Pulmonary Pharmacology & Therapeutics 48 (2018) 161–167

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Tiotropium inhibits mucin production stimulated by neutrophil elastase but not by IL-13

Kosaku Komiya[a,b,c], Shuichi Kawano[a], Isao Suzaki[a], Tomohiro Akaba[a], Jun-ichi Kadota[b], Bruce K. Rubin[a,*]

[a] Children's Hospital of Richmond at Virginia Commonwealth, Richmond, VA 23298, USA
[b] Respiratory Medicine and Infectious Diseases, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan
[c] Clinical Research Center of Respiratory Medicine, Tenshindo Hetsugi Hospital, 5956 Nihongi, Nakahetsugi, Oita 879-7761, Japan

ARTICLE INFO

Keywords:
Tiotropium
Mucus
Neutrophil elastase
IL-13
Bronchial epithelial cell

ABSTRACT

Tiotropium, a muscarinic antagonist, is approved for the treatment of chronic obstructive pulmonary disease and poorly controlled asthma. Because mucus hypersecretion is characteristic of both of these diseases, and muscarinic agonists stimulate mucus secretion, we hypothesized that tiotropium would attenuate airway MUC5AC expression. We grew normal human bronchial epithelial (NHBE) cells to a goblet cell phenotype with 1 or 5 ng/mL of IL-13 and exposed these cells to 10 nM tiotropium or excipient for the full 14 days. Normally differentiated NHBE cells (without IL-13) were exposed to neutrophil elastase (NE) 1×10^{-7} or 5×10^{-7} M for 1 h. MUC5AC was measured by quantitative PCR and ELISA. Acetylcholine production by the epithelium was evaluated by quantitative PCR and by choline/acetylcholine quantification. Tiotropium had no effect on IL-13-stimulated MUC5AC, but attenuated MUC5AC stimulated by NE ($p = 0.007$ at 5×10^{-7} M). IL-13 increased CarAT mRNA ($p < 0.001$ at 5 ng/mL) and acetylcholine concentration in the medium ($p = 0.018$ at 5 ng/mL), while NE had no effect. Tiotropium had no direct effect on IL-13 or NE-induced CarAT or acetylcholine concentration. Tiotropium decreased MUC5AC stimulated by NE, but had no effect on MUC5AC stimulated by IL-13. These results may be due to IL-13, but not NE, increasing acetylcholine production.

1. Introduction

Tiotropium is a long-acting muscarinic antagonist bronchodilator which has been primarily used to treat chronic obstructive pulmonary disease (COPD), and has been approved for treating poorly controlled asthma [1]. Poorly controlled asthma is a heterogeneous disease with endotypes usually classified on the basis of biomarkers including eNO, periostin, and airway cell predominance (ie eosinophilic, neutrophilic, mixed cellularity) [2]. Severe asthma is often associated with mucous cell hyperplasia and mucus hypersecretion. Acting by different pathways, both interleukin (IL)-13, a TH2 cytokine, and neutrophil elastase (NE), which is increased in COPD and neutrophil dominant asthma, can cause mucus secretion [3,4].

It is known that acetylcholine is primarily released from parasympathetic nerve endings and induces airway smooth muscle contraction. More recent studies have demonstrated that acetylcholine is also released from non-neuronal cells (e.g. epithelial cells or inflammatory cells) and can also influence airway inflammation and remodeling through muscarinic receptors expressed on these non-neuronal cells [5,6]. However, there are few studies focusing on the role of non-neuronal acetylcholine effects in the airway. To assess the impact of non-neuronal acetylcholine on mucus secretion from airway epithelial cells, we used a differentiated airway epithelial cell culture system that lacks nerve fibers.

Tiotropium inhibits acetylcholine from binding to the muscarinic 3 receptor (M3R) by competitively blocking the binding site [7]. While there is clinical evidence that tiotropium reduces sputum production in patients with COPD [8,9], its specific effects on IL-13 and NE induced mucus secretion have not been evaluated. We hypothesized that tiotropium would decrease mucin (MUC5AC) expression, and potentially prevent IL-13 stimulated goblet cell metaplasia in cultured human airway cells.

2. Methods

2.1. Reagents

The following reagents were purchased: recombinant human (rh) IL-

* Corresponding author. Dept. of Pediatrics, Virginia Commonwealth University School of Medicine, Children's Hospital of Richmond, 1000 East Broad St., Richmond, VA 23298, USA.
E-mail address: bruce.rubin@vcuhealth.org (B.K. Rubin).

https://doi.org/10.1016/j.pupt.2017.11.008
Received 28 September 2017; Received in revised form 15 November 2017; Accepted 17 November 2017
Available online 20 November 2017
1094-5539/

Abbreviation list

AChE	acetylcholine esterase
ALI	air-liquid interface
ANOVA	analysis of variance
CarAT	carnitine acetyltransferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBE	human bronchial epithelial
IL-13	interleukin-13
M1R	muscarinic 1 receptor
M2R	muscarinic 2 receptor
M3R	muscarinic 3 receptor
NE	neutrophil elastase
PAS	periodic acid-Schiff
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

13 (R&D Systems, Minneapolis, MN, USA); human NE (Innovative Research, MI, USA); DMEM, Ham's F12 medium (Gibco, Grand Island, NY, USA); human bronchial epithelial (HBE) cell growth medium, SingleQuotR® kit and Hanks' balanced salt solution (HBSS) (Lonza Walkersville Inc. Walkersville, MD, USA). Tiotropium was supplied by Boehringer Ingelheim (Rhein, Germany).

2.2. Culture and differentiation of HBE cells

The cultivation of HBE cells (Lonza Walkersville Inc.) and differentiation at air-liquid interface (ALI) have been previously reported (see the online supplement) [10,11]. Cell viability was evaluated using WST-8 assay (Cell Counting Kit-8, Dojindo, Kumamoto, Japan).

2.3. IL-13 stimulation

HBE cells were grown for 14 days at ALI with 0, 1, 5 ng/mL of IL-13 or IL-13 with 10 nM tiotropium by exposure from the basolateral side [12]. The medium was changed every 48 h. At day 14, supernatants, medium and cell lysates were collected for ELISA, choline/acetylcholine quantification assay, RNA for RT-PCR and Western blotting (Fig. 1). In addition, to compare with a short exposure to NE, the ciliated cells, which were grown for 14 days without IL-13, were exposed to IL-13 for 1 h.

2.4. NE exposure

The cells were grown for 14 days at ALI with 0, or 10 nM tiotropium in the basolateral medium; changed every 48 h. Neutrophils exist both in airway and in submucosa in neutrophil predominant asthma [13]. Therefore, HBE cells were exposed from the apical and basal side to 1×10^{-7} or 5×10^{-7} M of NE for 1 h at day 14 as previously reported [4,14]. Cell lysates were collected for RNA for RT-PCR and Western blotting (Fig. 1).

2.5. Real-time-PCR

Total RNA was extracted from the cells after IL-13 or NE exposure and prepared for real-time PCR as described in the online supplement. In addition to MUC5AC m RNA, we measured expressions of carnitine acetyltransferase (CarAT) an enzyme that promotes acetylcholine production and acetylcholine esterase (AchE) that catalyzes the breakdown of acetylcholine, and the muscarinic receptors expressed in the airway (M1, M2, and M3) [15]. The following primers were used [11,16].

MUC5AC forward: 5′-TACTCCACAGACTGCACCAACTG-3′
MUC5AC reverse: 5′-CGTGTATTGCTTCCCGTCAA-3′
CarAT forward: 5′-AAGAAGCTGCGGTTCAACAT-3′
CarAT reverse: 5′-GGGCTTAGCTTCTCCGACTT-3′
AchE forward: 5′—CCTCCTTGGACGTGTACGAT-3′
AchE reverse: 5′-CTGATCCAGGAGACCCACAT-3′
Muscarinic 1 receptor forward: 5′—CCGCTACTTCTCCGTGACTC-3′
Muscarinic 1 receptor reverse: 5′-GTGCTCGGTTCTCTGTCTCC-3′
Muscarinic 2 receptor forward: 5′-TACGGCTATTGCAGCCTTCT-3′
Muscarinic 2 receptor reverse: 5′-GCAACAGGCTCCTTCTTGTC-3′
Muscarinic 3 receptor forward: 5′-GGTCATACCGTCTGGCAAGT-3′
Muscarinic 3 receptor reverse: 5′-AGGCCAGGGTTAAGAGGAAG-3′
GAPDH forward 5′-TGAACGGGAAGCCACGG-3′
GAPDH reverse 5′-TCCACCACCCTGTTGCTGTA-3’.
ELISA for MUC5AC in cells supernatants

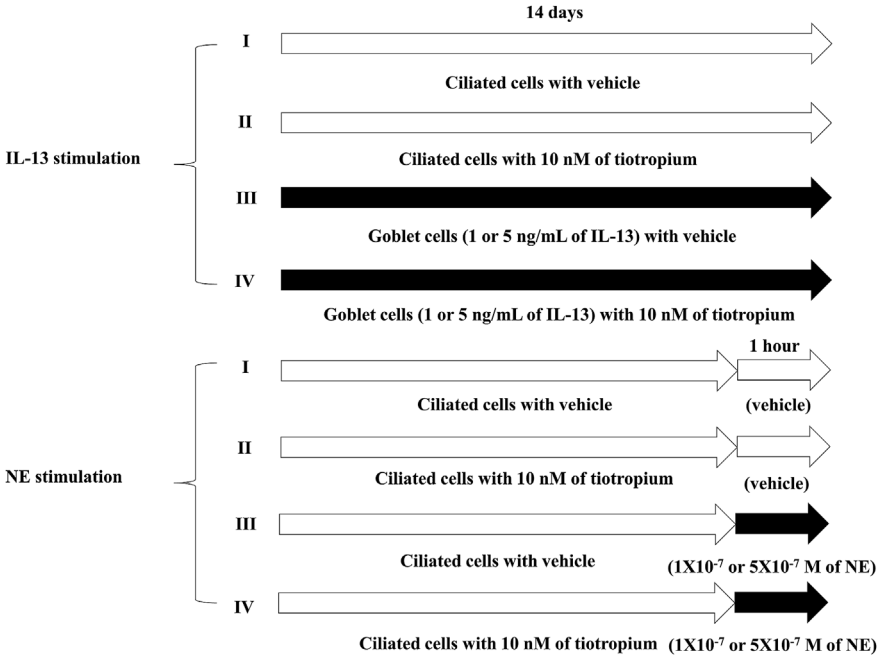

Fig. 1. Experimental design.
To evaluate the effect of tiotropium on IL-13 induced MUC5AC production, human bronchial epithelial cells (HBE) were grown for 14 days at air-liquid interface (ALI) with 0, 1, 5 ng/mL of IL-13 or IL-13 with 10 nM tiotropium exposure from the basolateral side. The medium was changed every 48 h. At day 14, supernatants and cell lysates were collected for ELISA, RNA for RT-PCR and Western blotting. For the effect on neutrophil elastase (NE) induced MUC5AC production, the cells were grown for 14 days at ALI with 0, or 10 nM tiotropium in the basolateral medium; changed every 48 h. HBE were exposed from the apical and basal side to 1×10^{-7} or 5×10^{-7} M of NE for 1 h at day 14.