Evidence for LHRH-Receptor Expression in Human Airway Epithelial (Calu-3) Cells and Its Role in the Transport of an LHRH Agonist

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Purpose. To determine whether LHRH-receptor is expressed in Calu-3, a human bronchial epithelial cell line, and to further determine whether this receptor plays a role in the transport of deslorelin, an LHRH agonist.

Methods. Using cultured monolayers of Calu-3 grown at air-interface, the presence and localization of LHRH-receptors in Calu-3 cells was determined using immunochemical methods. To determine the mechanisms of deslorelin transport, the directionality [apical-basolateral (A-B) and basolateral-apical (B-A)] of deslorelin transport across Calu-3 monolayers and the effects of temperature (37°C and 4°C) and an energy depletor (2,4-dinitrophenol) were investigated. To determine the role of LHRH-receptor in deslorelin transport across Calu-3 monolayers, the influence of an LHRH-receptor antisense oligonucleotide on the LHRH-receptor expression and deslorelin transport was studied. Also, the effect of a competing LHRH agonist, buserelin, on deslorelin transport was determined.

Results. Immunofluorescence studies indicated the predominance of LHRH-receptor in Calu-3 cells at the apical and lateral surfaces. Western blot and RT-PCR studies further confirmed the expression of LHRH-receptor in Calu-3 cells. Deslorelin transport across Calu-3 monolayers was vectorial, with the cumulative A-B transport (1.79 ± 0.29%) at the end of 240 min being higher than the B-A transport (0.34 ± 0.11%). Low temperature as well as 2,4-dinitrophenol abolished this directionality. LHRH-receptor antisense oligonucleotide decreased the receptor expression at the mRNA and protein level and reduced the A-B deslorelin transport by 55 ± 4%, without affecting the B-A transport, suggesting a role for LHRH-receptor in the vectorial transport of deslorelin. In addition, buserelin reduced the A-B deslorelin transport by 56 ± 5% without affecting the B-A transport.

Conclusions. Taken together, our results provide evidence that deslorelin is transported across the respiratory epithelium via the LHRH-receptor.

KEY WORDS: buserelin; deslorelin; LHRH-receptor antisense oligonucleotide; Calu-3 cells.

INTRODUCTION

Deslorelin, a nonapeptide with the amino acid sequence pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-ProNHEt, is a leutinizing hormone releasing hormone (LHRH) agonist, which is 144 times more potent than the native LHRH (1). Deslorelin is of therapeutic value in the treatment of endometriosis, uterine fibroids, precocious puberty, and breast and prostate cancers (2). LHRH agonists can be delivered to the systemic circulation via the respiratory routes. Indeed, two LHRH agonists, buserelin (Suprefact) and nafarelin (Synarel) are currently available as nasal sprays. Previously, we demonstrated that deslorelin is degraded by various mucosal tissues including the nasal tissue (3–5). Furthermore, we elucidated the pathways and kinetics of deslorelin degradation in Calu-1, a human airway epithelial cell line (6). Although it is well recognized that the respiratory epithelium constitutes a barrier against drug absorption following inhalation or nasal administration, the transport of deslorelin across respiratory epithelium has not been elucidated (7,8). Thus, the objective of this study was to elucidate the mechanism of deslorelin transport across Calu-3 monolayers, an *in vitro* model for the respiratory epithelium.

Calu-3 is a mucus-producing submucosal gland carcinoma cell line derived from human bronchial epithelium that is capable of forming tight, polarized, and well differentiated cell monolayers (8). Calu-3 cells also possess other functional characteristics such as asymmetric transferrin transport (9), presence of functional cytochrome P450 isozymes 1A1, 2B6, and 2E1, and the expression of cystic fibrosis transmembrane conductance regulator (CFTR) (10), similar to the respiratory epithelia *in vivo*. Indeed, the permeability characteristics of Calu-3 cells correlate well with primary cultured rabbit tracheal epithelial cells *in vitro* ($r^2 = 0.91$), and the rate of drug absorption from the rat lung *in vivo* ($r^2 = 0.94$), making this model a potentially useful one to predict absorption of molecules delivered by the respiratory routes (11). All of these factors along with its ability to differentiate in culture in a period of 10 days (12) makes Calu-3 an attractive *in vitro* model to study the transport mechanisms of peptide drugs across respiratory epithelia.

Transport of peptide drugs across respiratory epithelia has been postulated to occur primarily via the paracellular route (13). Previous *in vivo* studies indicated that molecular weight cutoff for nasal absorption is 2 kDa, with an inverse relationship between the nasal bioavailability and the molecular weight of the permeant (14). Clinical studies with healthy human volunteers indicated that the bioavailability of leuprolide, another LHRH agonist, following suspension and solution aerosol administration was 28 and 6.6%, respectively, and the nasal bioavailability was 2.4% (15). This is much greater than the oral bioavailability of leuprolide, which ranges between 0.1 and 0.2% (16). Thus, the respiratory routes are beneficial for LHRH agonist delivery and attempts have been made to improve the bioavailability of these molecules (17). Although one study assessed the transport of buserelin and LHRH across excised bovine nasal tissue and reported that the permeability of buserelin was 10 times greater than native LHRH (18), no studies exist on the transport mechanisms of LHRH agonists across the respiratory epithelium. A better understanding of the mechanisms of transport of LHRH agonists will likely facilitate approaches to improve the bioavailability of these drugs.

The aim of this study was to investigate the mechanisms of deslorelin transport across Calu-3 cells and to determine whether the LHRH-receptor plays a role in the transport of deslorelin across Calu-3 cells. There is no prior evidence for the transcellular route of transport for LHRH agonists across epithelial cells or any other cell type. For this purpose, we have investigated the directionality and energy-dependence

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of deslorelin transport, localized LHRH-receptor in Calu-3 cells, and used an antisense oligonucleotide directed toward the LHRH-receptor to decrease the receptor expression and investigate its effect on deslorelin transport. Furthermore, we have used a competing LHRH agonist of similar structure [(D-(tBu)Ser^6-Des Gly^{10}) LHRH ethylamide (buserelin)] and determined its influence on deslorelin transport across Calu-3 monolayers.

MATERIALS AND METHODS

Chemicals

Cell culture materials and reagents were obtained from Gibco (Grand Island, NY, USA) and Becton Dickinson Labware (Franklin Lakes, NJ, USA). Deslorelin was kindly provided by Balance Pharmaceuticals, Inc. (Santa Monica, CA, USA). Calu-3 cells were obtained from the ATCC (Manassas, VA, USA). Lipofectin was obtained from Invitrogen Corporation (Carlsbad, CA, USA), and trifluoroaceticacid (TFA) and all other chemicals were obtained from Sigma (St. Louis, MO, USA). Acetonitrile of high-performance liquid chromatography (HPLC) grade was obtained from Fisher Scientific (Pittsburg, PA, USA). The composition of the assay buffer (isotonic, pH 7.4) used in transport studies was reported previously (19).

Cell Culture

Calu-3 cells were obtained from the ATCC (ATCC no. HTB-55). Cells free of mycoplasma were maintained in culture at 37°C, 5% CO_2, and 95% O_2 in Minimum Essential Medium with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) supplemented with nonessential amino acids, 1% sodium pyruvate, 2% L-glutamine, and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (Life Technologies). Cells were plated in 75 cm^2 cell culture flasks (Corning Corporation, Corning, NY, USA) and subcultured at 90% confluence using a 0.25% trypsin/0.1% EDTA in maintenance medium. The culture medium was changed every 2 days and cells were split 1:2 during each passage. Cells of passages 25–36 were used in this study.

Confocal Laser Scanning Microscopy for LHRH-Receptor Visualization in Calu-3 Monolayers

For confocal microscopy, Calu-3 cells were grown on 6-well clear polyester Transwells (0.4 μm pore size; Costar, Corning, NY, USA) for improved microscopic visualization. On day 10, after removing the medium, cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4) and fixed during a 15 min incubation at room temperature (Histochoice; Sigma). The fixed cells were washed again and permeabilized with 0.1% Triton-X 100 followed by blocking of nonspecific binding sites for 1 h (1% w/v goat serum in PBS). Following this, cells were incubated for another 1 h at room temperature with a 1 μg/ml solution of primary LHRH-receptor antibody (GnRH-R Ab03, Lab Vision Corporation, Fremont, CA, USA), washed, and incubated with a secondary antibody conjugated with CY3 (Molecular Probes, Eugene, OR, USA). Images of LHRH-receptor immunostained cells were acquired under an oil immersion objective (×63) with a confocal laser microscope (Zeiss Confocal LSM410, Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) equipped with an argon-krypton laser. Negative controls were prepared similarly except that an isotypic IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL, USA) was used instead of the primary LHRH-receptor antibody.

Cellular Transport of Deslorelin Across Calu-3 Monolayers

Briefly, Calu-3 cells were plated at a density of 10^5 cells/cm^2 in collagen-coated Transwells (12-mm diameter, 0.4-μm pore size; Costar). After the cells had attached to the Transwells overnight, the media was removed from the apical compartment to allow the monolayers to grow at the air interface. Thereafter, the monolayers were allowed to differentiate under air-interface conditions by replenishing the medium in the basolateral compartment every other day. Air-interface conditions have been shown to improve the differentiation of primary cultures of human airway epithelia (20). Transport experiments were conducted using cultures of days 10–12. Prior to the actual transport studies, the cell culture medium was removed, and the cells were allowed to equilibrate in prewarmed (37°C) assay buffer. Following equilibration, the transepithelial electrical resistance of each monolayer was measured using EVOM chopstick electrodes (World Precision Instruments). The transepithelial electrical resistance was also monitored at the end of each experiment to assess the integrity of the cell monolayers. Both apical to basolateral (A-B) and basolateral to apical (B-A) transport studies were performed. The apparent permeability coefficient (P_{app}) and % deslorelin transport were calculated using Eqs. 1 and 2, respectively.

$$P_{app} = (dM/dt)/(A \times C_d) \quad (1)$$

$$\text{Deslorelin transport (\%)} = (\text{Cumulative deslorelin transport/Initial amount in donor chamber})* 100 \quad (2)$$

In Eq. 1, dM/dt is the slope of the cumulative amount of deslorelin transported vs. time, A is the monolayer area (1.13 cm^2) available for transport, and C_d is the initial donor drug concentration. Permeation data were corrected for dilution of the receiver solution with sample volume replenishment. Deslorelin transported was quantified by a RP-HPLC method as described latter in this section.

Effect of an Antisense Oligonucleotide on LHRH-Receptor Expression in Calu-3 Monolayers

Reverse Transcriptase-Polymerase Chain Reaction

LHRH-receptor mRNA expression in Calu-3 cells, excised bovine nasal tissue, rat trachea, and rat lungs was determined using reverse transcriptase-polymerase chain reaction (RT-PCR). Excised tissues were assessed in order to ensure the expression of LHRH-receptor in normal tissues. Bovine nasal mucosa was obtained from freshly slaughtered cattle at the local slaughterhouse (J & J Meats, Elkhorn, NE, USA). The nasal tissue was isolated and prepared for experiments as described previously (21). The skin covering the nose was removed, and the frontal part of the nasal conchae was dissected. The mucosal tissue was then carefully stripped from the cartilage using a pair of tweezers and used for expression studies. LHRH-receptor expression was assessed in the medium turbinate posterior (beyond 2.5 inches past the

tip of nares) region of the bovine nose. In case of rat tissues, following euthanization, an incision was made at the level of the esophagus, and the lung lobes were isolated along with the trachea. The trachea representative of upper airways, and lung lobes representative of lower respiratory tract, were used for expression studies. Following total RNA isolation using RNA STAT-60 RNA isolation kit (TEL-TEST, Friendswood, TX, USA), RT-PCR was performed with an Access RT-PCR system (Promega, Madison, WI, USA). RT-PCR for LHRH-receptors and 18s rRNA (internal control) was performed in a standard 50 μl reaction mixture containing 0.2 mM deoxytriphospahate nucleotides, 2 mM $MgCl_2$, 20 pmol each of sense and antisense primers, 5 U of Tfl DNA polymerase, and 5 U of avian myeloblastosis virus (AMV) reverse transcriptase. The amplification of the cDNA was performed as described previously (22). The amplified products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide and were subjected to DNA sequencing. The primers used for LHRH-receptors were forward, 5′-GACCTTGTCTGGAAAGATCC-3′ (bp 93-112); and reverse, 5′- CAGGCTGATCACCACCATCA-3′ (bp 392-411) (22). Densitometric analysis for LHRH-receptor mRNA expression was performed using Nucleovision Imaging System (Nucleotech, San Mateo, CA, USA). The amplified products were then isolated and sequenced to confirm the identity (University of Nebraska, DNA sequencing facility, Omaha, NE, USA). A Blast sequence search revealed that the observed band corresponded to a mammalian LHRH-receptor.

Western Blot

Cell monolayers of Calu-3 were solubilized in phosphate-buffered saline (pH 7.4) containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN, USA) and 1% sodium dodecyl sulfate (SDS), and the total protein content of each of the cell lysates was estimated. For separation, lysate samples (50 μg) were loaded onto preformed 10% polyacrylamide gels (Bio-rad, Hercules, CA, USA). Cell lysates of prostate carcinoma cell line, LnCap, loaded at 30 μg were used as positive controls (22). Molecular weight markers ranging from 220 to 14.3 kDa (Amersham Life Science, Arlington Heights, IL, USA) were used to identify the LHRH-receptor and β-actin protein bands. The proteins on the gels were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run at 60 V for 10 min and then continued at 120 V for another 1.5 h. The proteins were then transferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 4°C using a 480 mA current. The immunoblots were done using the LHRH-receptor specific monoclonal antibody GnRHAb-3 (Neomarkers, LabVision, CA, USA) or the monoclonal anti β-actin antibody (Sigma, St Louis, MO, USA). The transferred proteins were treated with the blocking buffer, which contained 0.3% Tween-20 and 1% BSA and incubated with the specific antibodies overnight at 4°C. The antibody was used at 1:100 dilutions. Following a series of washes with the washing buffer, a secondary horseradish peroxidase conjugated mouse immunoglobulin (Ig) antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) was added (1:1500) and incubated at 4°C for 1 h. LHRH-receptor bands were visualized using a chemiluminescence kit (Amersham Life Science).

Decreased LHRH-Receptor Expression and Effect on Deslorelin Transport

This set of experiments were designed to elucidate the effect of antisense oligonucleotide of LHRH-receptor mRNA on LHRH-receptor expression and the role of LHRH-receptor in deslorelin transport across Calu-3 cells. For this purpose, phosphorothioated antisense oligonucleotide (AON) directed against the LHRH-receptor 5′ GCATTGTTAGCCATATTTCTCC 3′ (22-mer) and sense oligonucleotide (SON) 5′- GGAGAAATATGGCTAACAATGC-3′ (22-mer) were synthesized (23). These oligonucleotides were previously validated by Seong *et al.* for reducing LHRH-receptor expression (23). A GENBANK search for the alignment of these oligonucleotides indicated a 100% match for binding with LHRH-receptor mRNA. No significant matches were found for the binding of these oligonucleotides with other RNA species. Briefly, Calu-3 cells plated in t-25 flasks were treated with 10 μM AON and lipofectin (10 μg/ml) in serum-free medium for 24 h, after which the cells were placed in 2% serum containing medium for 12 h. At the end of 36 h after the start of treatment, mRNA expression of LHRH-receptors was determined by RT-PCR, and protein expression was determined using Western blot as described above. To assess the effect of decreased LHRH-receptor expression on deslorelin transport, Calu-3 cells grown in Transwell inserts were similarly treated with AON and sense oligonucleotide on the donor side, and the deslorelin transport across these monolayers was determined as described above.

High-Performance Liquid Chromatography for the Simultaneous Analysis of Deslorelin and Buserelin

Deslorelin and buserelin, either alone or in combination, were isolated and quantified by high-performance liquid chromatography using a Waters HPLC system composed of a Waters 600 S controller, Waters TM 616 solvent delivery pump, and a Waters 717 plus auto injector. The peptide molecules were detected using a Waters 474 scanning fluorescence detector with an excitation wavelength of 220 nm and an emission wavelength of 350 nm, and the peak areas were integrated using Millennium software (version 2.15.01). A Symmetry (Waters Corporation, Milford, MA, USA) C-18 column, (WAT200632; 150 × 4.6 mm), with a 19% carbon loading, particle diameter of 3.5 μm and a pore size of 100 Å was used. For the simultaneous detection of deslorelin and buserelin, a gradient elution was run with (A) 0.1% TFA in acetonitrile and (B) 0.1% TFA in distilled water, at a flow rate of 1 ml/min. Elution started with an isocratic flow of 80% B + 20% A for 5 min followed by a linear increase of A from 20 to 35% over 20 min and holding it at 35% for 10 min. The column was then re-equilibrated at 80% B + 20% A for 10 min before the next injection.

Statistical Analysis

Data in all cases are expressed as mean ± standard deviation. Comparison of mean values between the different treatments were done using two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis using SPSS (version 8.0) software. Differences were considered statistically significant at $p < 0.05$.

RESULTS

LHRH-Receptor Is Present in the Calu-3 Cell Membrane

LHRH-receptor distribution was examined in Calu-3 cells using immunofluorescent staining by a monoclonal antibody against LHRH-receptor and a CY3 tagged goat anti-mouse antibody. There was no red fluorescence in the negative controls, wherein the cells were processed similar to controls except that an isotypic IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL, USA) was added instead of the primary LHRH-receptor monoclonal antibody (Fig. 1a). However, in cells incubated with the primary antibody, there was a clear red staining pattern and the intensity of staining was strongest at the apical membrane and minimal at the basal membrane, indicating the predominance of LHRH-receptors in the apical and lateral membranes of Calu-3 cells (Figs. 1b–1d). No fluorescent endocytotic vesicles at the level of plasma lemma or within the cells could be observed in these sections possibly because the relative number of receptors endocytosed at any point of time may be very less compared to those present on the membrane, thereby making their visualization difficult. Also, the cross section of the monolayers indicated a strong signal along the apical membrane (Fig. 1e). Thus, these studies for the first time demonstrate the expression and membrane localization of LHRH-receptors in Calu-3 cells. Also, LHRH-receptor is expressed in normal excised bovine nasal tissue, rat trachea, and rat lungs (Fig. 2).

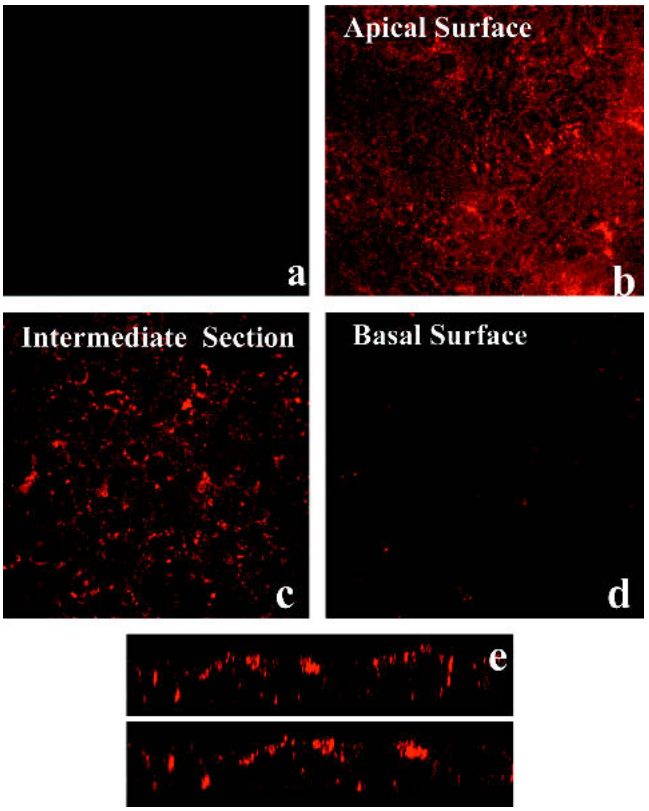

Fig. 1. Representative confocal photomicrographs showing immunostaining for the LHRH-receptor (red) in Calu-3 cells. The cells were grown for 10 days on clear Transwell inserts and treated as described in the "Materials and Methods" section. The LHRH-receptor primary antibody (Ab03) was visualized by a secondary antibody labeled with Cy3. (a) Negative controls with an isotypic IgG primary antibody. (b), (c), and (d) Z sections of Calu-3 cells treated with LHRH-receptor primary antibody and stained with a Cy3-conjugated secondary antibody at the apical membrane, middle of the cell (6 μm depth from the apical membrane), and the basal membrane (12 μm depth from the apical membrane), respectively. (e) Vertical XZ cross-sections of stained Calu-3 cell monolayers from the apical to the basal direction.

Deslorelin Transport Across Calu-3 Is Directional and Temperature- and Cellular Energy–Dependent

Data from the transport studies clearly showed that deslorelin transport across Calu-3 monolayers was polarized in the A-B direction. The cumulative deslorelin transported at 37°C at the end of 4 h across Calu-3 monolayers in the A-B direction was 1.79 ± 0.29%, whereas the transport in the B-A direction was 0.34 ± 0.11% (Fig 3a). Thus, the A-B deslorelin transport was 5.4-fold greater than the B-A transport. P_{app} for A-B deslorelin transport was 1.63 ± 0.18 × 10^{-6} cm/s, which was 7.3-fold greater than the B-A permeability (0.225 ± 0.057 × 10^{-6} cm/s). At low temperature (4°C), the cumulative deslorelin A-B transport was 0.2 ± 0.087%, which was 9-fold lower compared to the A-B transport at 37°C and equal to the B-A transport at 4°C (Fig. 3b). Furthermore, with the use of 2,4-dinitrophenol, a cellular energy depletor (0.1 mM), the A-B cumulative deslorelin transport was 0.36 ± 0.12%, which was 84% lower than the control, and the vectorial deslorelin transport was abolished (Fig. 3b). Thus, deslorelin transport across Calu-3 monolayers was directional and temperature- and cellular energy–dependent.

LHRH-Receptor Antisense Oligonucleotide Decreased the LHRH-Receptor Expression at the Protein and mRNA Level

Following treatment with LHRH-receptor AON (as described in the "Materials and Methods" section), the LHRH-receptor expression in Calu-3 cells was monitored both at the

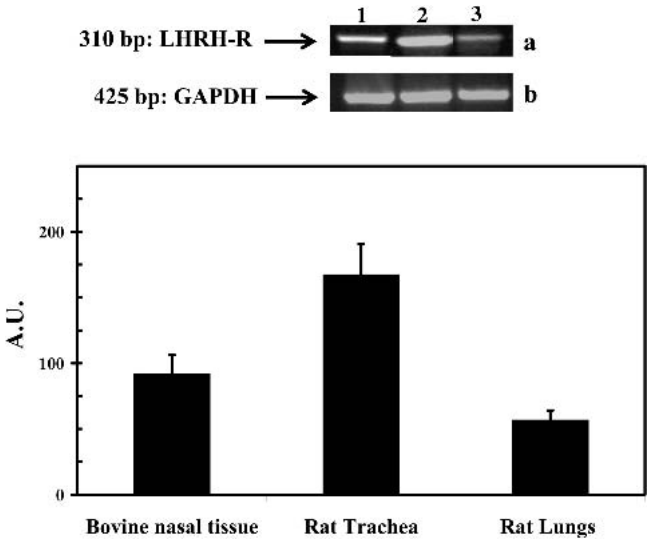

Fig. 2. Representative RT-PCR bands for (a) LHRH-receptor and (b) GAPDH expression in normal excised bovine nasal tissue (lane 1), rat trachea (lane 2), and rat lungs (lane 3). The densitometric data is presented as mean ± SD for n = 4 animals.

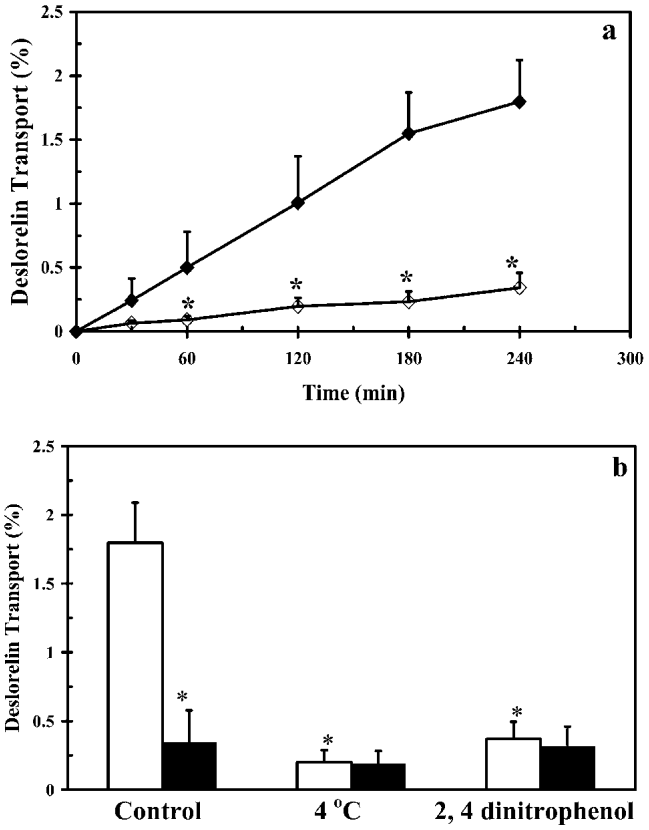

Fig. 3. (a) Vectorial transport of deslorelin across Calu-3 monolayers. Cumulative percent of deslorelin transported across Calu-3 monolayers in the (♦) apical to basolateral and (◊) basolateral to apical directions. Data are expressed as mean ± standard deviation for n = 4. *Indicates significant difference compared to apical to basolateral transport ($p < 0.005$). (b) Effect of temperature and 2,4-dinitrophenol (100 μm), a cellular energy depletor, on deslorelin transport across Calu-3 monolayers. Cumulative percent of deslorelin transported at the end of 4 h at 37°C (Control), at 4°C, and in the presence of 2,4 dinitrophenol (2,4 DNP) at 37°C in the apical to basolateral (open bars) and basolateral to apical (closed bars) directions. Data are expressed as mean ± standard deviation for n = 4. *Indicates significant difference compared to control apical to basolateral transport at $p < 0.005$.

mRNA and at the protein level. RT-PCR analysis indicated the presence of a 310-bp LHRH-receptor in Calu-3 cells. Following 24 h pretreatment with LHRH-receptor AON (10 μM), a decrease in the LHRH-receptor mRNA expression was observed compared to controls (Fig. 4a). 18S rRNA expression served as internal control (Fig. 4b), and the band intensities of LHRH-receptor were normalized to 18S rRNA. Also, Western blot analysis of the LHRH-receptor protein revealed the presence of protein band of approximately 64 kDa, which corresponded to the human pituitary LHRH-receptor. Upon LHRH-receptor AON treatment, a decrease was observed in this protein band (Fig. 4c). β-actin protein expression served as internal control and the band intensities of LHRH-receptor were normalized to β-actin expression. Densitometric analysis of the bands normalized to controls at 100% (Fig. 4e) indicated that the AON reduced the LHRH-receptor mRNA expression by 73 ± 10.2% and the protein expression by 42 ± 4.8%.

LHRH-Receptor Antisense Oligonucleotide Decreased Deslorelin Transport

For studying the involvement of the LHRH-receptor in the polarized transport of deslorelin across Calu-3 monolayers, we pretreated monolayers with the LHRH-receptor AON to decrease LHRH-receptor expression as described in "Materials and Methods." The deslorelin transport was then assessed across these monolayers. Following treatment with the LHRH-receptor AON, the cumulative A-B deslorelin transport was significantly reduced by 56% from 1.79 ± 0.29% (control) to 0.78 ± 0.14% (AON treated) (Fig. 5a). Interestingly, this decrease is consistent with the decrease in the LHRH-receptor expression observed above. Also, the directionality of deslorelin transport was abolished by AON and the percent deslorelin transported in the A-B direction (0.78 ± 0.143) was not significantly different from that in the B-A direction (0.72 ± 0.17) (Fig. 5b). However, when cells were treated with the LHRH-receptor SON, the A-B deslorelin transport was not significantly different compared to controls (Fig. 5c). The LHRH-receptor SON did not alter the expression of LHRH-receptor (Fig. 4a). Thus, these results indicate a role for LHRH-receptor in the vectorial transport of deslorelin across Calu-3 monolayers.

Buserelin Reduced the A-B Deslorelin Transport Across Calu-3 Monolayers

To understand the effect of buserelin on deslorelin transport, equal concentrations of buserelin and deslorelin were added to the apical or basolateral surfaces of Calu-3 monolayers, and their A-B or B-A transport was assessed. Buserelin is structurally similar to deslorelin with difference in only one amino acid: the D-tryptophan at the sixth position in deslorelin is replaced by a *t*-(Bu)-D-Serine in buserelin. Compared to controls, co-treatment with buserelin reduced the cumulative A-B deslorelin transport significantly ($p = 0.0035$) by 56.73 ± 4.2% (Fig. 6a). Also, the percent deslorelin transport in the A-B direction was reduced from 1.25 ± 0.26 to 0.5 ± 0.068%. However, the B-A deslorelin transport in the presence of buserelin was not significantly different from controls (Fig. 6b). Also, in the presence of deslorelin we observed vectorial transport for buserelin with the cumulative A-B transport (0.71 ± 0.086%) being greater than the B-A transport (0.23 ± 0.14%). The observed reduction in deslorelin transport in the presence of buserelin is consistent with competition between the two agonists for the LHRH-receptor.

Effect of Various Treatments on the Transepithelial Electrical Resistance

The TEER of Calu-3 monolayers was measured right before the start and at the end of each experiment to ensure that the integrity of the monolayers was unaffected during the experiment. Compared to controls, the various treatments including 2,4-dinitrophenol and buserelin did not alter the TEER significantly (Table I). With pretreatments, the TEER was also measured before and after pretreatment (Table II). The mean TEER of cell monolayers before treatment with LHRH-receptor antisense oligonucleotide and lipofectin was 432 ± 48 ohm · cm^2, which decreased to 336 ± 20 ohm · cm^2 upon 24 h treatment. This decrease was significant, and simi-

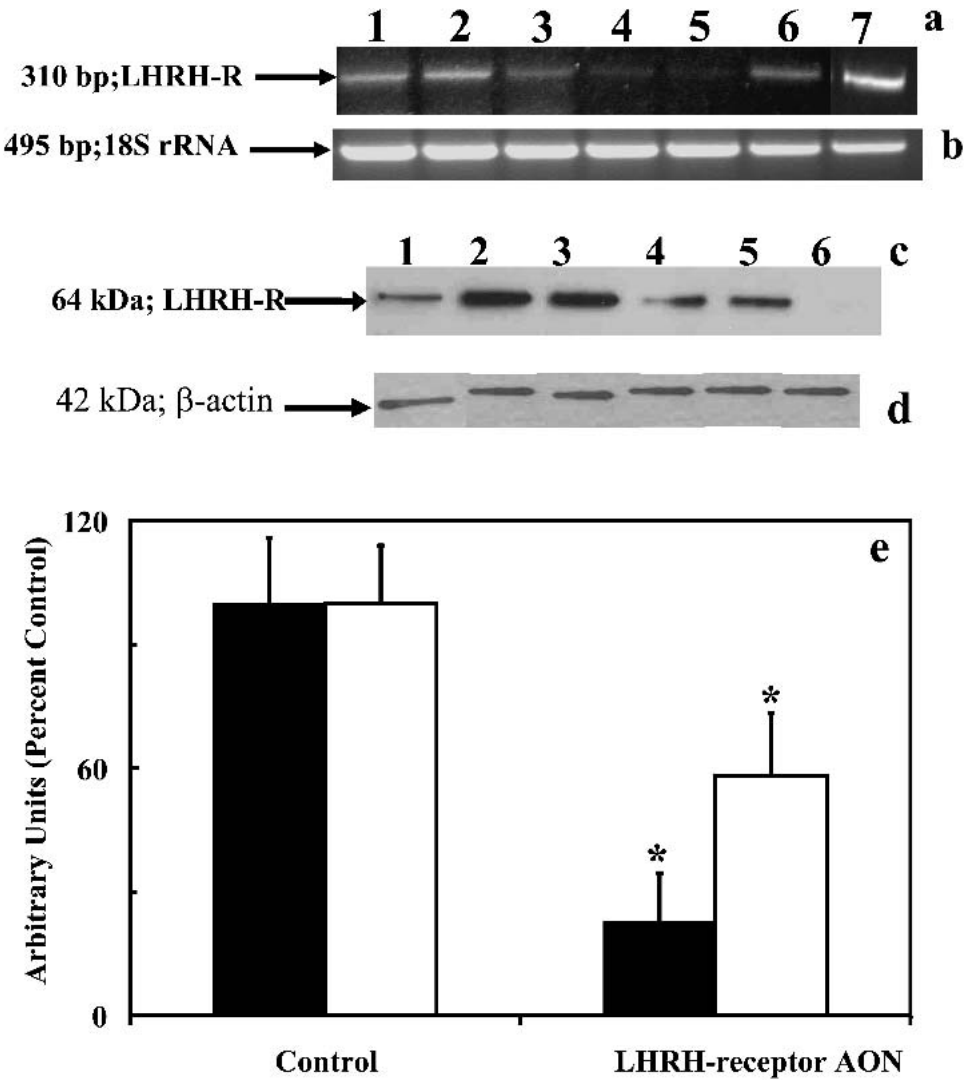

Fig. 4. Effect of LHRH-receptor antisense oligonucleotide (AON) on the LHRH-receptor expression in Calu-3 cells. (a) LHRH-receptor and (b) 18S rRNA mRNA expression. Key: lanes 1, 2, and 3, controls; lanes 4, 5, and 6, cells treated with LHRH-receptor AON (10 μM) for 24 h; lane 7, cells treated with LHRH-receptor sense oligonucleotide for 24 h. (c) LHRH-receptor and (d) β-actin protein expression. Key: lanes 1, 2, and 3, controls; lanes 4, 5, and 6, cells treated with LHRH-receptor AON (10 μM) for 24 h. All lysates were loaded at 50 μg protein. (e) Densitometric analysis of LHRH-receptor expression. Data normalized to internal controls for LHRH-receptor mRNA (closed bars) and protein (open bars) expression are presented as mean ± standard deviation for n = 3. *Indicates significant difference compared to controls at $p < 0.05$.

lar observations were made with lipofectin plus LHRH-receptor sense oligonucleotide pretreatment (Table II). This is consistent with the known ability of lipofectin to impair epithelial integrity (24). Interestingly, the decreased TEER recovered at the end of 12-h recovery period in low serum (2%) containing growth medium devoid of oligonucleotides and lipofectin (Table II). After the recovery period, the monolayers were used for deslorelin transport studies (Table I).

DISCUSSION

For the first time, the results of the current study indicate that LHRH-receptor is expressed in Calu-3 cell monolayers and that it mediates the energy-dependent apical to basolateral transport of an LHRH-agonist, deslorelin. To the best of our knowledge, no previous studies exist supporting a receptor-mediated transepithelial transport for LHRH agonists in any cell type. Our immunofluorescence, Western blot, and RT-PCR studies confirmed the expression of LHRH-receptor in Calu-3 cells. Immunofluorescence studies indicated a predominantly apical localization of the LHRH-receptor. An A-B/B-A deslorelin transport ratio greater than 1 (5.3) in controls indicated vectorial transport of deslorelin (Fig. 7). Interestingly, this ratio was reduced by low temperature (1.1), 2,4-dinitrophenol (1.2), an LHRH-receptor antisense oligonucleotide pre-treatment (1.0), and buserelin (2.9), suggesting energy-dependent vectorial transport of deslorelin via the LHRH-receptor. The internalization of a LHRH-receptor agonist was previously shown to be energy-dependent and inhibited at 4°C in pituitary membrane preparations (25).

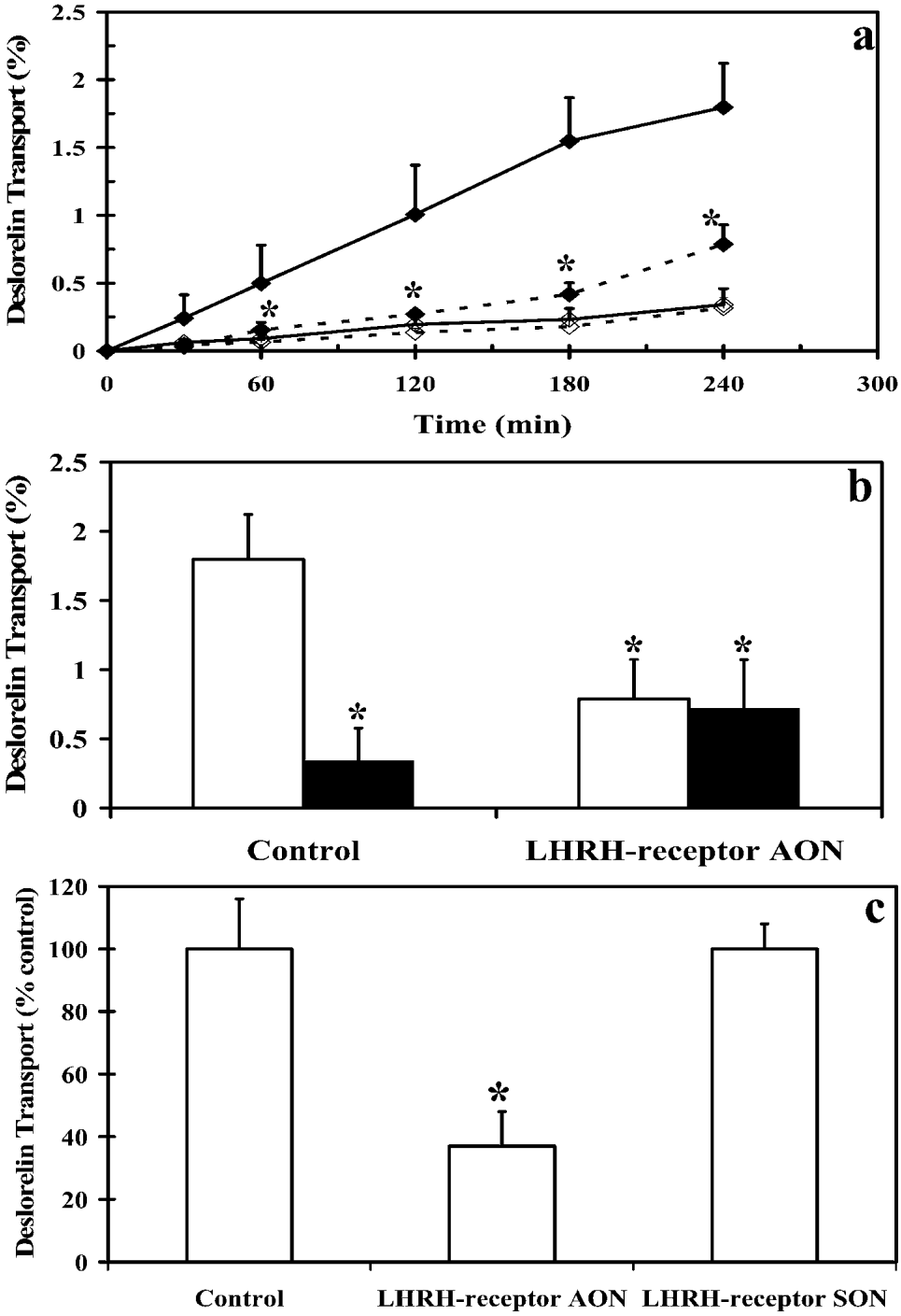

Fig. 5. Effect of LHRH-receptor antisense oligonucleotide (AON) on deslorelin transport across Calu-3 monolayers. (a) Cumulative percent of deslorelin transported in the (♦) apical to basolateral and (◊) basolateral to apical direction with (broken lines) or without (solid lines) pretreatment with LHRH-receptor AON. (b) Percent cumulative deslorelin transport at the end of 4 h across Calu-3 monolayers in the apical to basolateral (open bars) and basolateral to apical direction (closed bars). (c) Percent reduction of cumulative deslorelin A-B transport at the end of 4 h by LHRH-receptor AON and sense oligonucletide (SON). Data are presented as mean ± standard deviation for n = 4. *Indicates significant difference compared to control apical to basolateral deslorelin transport at $p < 0.05$.

Thus, it appears that the net A-B deslorelin flux is LHRH-receptor mediated.

Apart from pituitary tissues, LHRH-receptor is expressed in various reproductive tissues including the ovary, testes, and the prostate and nonreproductive tissues including liver, heart, skeletal muscle, kidney, and placenta (26). Our studies indicated that Calu-3 cells express LHRH-receptor. Because it can be argued that cell lines may exhibit receptor

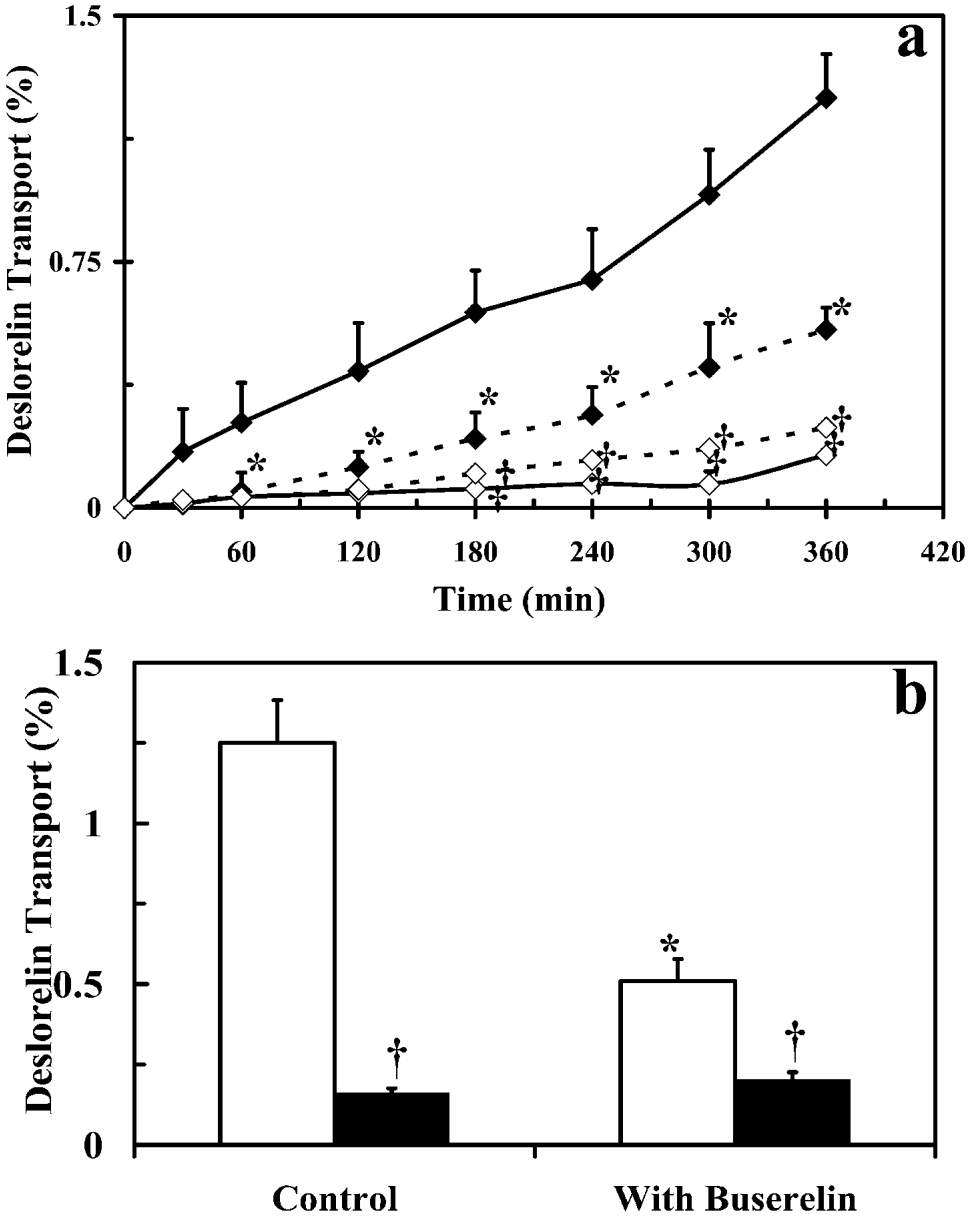

Fig. 6. Effect of buserelin, a competing LHRH agonist, on deslorelin transport across Calu-3 monolayers. (a) Cumulative percent of deslorelin transported in the (♦) apical to basolateral and (◊) basolateral to apical direction in the absence (solid lines) or presence (broken lines) of buserelin. (b) Percent cumulative deslorelin transport at the end of 6 h across Calu-3 monolayers in the apical to basolateral (open bars) and basolateral to apical (closed bars) directions for deslorelin alone (control) and in the presence of buserelin. Data are presented as mean ± standard deviation for n = 4. *Indicates significant difference compared to control apical to basolateral transport at $p < 0.05$. †Indicates significant difference compared to apical to basolateral deslorelin transport of the same treatment group, at $p < 0.05$.

expression different from normal tissue, we assessed the LHRH receptor expression in normal tissues from bovine nose, rat trachea, and rat lungs. Interestingly, all these tissues expressed the LHRH-receptor (Fig. 2). This receptor is also overexpressed in the tumors of breast, ovary, endometrium, and prostate (27,28). This overexpressed LHRH-receptor serves as a useful target to achieve high intracellular concentrations of anti-tumor drugs and to reduce side effects of cytotoxic drugs. For instance, conjugation of LHRH-agonists such as D-Lys^6-LHRH to doxorubicin and 2-pyrrolinodoxorubicin facilitated intracellular accumulation of these cytotoxic agents (29). The role of LHRH-receptors in the action of LHRH agonists in conjunction with the drug delivery opportunities via LHRH-receptors provides an impetus for understanding the interaction of LHRH agonists with LHRH-receptors.

LHRH agonists act via LHRH-receptors, which are members of the G-protein–coupled receptor (GPCR) superfamily. It has been known since 1980 that agonist occupancy of the LHRH-receptor results in patching, capping, and in-

Table I. Effect of Various Treatments on the Transepithelial Electrical Resistance (TEER) of Calu-3 Cell Monolayers

Treatments	Resistance (ohm · cm^2) (Mean ± S.D.)			
	Apical-basolateral (A-B)		Basolateral-apical (B-A)	
	Before experiment	After experiment[a]	Before experiment	After experiment[a]
Control (37°C)	432 ± 14.2	485.2 ± 38	480.2 ± 56.5	457.8 ± 56.7
Control (4°C) (measured with 4°C medium)	668 ± 32	615 ± 62	674 ± 93	705 ± 101
Control (4°C) (measured with 37°C medium)	435 ± 27	491 ± 53	463.3 ± 36.5	488 ± 38.5
2,4-Dinitrophenol (0.1 mM) co-treatment	487 ± 48	492 ± 12.5	507.2 ± 26.5	466 ± 32
LHRH-receptor AON pretreatment followed by 12 h of no treatment	483 ± 68	497 ± 12.2	492 ± 18	464 ± 32
LHRH-receptor SON pretreatment followed by 12 h of no treatment	527 ± 23	513 ± 38	480 ± 57	456 ± 35
Buserelin co-treatment	443 ± 34	449 ± 48	473 ± 8.9	474.66 ± 32

Data are expressed as mean ± SD for n = 4. Differences were considered significant at $p < 0.05$. LHRH-receptor AON, LHRH-receptor antisense oligonucleotide; LHRH-receptor SON, LHRH-receptor sense oligonucleotide.

[a] There was no statistically significant change in the TEER values at the end of the experiment in any of the treatments.

ternalization of the receptor agonist complex (30). Thus far, studies characterizing the LHRH-receptor–agonist complex indicated that upon activation by an LHRH-agonist, the LHRH-receptor initially localized to the plasma membrane undergoes phosphorylation, primarily mediated by specific G-protein receptor kinases, second messenger–regulated kinases [e.g., protein kinase C (PKC) or PKA], (31) or by casein kinases (27,32). Subsequently, the LHRH-receptor–agonist complexes are endocytosed via clathrin-coated vesicles (CCVs), and then the receptor is recycled to the plasma membrane (33). However, in all of these studies, the fate of the agonist remained unknown, and no attempts were made to determine whether internalized agonist is exocytosed. Though some investigators have postulated that the agonist is degraded within the lysosomes (34), others have suggested that it may be released within the cytoplasm in the perinuclear space; yet others have identified vesicles containing free LHRH agonist within LHRH-receptor transfected (GH3) cells of pituitary origin (34). Based on our studies, we postulate that part of the endosomes containing the ligand or the receptor agonist complex reach the basolateral surface, leading to the transcytosis of LHRH agonists. Indeed, such a transcellular transport has been indicated for gonadotrophins, lutropin (leutinizing hormone), and chorionic gonadotropin in endothelial cells (35).

Structure-activity relationship studies revealed that the N-terminal domain, pGlu1-His2-Trp3, and the C-terminal domain, Pro9-Gly10-NH2, of LHRH are important in receptor binding and activation. Although both domains are involved in receptor binding, residues in the N-terminal are predominantly responsible for receptor activation. Arg8 is required for high-affinity binding to the mammalian receptor (36). Also, substitutions at the sixth position with D-amino acids having bulky hydrophobic side chains, particularly aromatics, result in increased binding affinity and activity. For this reason, most analogs including deslorelin and buserelin have substitutions at the sixth amino acid and they both bind to LHRH-receptor. Also, a type II β-turn conformation, which is important for receptor binding, has been identified in the middle region (Tyr5-Gly6-Leu7-Arg8) of LHRH structure. This may also be the reason why D-amino acid substitutions, which stabilize the β-turn conformation, result in high receptor binding affinities for deslorelin and buserelin, compared to LHRH (37).

In our transport studies across intact monolayers, we did not observe very significant metabolism, and percent deslore-

Table II. Effect of Various Pretreatments on the Transepithelial Electrical Resistance (TEER) of Calu-3 Cell Monolayers[a]

Treatments	Resistance (ohm · cm^2) (mean ± SD)					
	Apical-basolateral (A-B)			Basolateral-apical (B-A)		
	Before pretreatment	After pretreatment	After recovery	Before pretreatment	After pretreatment	After recovery
LHRH-receptor AON	432 ± 49	336.5 ± 20[b]	518 ± 31[c]	452 ± 38	380 ± 17[b]	532 ± 47[c]
LHRH-receptor SON	495 ± 38	406 ± 21[b]	550 ± 42[c]	515 ± 52	444 ± 16[b]	563 ± 45[c]

Data are expressed as mean ± SD, for n = 4.

[a] These monolayers are distinct from those used in Table I. LHRH-receptor AON, LHRH-receptor antisense oligonucleotide; LHRH-receptor SON, LHRH-receptor sense oligonucleotide.

[b] Significantly different compared to the values before pretreatment at $p < 0.05$.

[c] No significant difference compared to the values before pretreatment.

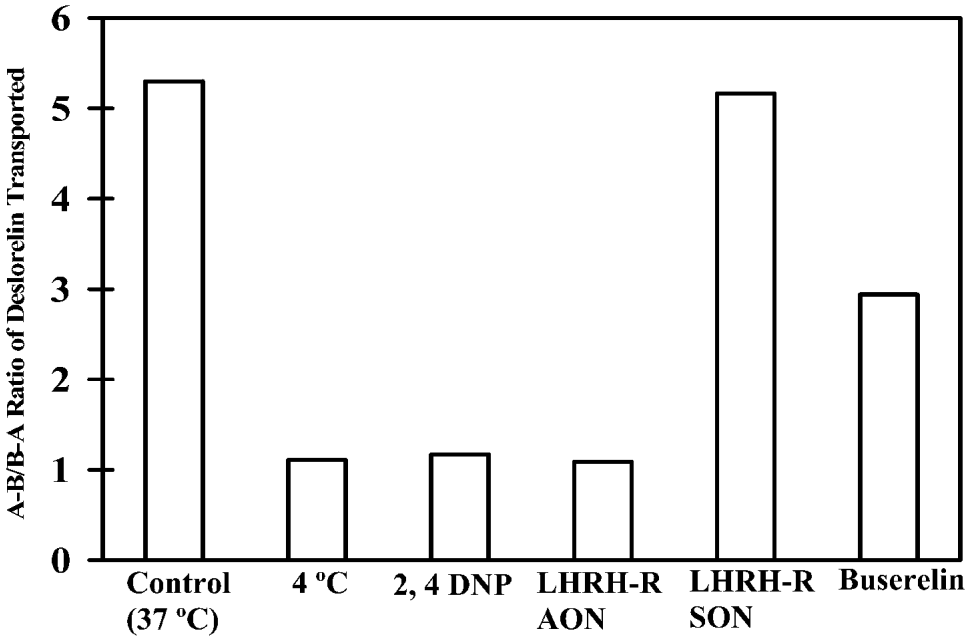

Fig. 7. Effect of different treatment conditions on the apical-basolateral/basolateral-apical ratios of cumulative deslorelin transported at the end of 4 h. Key: 2,4 DNP, 2, 4 dintrophenol; LHRH-R AON, LHRH-receptor antisense oligonucleotide; LHRH-R SON, LHRH-receptor sense oligonucleotide.

lin remaining in the donor chambers at the end of the transport study was 95.9 ± 6.6%. The chromatograms yielded minor metabolite peaks (Fig. 8), which were not quantified. However, our previous studies with cell lysates indicated that the major deslorelin-metabolites in respiratory cells are (i) pGlu1-His2-Trp3, (ii) Ser4-Tyr5-DTrp6-Leu7-Arg8-Pro9NHEt, and (iii) Tyr5-DTrp6-Leu7-Arg8-Pro9NHEt (38). We have not determined the receptor binding of any of these metabolites. However, based on the discussion above, because metabolite 1 cannot form a type II β-turn, it is less likely to bind to the receptor compared to the parent compound. Metabolites 2 and 3 are capable of forming the β-turn structure and hence may bind to the receptor. However, because they lack the N-terminal region, they are less likely to activate receptor internalization. Indeed, LHRH agonists lacking the N terminal domain (pGlu-His-Trp) exhibited very low receptor activation (39). Thus, the above metabolites are less likely to use the LHRH-receptor mediated transport. Given the low extent of degradation of deslorelin in the current study, it is likely that the metabolites did not interfere significantly with deslorelin transport.

Competition studies with molecules of similar structure are a useful approach to investigate the involvement of a specific receptor in transport. The relative binding affinities of buserelin and deslorelin to LHRH-receptors in Calu-3 cells appear to be similar as there is no significant difference between cumulative A-B transport of deslorelin (0.509 ± 0.07%) and buserelin (0.710 ± 0.086%). Indeed, it has been observed previously that LHRH agonists of different potencies bind to pituitary membrane receptors with similar affinity (40). In the presence of buserelin, the deslorelin A-B transport was reduced by 49 ± 3.5%, indicating that buserelin competes with deslorelin for the LHRH-receptor. Another possible reason for decreased deslorelin transport could be a preferential metabolism of deslorelin, leading to reduction in amount available for transport (41). However, the percent deslorelin remaining in the apical compartment in controls (95.9 ± 6.6) at the end of 6 h was not significantly different from that in the presence of buserelin (93.8 ± 4.2), suggesting that reduced degradation does not explain the observed differences in the A-B deslorelin transport. Our data indicate that upon dinitrophenol treatment, the A-B deslorelin transport decreased, but the B-A transport was similar to controls (Fig. 3b). This cannot be due to reduced paracellular diffusion because B-A transport was unaffected. Another reason for the observed alteration in deslorelin transport could be reduced transcellular passive diffusion of deslorelin. If dinitrophenol is capable of reducing passive transcellular transport, both A-B and B-A transport should have reduced. However, in our studies, dinitrophenol decreased the A-B transport and the B-A transport was unaffected. Thus, transcellular diffusion alteration does not likely contribute to the observed results.

A number of approaches can be used to inhibit receptor-mediated endocytosis including the use of nonspecific inhibitors such as cytochalasin B and monensin. However, these molecules decrease the resistance and increase the passive paracellular permeability (42) making it more difficult to discern net fluxes. To circumvent this problem and understand the role of LHRH-receptors in deslorelin transport, we used a 22-mer antisense oligonucleotide for the LHRH-receptor mRNA (23). This phosphorothioate modified oligonucleotide, when compared to standard phosphodiester oligonucleotides, offers a number of advantages including ease of synthesis, a substantial degree of resistance to exonucleases, and the ability to activate RNase H that efficienctly degrades the RNA (43). An antisense approach was previously used in investigating the role of LHRH-receptor expression in the steroid-induced LH surge (44). Furthermore, unlike nonspecific inhibitors of protein synthesis such as cycloheximide, an

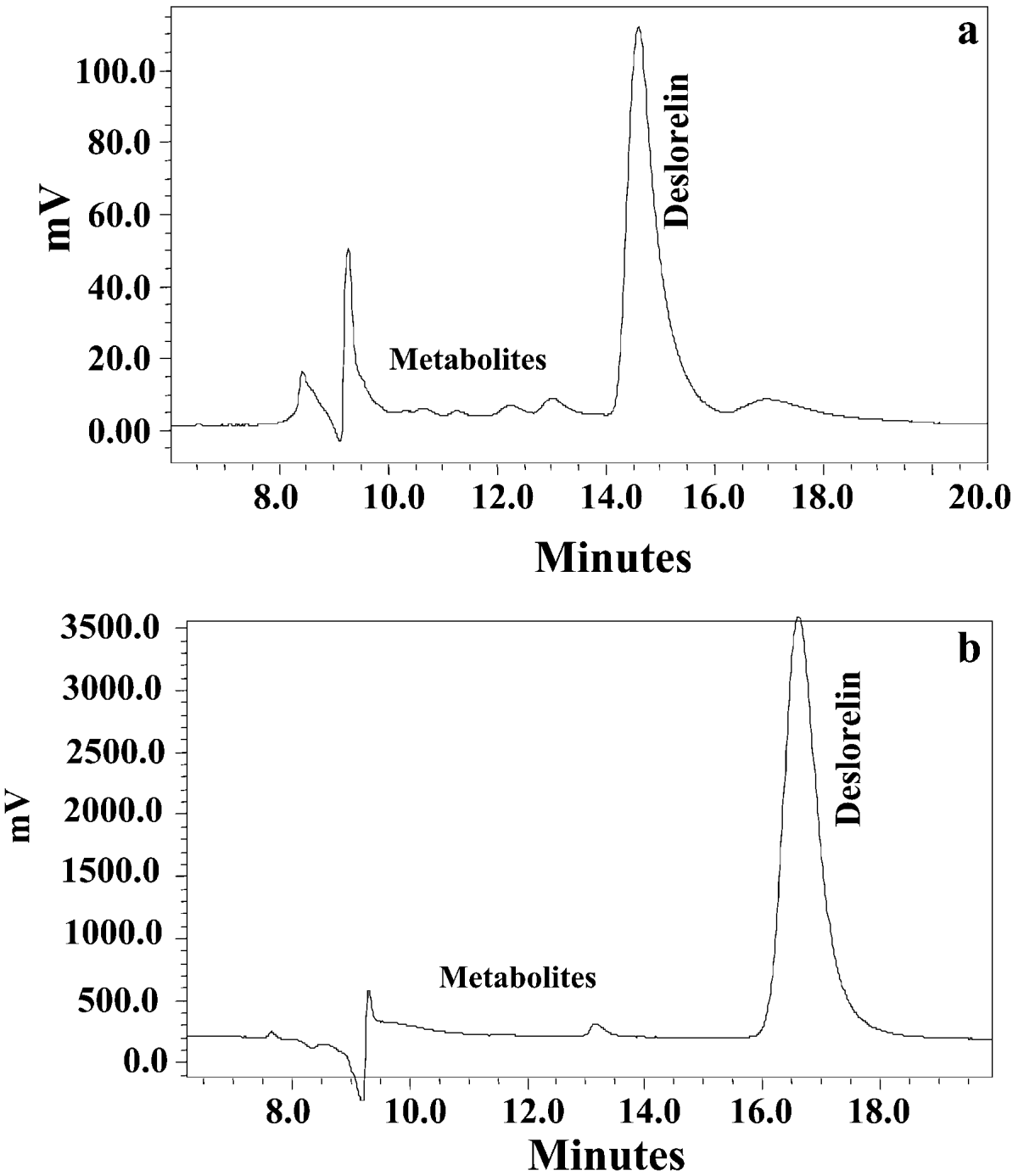

Fig. 8. Representative chromatograms showing deslorelin and metabolite peaks (a) in the receiver fluid and (b) in the donor fluid at the end of 6 h in Calu-3 transport study.

antisense oligonucleotide specifically downregulates the target mRNA and protein without decreasing the transepithelial electrical resistance (TEER) (24). We observed that the antisense oligonucleotide but not the sense oligonucleotide reduced the LHRH receptor expression as well as deslorelin transport across Calu-3 monolayers. Neither of the oligonucleotide treatments as used resulted in monolayers with altered TEER, suggesting the specificity of their effects on the receptor-mediated transport of deslorelin.

The observed deslorelin A-B permeability is much greater than what we would obtain if the transport is primarily paracellular as is the case with molecules such as mannitol. Our observed TEER values for Calu-3 cells cultured at air interface conditions are similar to those observed in previous literature reports (400-600 ohm·cm^2) (11,45). Also, the apparent permeability of deslorelin in our studies is greater than that reported for mannitol ($5.2 \pm 0.92 \times 10^{-7}$ cm/s) by Pezron *et al.* in Calu-3 monolayers (46). Thus, despite its higher molecular weight (1282 Da), deslorelin exhibited much higher A-B permeability ($1.52 \pm 0.098 \times 10^{-6}$ cm/s) compared to mannitol (182 Da). The observed deslorelin permeability coefficient is also higher than those reported for Na-fluorescein

(376 Da) and Lucifer yellow (479 Da) in Calu-3 monolayers (11,45).

In summary, this study demonstrated the expression and predominantly apical localization of a mammalian LHRH-receptor in Calu-3 cells. The current data indicate that LHRH-receptor mediated endocytosis contributes to the active apical to basolateral transport of deslorelin across Calu-3 cells. This extends our understanding of the role of the LHRH-receptor to the vectorial transport of LHRH agonists across epithelial cells. It remains to be seen whether epithelial regions with high LHRH-receptor density can be good portals for LHRH delivery.

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