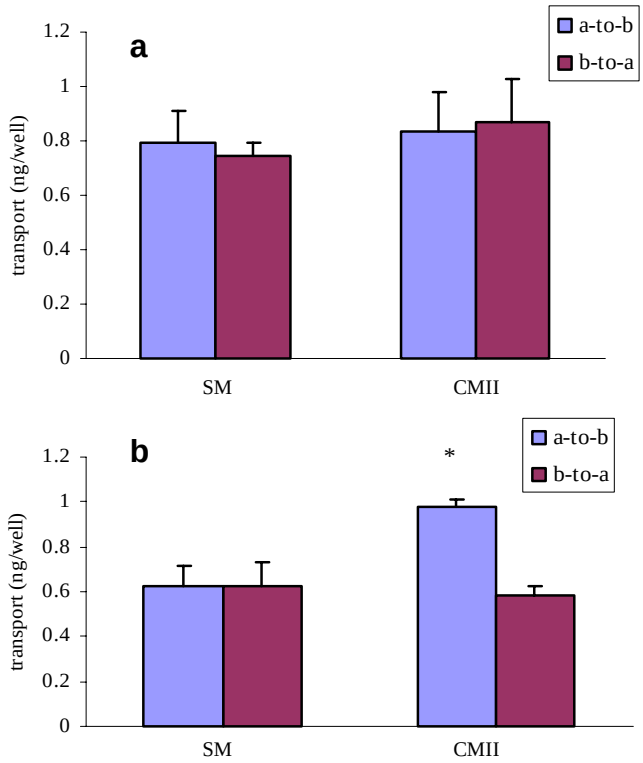

Fig. 2. Comparison of insulin transport across RAECM-I and RAECM-II. Apical-to-basolateral (a-to-b) and basolateral-to-apical (b-to-a) transport of intact ^{125}I-insulin measured at donor concentration of 4 μg/ml across **a** RAECM-I and **b** RAECM-II in culture medium containing 10% NBS (SM) and conditioned apical medium from RAECM-II (CMII). Data represent mean ± SD. * indicates $p<0.05$ from all others as determined by one-way analysis of variances ($n=3$).

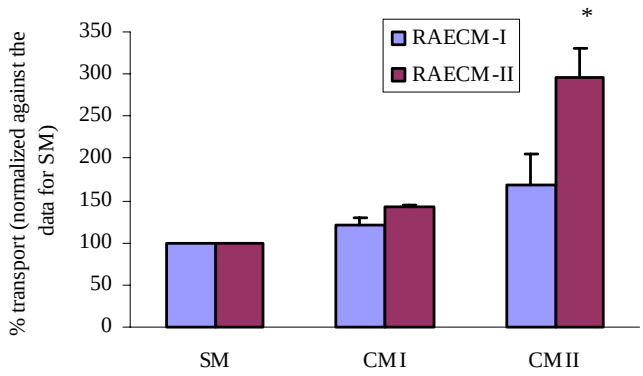

Fig. 3. Comparison of insulin transport across RAECM-I and RAECM-II. Apical-to-basolateral transport of intact ^{125}I-insulin measured at apical donor concentration of 4 μg/ml across RAECM-I and RAECM-II in culture medium containing 10% NBS (SM), conditioned apical medium from RAECM-I (CMI) and conditioned apical medium from RAECM-II (CMII). Data represent mean ± SD. * indicates $p < 0.05$ from all others as determined by one-way analysis of variances ($n=3$).

either RAECM-I or RAECM-II. Furthermore, conditioned medium from apical compartment of RAECM-I (CMI) did not have any effect on apical-to-basolateral transport of ^{125}I-insulin in either RAECM-I or RAECM-II (Fig. 3). Based on these observations, subsequent studies were focused only on CMII for the enhancement of apical-to-basolateral transport of insulin across RAECM-II.

Biochemical and Physical Characterization of CMII

To determine a molecular weight range for the factor(s) causing enhanced insulin transport across RAECM-II, CMII was centrifuged in a Centricon device (50 kD molecular weight cutoff) and ^{125}I-insulin was dosed in the resultant filtrate (filtCMII). Figure 1 shows that enhanced insulin transport was not seen when filtCMII was used for dosing, supporting our observation that the enhancing effect of CMII resides with soluble factor(s) whose molecular weight equals to or is greater than 50 kDa. Next, when CMII was heated at 80°C for 15 min, a complete loss of effect on insulin transport across RAECM-II was observed (Table I), indicating that the enhancing factor(s) may be protein(s). Moreover, when ammonium sulfate was added to CMII, about 95% of enhancement activity of CMII was retained in the precipitated fraction, suggesting that the enhancing effect for insulin transport is afforded by protein(s) in CMII.

We next investigated the effects of supplementing serum on alveolar epithelial elaboration of the factor(s) for enhancing insulin transport. After 6 days of culture with 1% newborn bovine serum (NBS) in lieu of 10% serum, culture medium from apical compartment of RAECM-II was collected and tested for the enhancing effects on insulin transport across RAECM-II. Figure 4 shows that when conditioned medium collected from monolayers cultured in 1% NBS supplementation instead of 10% NBS (denoted as 1 and 10% CMII, respectively), 1% CMII had a significantly ($p < 0.05$) lower enhancement of insulin transport across RAECM-II compared to that of 10% CMII. This latter data set indicates that the production of enhancing factor(s) by RAECM-II may require component(s) present in NBS.

Effects of Putative Factor(s) in CMII for Enhancing Insulin Transport on Paracellular vs Transcellular Pathways

The effect of CMII on transport of a paracellular marker (mannitol) and a fluid-phase endocytosis marker (horseradish peroxidase), were also investigated. Mannitol dosed in SM, CMI and CMII all showed similar magnitude of transport across RAECM-II (Fig. 5a), with no significant changes in TEER (2,500 Ω.cm^2). Horseradish peroxidase transport (presumably occurring via fluid-phase transcytosis) across RAECM-II was similar in magnitude, irrespective of medium used for dosing (i.e., SM, CMI and CMII) (Fig. 5b). Further implications of CMII factor(s) on endocytosis in RAECM-II were assessed by incubating radiolabeled insulin in either CMII or SM at 37, 16 or 4°C. Figure 6 shows that insulin transport at 16 and 4°C was decreased 90% from that observed at 37°C. In contrast, the amount of insulin transport when dosed with SM, was decreased by only 40 and 70% at 16 and 4°C, respectively.

Effect of CMII on ^{125}I-Insulin Degradation

Throughout the transport experiments (apical-to-basal) across RAECM-II, it was observed that a smaller fraction of intact radiolabeled insulin (measured by trichloroacetic acid precipitation) was present in the basolateral compartment when dosed in CMII compared to that of SM (5 vs 10%, respectively). Incubation of ^{125}I-insulin in CMII or SM for 2 h at 37°C did not yield significant degradation in either medium (data not shown), indicating that the factor(s) in CMII was (were) not a contributing source of ^{125}I-insulin degradation. However as shown in Fig. 7, the addition of a protease inhibitor cocktail, which included mostly cytoplasmic and lysosomal protease inhibitors, to CMII resulted in a significantly higher ($p < 0.01$) percent of intact ^{125}I-insulin in the basolateral compartment of RAECM-II compared to that of CMII devoid of inhibitors (20 vs 5%). On the other hand, there was no significant difference in the amount of intact ^{125}I-insulin transported in SM in the presence or absence of protease inhibitors.

DISCUSSION

Our findings demonstrate that conditioned medium (CMII), obtained from cultured type II cell-like alveolar epithelial monolayers (RAECM-II), contains (a) protein factor(s) which can increase transport of insulin across the alveolar epithelial barrier. This conclusion is based on the following observations: (1) neither newborn bovine serum components nor keratinocyte growth factor (KGF) per se, both used in culture medium, produced increases in trans-alveolar epithelial transport of insulin; (2) when centrifuged using a 50 kDa molecular weight cut-off Centricon filter, enhancement of transport was only in the retentate of the CMII; (3) the factor(s) in CMII was (were) heat labile and precipitated in 50%

Table I. Characterization of Conditioned Medium from Rat Alveolar Epithelial Type II Cell-like Monolayer Cultures (CMII)

Treatment	% Activity Remaining
Heat (80°C, 15 min)	0%±0.04% ($n=3$)
Protein precipitation 4M $(NH_4)_2SO_4$	95%±2% ($n=3$)

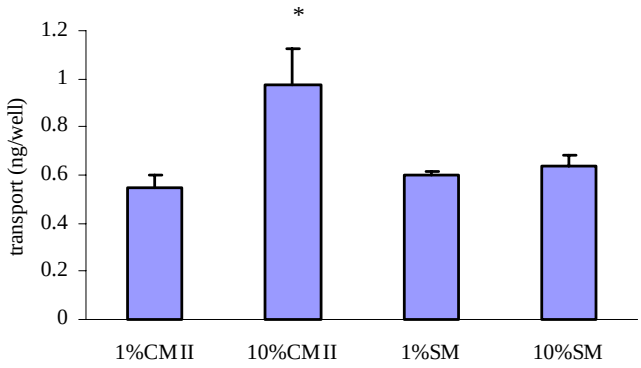

Fig. 4. Effect of culture medium containing 1 or 10% newborn bovine serum (SM) on insulin transport across RAECM-II. After 6 days of culturing RAECM-II in presence of 1 or 10% SM, apical fluids were collected and designated as 1% CMII or 10% CMII, respectively. Apical-to-basal transport of intact insulin measured at apical donor concentration of 4 μg/ml across RAECM-II in either 1 and 10% SM, or 1 and 10% CMII, respectively. Data represent mean ± SD. * indicates $p<0.05$ from all others as determined by one-way analysis of variance ($n=3$).

ammonium sulphate; (4) conditioned medium from type I cell-like alveolar epithelial cell monolayers (RAECM-I), CMI, did not increase peptide transport, suggesting the absence in CMI of (a) factor(s) secreted predominantly by RAECM-II; and 5) the increase of insulin transport enhanced by CMII was observed only across RAECM-II, but not RAECM-I.

Absorption enhancers in protein/peptide delivery have been studied extensively (3,23). Some of the enhancers act by loosening tight junctions and allowing increased paracellular transport (ethanol, chitosan, bile salts) (24–26). However, CMII did not change permeability of mannitol (a paracellular marker) when compared to SM and, in addition, did not cause any significant decrease in TEER, indicating that CMII most likely acts on transcellular routes for insulin absorption. The use of protease inhibitors has been successful in enhancing bioavailability of various peptides (5,6,26,27). Insulin, which transports paracellularly at baseline (4), when administered through the lungs, is likely to be most susceptible to membrane-associated aminopeptidase (28). Although peptide degradation is a major concern, incubating insulin in SM and CMII for 2 h at 37°C showed no significant degradation upon TCA precipitation. Therefore, possible degradation of insulin takes place either on the surface cell membrane or within the cell after insulin uptake. In this study, using a protease inhibitor cocktail protected insulin from degradation in CMII rather than SM. This might seem to contradict what has been published regarding insulin absorption enhancement in presence of protease inhibitors (5,28); however, the inhibitor cocktail composition in our study, which mainly protected against cytosolic and lysosomal proteases, was different from reagents used in other studies (5,9,28) and might not have had the same protective activity against insulin degradation by aminopeptidase present on cell membrane surface. These results may indicate a partial rerouting of insulin transport through the transcellular pathway, exposing it to degradation by lysosomal proteases or cytosolic insulin degrading enzymes known to be present in cultured type II alveolar cells (29). Regardless of the high degradation, this transcellular pathway can maintain the amount of insulin transport at a higher level than that in cells incubated with SM.

Bile salts and lipid surfactants have also been studied as enhancers in protein formulations for pulmonary delivery (4,11,18). The mechanism of action has been attributed to opening of tight junctions, production of insulin monomers, and regional phase changes and/or pore formation due to reverse micelle formation in cell membranes (7,30,31). When 1,2-dipalmitoyl phosphatidylcholine (DPPC), a component of pulmonary surfactants, was incorporated with a mixture of liposome and insulin and delivered via pulmonary routes, a significant hypoglycemic effect was observed, suggesting a possible effect of the lipid surfactant on membrane perturbation (20). The results of the current study, however, indicate that the factor is a protein. Whether this factor can cause de-aggregation of insulin hexamers or otherwise cause phase changes in biomembranes remains to be elucidated. Moreover, membrane re-arrangement or fluidization seems unlikely since enhanced insulin transport was observed only in the apical-to-basal direction and not the other way around. Furthermore, CMII from the apical but not basolateral compartment of RAECM-II showed enhancement of insulin transport, indicating the possibility that the factor(s) were solely secreted and may also be recycled at the apical membrane.

The involvement of endocytosis as an enhancement mechanism for transalveolar epithelial insulin transport in the presence of conditioned medium from RAECM-II was

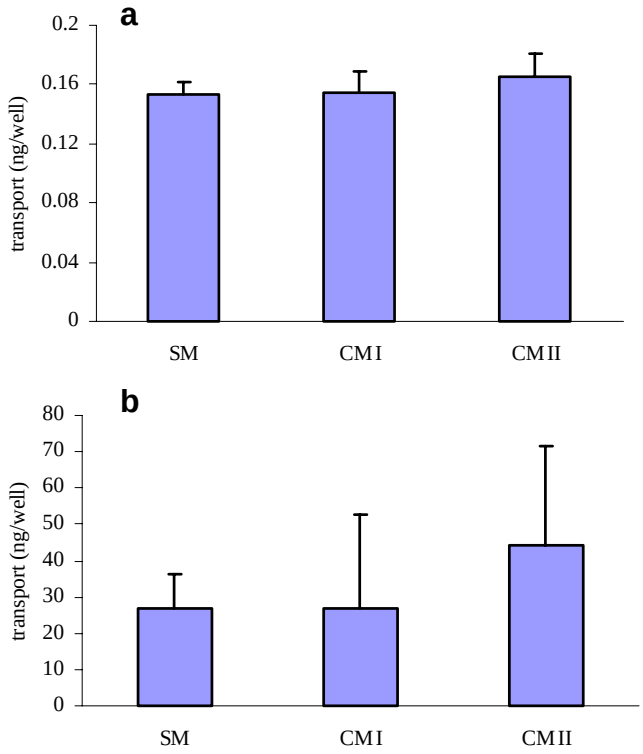

Fig. 5. Effect of CMII on paracellular transport and fluid-phase transcytosis across RAECM-II. Apical-to-basal transport of **a** ^{14}C-mannitol measured at apical donor concentration of 0.02 μg/ml and **b** horseradish peroxidase measured at apical donor concentration of 1 mg/ml across RAECM-II in culture medium containing 10% NBS (SM), conditioned apical medium from RAECM-I (CMI) and conditioned apical medium from RAECM-II (CMII) ($n=3$).

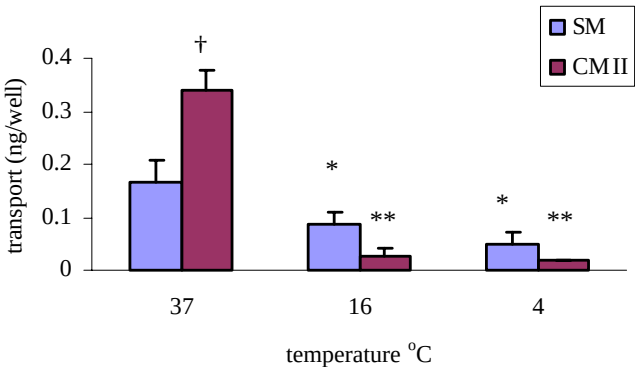

Fig. 6. Temperature dependence of CMII-enhanced insulin transport across RAECM-II. Apical-to-basal transport of insulin measured at apical donor concentration of 4 μg/ml across RAECM-II in culture medium containing 10% NBS (SM) and conditioned apical medium from RAECM-II (CMII) at 37, 16 and 4°C. Data represent mean ± SD. * indicates $p<0.05$ compared to the data observed at 37°C with SM; ** indicates $p<0.01$ compared to the data observed at 37°C with CMII; † indicates $p<0.01$ compared to the data observed at 37°C with SM as determined by one-way analysis of variances ($n=4$).

also investigated. Although CMII did not have marked effects on triggering fluid-phase endocytosis as determined by the marker horseradish peroxidase, effect of CMII on insulin transport was abolished when incubated at 16 and 4°C. By contrast, insulin dosed in SM followed the conventional rule of passive diffusion with a decrease of ~50% when incubated at 16°C (32). Taken together, these results, along with the effect of the protease inhibitor cocktail on insulin transport, may suggest that the factor(s) present in CMII could either be interacting with insulin or influencing the cellular membrane thereby ultimately leading to a rerouting of insulin transport from a paracellular pathway to a transcellular pathway other than nonspecific pinocytosis (i.e, possibly through macro-pinocytosis or receptor-mediated endocytosis). To date, studies involving receptor-mediated endocytosis as a target mechanism for enhancing peptide delivery have used ligand-based conjugates such as transferrin conjugated to insulin, GCSF or HRP (14,15,33). However, whether or not insulin is transported via receptor-mediated transcytosis when complexed with factor(s) in CMII, remains to be determined.

It is known that type II pulmonary epithelial cells secrete lipids and protein surfactants, a characteristic which is maintained for the length of cell culture (seven days) upon supplementation of culture medium with KGF (19). To determine whether these secreted factor(s) are implicated in enhancing peptide transport, it will be necessary to at least partially purify conditioned medium and narrow down the range of proteins involved. However, the major limitation is the presence of newborn bovine serum proteins in CMII that makes it difficult to accomplish such a step. Culturing alveolar cells in medium supplemented with only 1% instead of 10% serum abolished the effect of CMII requiring a different approach for this study.

Relevant to protein and peptide drug delivery, the lungs have proven to be useful for systemic absorption. Established reasons are inherent to the lung's large surface area, thin epithelium, rich blood supply, and extracellular protease inhibitory activity (3). However, studies regarding mechanistic information of interplay between the lung epithelium and its microenvironment in drug delivery are still lacking. In particular, the discrepancy between the low *in vitro* peptide transport across cultured RAECMs and the high *in vivo* bioavailability of several peptide and protein drugs is still largely unsolved.

Our results showed that CMII enhanced insulin transport across RAECM-II but not RAECM-I. These observations may be due to the absence of a receptor (on RAECM-I) which is involved in endocytosis of the factor(s) or the presence of a receptor with either low abundance and/or affinity, since several phenotypic changes are known to occur when RAECM-II transdifferentiate into RAECM-I *in vitro* (19,34). Given that alveolar type II cells cover about 5% of the total alveolar surface, the additional effect afforded by an endogenous factor may contribute far less to total net absorption of insulin *in vivo*. This scenario may also be true if peptides are transported across predominately type I cells of the *in vivo* lung. However, there have been limitations in translating *in vitro* data on drug transport to *in vivo* situation, evident in the studies utilizing IgG or albumin in the context of their cognate receptors, FcRn and gp60, respectively (35). Since the mechanisms of enhancement are not entirely clear yet, the *in vivo* implications remain to be determined. In summary, we show that conditioned medium from rat alveolar type II cell-like monolayers contain (a) protein

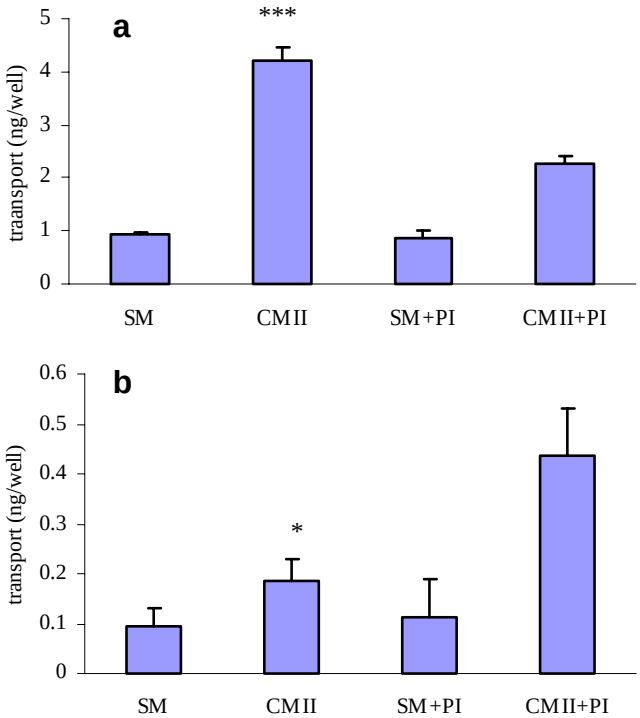

Fig. 7. Effect of protease inhibitors (PI) on insulin transport across RAECM-II. Apical-to-basal transport of **a** total and **b** intact ^{125}I-insulin measured at apical donor concentration of 4 μg/ml across RAECM-II in culture medium containing 10% NBS (SM) and conditioned apical medium from RAECM-II (CMII) without or with protease cocktail inhibitor (SM+PI and CMII+PI, respectively). Data represent mean ± SD. * indicates $p<0.05$ compared to the data for SM; ** indicates $p<0.01$ from all others; *** indicates $p<0.001$ from all others as determined by one-way analysis of variances ($n=3$).

factor(s) which facilitate(s) transcellular transport of insulin across primary RAECM-II. Underlying mechanisms and identification of the key protein(s) in the protein/peptide drug transport across lung alveolar epithelial cells await further studies.

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