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Rapid nongenomic actions of inhaled corticosteroids on long-acting β_2-agonist transport in the airway

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ABSTRACT

Corticosteroids inhibit organic cation transporters (OCTs) that play an important role in drug absorption, tissue distribution and elimination. Corticosteroid sensitivity of bronchodilator trafficking in the airway tissue, however, is poorly understood. To assess the effects of inhaled corticosteroids on airway absorption and disposal mechanisms of long-acting β_2-agonists, human airway epithelial and smooth muscle cell uptake of tritiated formoterol and salmeterol was measured *in vitro*. Corticosteroids caused a rapid, concentration-dependent inhibition of uptake of the cationic formoterol by airway smooth muscle cells, but not airway epithelial cells. Uptake of the non-charged lipophilic salmeterol was corticosteroid-insensitive in both cell types. In smooth muscle cells, inhaled corticosteroids inhibited formoterol uptake with a novel potency rank order: *des*-ciclesonide > budesonide > beclomethasone 17-monopropionate > beclomethasone dipropionate > ciclesonide > fluticasone. The inhibitory action was rapidly reversible, and was not enhanced by prolonged corticosteroid exposure or sensitive to a transcription inhibitor. Suppression of OCT3 expression using lentivirus-mediated production of shRNA reduced corticosteroid sensitivity of formoterol uptake by smooth muscle cells. Our data support a corticosteroid insensitive absorption and a corticosteroid-sensitive disposition mechanism for cationic long-acting β_2-agonist bronchodilators in the airway. Potency rank order and other ‘classical’ features of anti-inflammatory effects do not apply to inhaled corticosteroids’ rapid drug transport actions.

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1. Introduction

Corticosteroid/long-acting β_2-agonist combination inhalers are increasingly used in asthma management [1,2]. Combined drug administration provides more effective control in mild-to-moderate asthma: patients have better lung function, better symptom control, and lower incidence of exacerbation compared with those given either drug alone [3]. Combination inhalers can simultaneously deliver drugs that have complementary actions on asthma pathophysiology: corticosteroids effectively suppress airway inflammation, whereas long-acting β_2-agonists, acting as bronchodilators, provide long-lasting relaxation of airway smooth muscle [4]. Importantly, drug–drug interactions are now recognized to have a major role in the superior effects of combined drug administration; however, the molecular mechanisms of the interactions have not been fully elucidated [5]. In addition, it is still uncertain whether the choice of inhaled corticosteroid and long-acting β_2-agonist in combination preparations makes a difference to these beneficial interactions.

There is a growing body of evidence that inhaled corticosteroids, in addition to anti-inflammatory actions, could prevent the loss of function and improve the effects of β_2-agonist bronchodilators. Among other ‘classical’ delayed (transcriptional) actions, corticosteroids could increase β_2-receptor numbers and restore β_2-receptor coupling to G proteins that mediate adenylyl cyclase stimulation [6,7]. In contrast, corticosteroids may also improve β_2-adrenergic bronchodilation through rapid (non-transcriptional) mechanisms [8,9]. We showed that inhaled corticosteroids may limit the vascular clearance of inhaled bronchodilators by causing rapid vasoconstriction in the airway [10]. Furthermore, we recently demonstrated that corticosteroids rapidly interfere with the

Abbreviations: OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; MPP^+, *N*-methyl-4-phenylpyridinium.

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disposal of cationic β_2-agonist bronchodilators by smooth muscle cells in the airway [11]. By rapidly potentiating β_2-adrenergic bronchodilation, these mechanisms could be relevant to the use of combination inhalers both as maintenance therapy and rescue medications for symptom relief [12].

In our prior study, we showed expression of a corticosteroid-sensitive cationic drug transport machinery in human airways, and the inhibitory corticosteroid effect on cationic drug uptake by airway vascular and bronchial smooth muscle [11]. To further understand corticosteroid sensitivity of inhaled drug trafficking (i.e. absorption and disposal mechanisms) in the airway, we examined here how inhaled corticosteroids influence uptake of two long-acting β_2-agonists, the cationic formoterol and the lipophilic salmeterol, by human airway epithelial and smooth muscle cells. We also assessed whether drug transport actions of inhaled corticosteroids are mediated via the classical transcriptional pathway of steroid action.

2. Material and methods

2.1. Human tissues and airway epithelial cell isolation

Tracheas and large bronchi (≥third-generation) were obtained from organ donors whose lungs were rejected for transplantation because they failed to meet standard selection criteria [13]. Consent for research was obtained by the Life Alliance Organ Recovery Agency of the University of Miami. All consents were IRB-approved and conformed to the Declaration of Helsinki. Primary airway epithelial cells were isolated from airway tissues as previously described [14]. Briefly, the mucosa was dissected from the underlying cartilage under sterile conditions and incubated in 0.05% protease (type XIV; Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) overnight at 4 °C. After protease treatment, epithelial cells were released by vigorous shaking and cells were harvested by centrifugation. Human tracheobronchial epithelial cells were counted, and their viability was determined by Trypan Blue exclusion (viability was always > 80%).

2.2. Cell cultures

Freshly isolated airway epithelial cells were deposited onto human placental (type IV) collagen-coated culture dishes at a density of 0.5×10^6 cells/cm^2, and maintained ≤48 h in DMEM supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin within a humidified atmosphere containing 5% CO_2 at 37 °C [15].

Primary human bronchial smooth muscle cells were purchased from Lonza (Walkersville, MD), and grown in their recommended optimal medium (Smooth Muscle Cell Growth Medium; Lonza) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin within a humidified atmosphere containing 5% CO_2 at 37 °C. Smooth muscle cells were serum-deprived for 24 h before transport measurements.

2.3. Transport assays

To examine drug transport *in vitro*, uptake of the cationic [^{3}H]-formoterol (7.03 Ci/mmol; AstraZeneca, Loughborough, United Kingdom), the non-charged lipophilic [^{3}H]-salmeterol (19.1 Ci/mmol; AstraZeneca), and the organic cation transporter-carried [^{3}H]-*N*-methyl-4-phenylpyridinium (MPP$^+$; 81 Ci/mmol; PerkinElmer, Boston, MA) was measured in human airway cells. Experiments were performed at 37 °C by applying 200 nmol/l [^{3}H]-formoterol or [^{3}H]-salmeterol, or 5 nmol/l [^{3}H]-MPP$^+$ to monolayer cell cultures. Beclomethasone dipropionate and beclomethasone 17-monopropionate were generously provided by Chiesi Farmaceutici (Parma, Italy), and ciclesonide and *des*-ciclesonide by Nycomed (Konstanz, Germany). Other corticosteroids were purchased from Sigma. Corticosteroids were dissolved in ethanol (final concentration ≤ 0.1%), a solvent without significant effect on drug transport levels as confirmed by controls using vehicle only. Incubation was stopped after 15 min by rinsing the cells with ice-cold PBS. Subsequently, the cells were solubilized and radioactivity was measured by liquid scintillation counting. Protein was measured by the BCA protein assay (Pierce, Rockford, IL).

2.4. Organic cation transporter 3 (OCT3) silencing with shRNA lentiviruses

We used a third-generation, propagation-deficient HIV-pseudotyped lentivirus expressing OCT3 targeted shRNA to knock-down OCT3 gene expression. Proviral plasmids (pLKO.1) with anti-OCT3 shRNA sequences (shRNA set ID RHS4533-NM_021977) were purchased from Open Biosystems (Huntsville, AL). In preliminary experiments, one sequence, GCACCAAACTTCCCTGTGTTT (Clone ID TRCN0000038609), was found to be highly effective and thus chosen for the construction of lentivirus for transduction and shRNA expression. Same vector without OCT3 targeted shRNA sequence was used as control. The pLKO.1 lentiviral vector contained a selectable marker for resistance to puromycin. Replication-deficient lentiviruses were prepared by co-transfecting vector and the packaging plasmids pMDLg/pRRE#54 pRSV-Rev and pMDLgVSVG, into HEK 293T cells by calcium phosphate coprecipitation, as previously described [16]. Viruses were collected daily for 3 days beginning 24 h after removing the precipitates. Viruses were concentrated by precipitation with the addition of polyethylene glycol to 11% and centrifugation. Virus titers were estimated by measuring p24 by ELISA (Perkin–Elmer, Shelton, CT).

Human bronchial smooth muscle cells were infected with OCT3 lentiviruses, selected with 1 μg/ml puromycin until uninfected cells were dead, typically 3–5 days. Date and lung donor matched cultures that were infected with non-targeted shRNA lentiviruses (selected with puromycin) were used as controls.

2.5. OCT3 mRNA measurement

Total RNA was extracted from primary bronchial smooth muscle cells using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA). RNA samples were treated with RNase-free DNase (Qiagen) and quantified spectrophotometrically at 260 nm. RNA integrity was confirmed using RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA) and a bioanalyzer (model 2100, Agilent Technologies) provided by the University of Miami DNA Microarray Facility.

First-strand cDNA was synthesized using the Quantitect Reverse Transcription Kit (Qiagen). OCT3 mRNA expression levels were measured with quantitative, real-time PCR and our custom-designed gene-specific primers as described previously [15]: 5′-TGC CTA CTT CAT CCC CAA CTG G (forward) and 5′-TTC CGA GTA ATC AGC CAA CGG G (reverse). Real-time PCR amplification reactions were performed with Quantitect SYBR Green PCR Kit (Qiagen). The cycling conditions comprised of 15 min polymerase activation at 95 °C, and 45 cycles at 95 °C for 15 s, 57 °C for 30 s, and at 72 °C for 30 s. Reactions were run on an iCycler IQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Control reactions were performed in the absence of RT. Specificity of the amplificons were confirmed by purification on QiAquick PCR Purification Kit silica spin columns (Qiagen) and sequencing (DNA Core Laboratory, University of Miami). Gene expression levels were calculated by normalizing the

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