Life Sciences 89 (2011) 36–43

Contents lists available at ScienceDirect

Life Sciences

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

Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro

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ARTICLE INFO

Article history:
Received 8 November 2010
Accepted 27 April 2011

Keywords:
Muscarinic receptors
Leukotriene B4
Bronchial epithelial cells
Anticholinergic drugs

ABSTRACT

Aims: Acetylcholine (ACh) is synthesized by Choline Acetyl-Transferase (ChAT) that exerts its physiological effects in airway epithelial cells via muscarinic receptor (MR) activation. We evaluate the effect of ACh stimulation on human bronchial epithelial cells (16-HBE) and test whether cigarette smoke extract (CSE) can modify the basal cellular response to ACh affecting the non-neuronal cholinergic system signalling.
Main methods: ACh stimulated 16-HBE were tested for ACh-binding, Leukotriene B_4 (LTB_4) release and ERK1/2 and NFkB pathway activation. Additionally, we investigated all the aforementioned parameters as well as ChAT and MR proteins and mRNA expression and endogenous ACh production in CSE-treated 16-HBE.
Key findings: We showed that ACh induced in 16-HBE, in a concentration-dependent manner, LTB_4 release via the activation of ERK1/2 and NFkB pathways. The addition of Tiotropium (Spiriva®), Gallamine, Telenzepine and 4-DAMP (muscarinic receptor antagonists), as well as of PD 098059 (MAPKK inhibitor) and BAY117082 (inhibitor of IkBα phosphorilation), down-regulated the ACh-induced effects. Additionally, CSE treatment of 16-HBE increased the binding of ACh, and shifted the LTB4 release from the concentration ACh 1 μM to 10 nM. Finally, we observed that the treatment of 16-HBE with CSE increased the expression of ChAT, M_2 and M_3 and of endogenous ACh production in 16-HBE. Tiotropium regulated the LTB4 release and ACh production in CSE treated 16-HBE.
Significance: CSE increases the pro-inflammatory activity of human bronchial epithelial cells, and promotes the cellular response to lower concentrations of ACh, by affecting the expression of ChAT and MRs. Tiotropium might prevent pro-inflammatory events generated by ACh together with CSE.
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Introduction

Acetylcholine (ACh) has recently been demonstrated to be involved in airway inflammation and remodeling processes (Gosens et al., 2004; Koyama et al., 1998, Sato, 1998 #57, Profita, 2005 #143). It is synthesized by Choline Acetyl-Transferase (ChAT) in different cell types (macrophages, T-lymphocytes, fibroblasts, and epithelial cells) acting as an autocrine/paracrine growth factor in regulating various aspects on the innate mucosal defence mechanisms including mucociliary clearance and regulation of macrophage function (Kummer et al., 2008).

Non neuronal ACh is involved in the activation of bronchial epithelial cells and alveolar macrophages as well as in the release of chemotactic mediators for eosinophils and neutrophils. In this manner ACh contributes to the inflammatory processes of Chronic Obstructive Pulmonary Disease (COPD) via the activation of muscarinic M_1, M_2, M_3 receptors (MRs) (Gosens et al., 2006; Profita et al., 2008; Profita et al., 2005).

The epithelium is a target for factors released by infiltrating inflammatory cells and it has been shown to serve as an effector of such inflammation regulating in turn the production of secondary inflammatory mediators such as LTB_4 (Jame et al., 2007). LTB4 appears to play an important role in the pathogenesis of COPD regulating neutrophil accumulation in the airways of these subjects (Profita et al., 2000). Additionally, it has been observed that MRs may be involved in airway inflammation through ACh-induced, ERK1/2-dependent LTB_4 release from induced sputum cells of COPD subjects (Profita et al., 2005).

Cigarette smoke (CS) exposure represents the major risk factor for COPD and, together with Interleukin-1β (IL-1β) and Tumor Necrosis Factor alpha (TNF-α), is involved in the activation of airway epithelial cells (Pettersen and Adler, 2002). Pro-inflammatory IL-1β and TNF-α are able to increase ChAT and M_3 and to down-regulate M_2 in human embryonic lung fibroblasts (Hela 299 cells), while cigarette smoke

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0024-3205/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.04.025

extracts (CSE) are able to increase ChAT and M_3 expression in human fetal lung fibroblasts (HFL-1) (Profita et al., 2009).

The aim of this study is to better understand the mechanism of airway inflammation involving airway epithelial cells and non-neuronal cholinergic system signalling in COPD. For this reason, we first evaluate whether ACh might be able to promote the release of LTB_4 via the activation of ERK1/2 and NFkB by MRs, using an in vitro model of human bronchial epithelial cell line (16-HBE). Then we tested whether cigarette smoke, the major risk factor of COPD, increases the pro-inflammatory activity of human bronchial epithelial cells by affecting the expression of ChAT and MRs and ACh production and binding.

Methods

Epithelial cell cultures

The SV40 large T antigen-transformed 16-HBE cell line (16-HBE) was used for these studies. 16-HBE is a cell line that retains the differentiated morphology and function of normal airway epithelial cells. The cells represent a clonal diploid ($2n = 6$) cell line isolated from human lung previously used to study the functional properties of bronchial epithelial cells in inflammation and repair processes. 16-HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 1% MEM (non-essential aminoacids, Euroclone), 2 mM L-glutamine and gentamicin 250 μg/ml.

16-HBE stimulation with ACh

To investigate the ACh-mediated pro-inflammatory mechanisms in airway epithelial cells, 16-HBE were plated until confluence with MEM 10% FBS. Under these conditions, cells were treated with trypsin, and approximately 5×10^5 cells were washed in cold PBS and incubated for 1 h at 4 °C with ACh (from 10 nM to 10 μM) to test the concentration response of the exogenous ACh binding. Additionally, the confluent cells, after 24 h under FBS-free conditions (5% CO_2 at 37 °C), were then stimulated with various concentrations of ACh (0.1 nM to 10 μM) (Sigma St. Louis, MO) for 18 h to evaluate LTB_4 production in cell culture supernatants and with ACh 1 μM for 30 min to test ERK1/2 and NFkB pathway activation. Cell viability was determined by light microscopy and trypan blue exclusion. Cell numbers were measured by directly counting the cells using a hemocytometer.

Stimulation of 16-HBE with drugs

In order to understand whether the activation of 16-HBE was related to M_1, M_2 or M_3, the effects of the following anticholinergic compounds were evaluated on ACh binding and on LTB_4 production: Tiotropium (non selective MR antagonist 0.1 μM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany), Telenzepine (described as M_1 antagonist, 10 nM) (Sigma Aldrich s.r.l., Milan Italy), Gallamine (described as M_2 antagonist, 10 nM) (Sigma Aldrich s.r.l., Milan Italy), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (described as M_3 antagonist, 10 nM) (Sigma St. Louis, MO). Additionally, in order to identify whether ACh activation of 16-HBE involves ERK1/2 and NFkB pathways, the effects of the following compounds were evaluated on LTB4 production: 2-(2-Amino-3methoxyphenyl)-4h-1benzopyran-4-one (PD O98059; an inhibitor of MAPKK activation, 25 μM) (Sigma Aldrich s.r.l., Milan Italy) and (E)-3-(4-methylphenylsulfonyl)-2-propenetrile (BAY 11–7082; an inhibitor of IkBα phosphorilation, 50 μM) (Sigma Aldrich s.r.l., Milan Italy). All these drugs were added to 16-HBE after 24 h under FBS-free conditions (5% CO_2 at 37 °C) and 1 h before the stimulation with ACh 1 μM. Finally, in order to verify whether anticholinergic drugs affect the ERK1/2 and NFkB pathway activation, measurements of ERK1/2 and NFkB activation were performed in 16-HBE stimulated with ACh 1 μM for 30 min in the presence or absence of Tiotropium added to the cells as previously described.

Preparation of CSE

Commercial cigarettes (Marlboro; Philip Morris USA, Richmond, VA) were used in this study. CSE was prepared as described previously (Luppi et al., 2005) with minor modifications (Profita et al., 2009) and further diluted to the required concentration in fresh culture medium. The viability of the cells exposed to CSE was evaluated by trypan blue exclusion dye assay.

Stimulation of 16-HBE with CSE

The cells were plated until confluence with MEM 10% FBS, followed by an additional 24 h under FBS-free conditions (5% CO_2 at 37 °C). Then, 16-HBE cells were treated for 18 h with CSE (10%) or medium alone under FBS-free conditions (5% CO_2, 37 °C). These cell cultures were then further incubated with and without ACh (0.1 nM to 10 μM) for 18 h to evaluated LTB_4 production in the culture supernatants. 5×10^5 16-HBE incubated with CSE 10% were treated with trypsin, washed in cold PBS and incubated for 1 h at 4 °C with ACh (10 nM) to test the exogenous ACh binding. 16-HBE treated with CSE 10% were also stimulated with ACh (0.1 nM to 10 μM) for 30 min at 37 °C for ERK1/2 and NFkB activation in cell lysates. Additional experiments were performed with 16-HBE stimulated with CSE (10%) for 18 h in the presence or absence of Tiotropium (0.1 μM) to evaluate the ACh measurements in cell supernatant and cell lysates. Finally, 16-HBE treated with CSE and Tiotropium were also stimulated with ACh (10 nM) for 18 h to evaluate LTB4 release.

Binding of ACh to 16-HBE cells

The cells stimulated as previously described were washed in cold PBS and incubated for 1 h at 4 °C with a rabbit polyclonal anti-ACh antibody (Abcam, Cambridge, UK). The antibody specifically recognized ACh bound by glutaric acid and it is a tool to visualise ACh bound to biological structure such as receptors. Anti rabbit IgG was used as an isotype negative control antibody (Dako LSAB, Glosgroup, Denmark). No specific binding and background fluorescence were detected by analyzing negative control samples. After washing the cells with cold PBS, a FITC-conjugated polyclonal swine anti-rabbit Ig (Dako LSAB, Glostrup, Denmark) was added to the cells for 30 min at 4 °C. Fluorescence-positive cells were analyzed using a FACStar Plus (Becton Dickinson, Mountain View, CA, USA) analyzer. The percentages of positive cells were determined from forward (FS) and sideways (SS) scatter patterns.

Measurement of LTB_4 release in 16-HBE cells

The release of LTB_4 was determined in 16-HBE culture supernatants using a commercially available enzyme-linked immunosorbent assay (ELISA) (GE Healthcare UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK), according to the manufactures' specifications. The lower detection limit was 6 pg/ml.

Measurements of ERK1/2 and NFkB activation

To assess ERK1/2 and NFkB activation in cell lysates from 16-HBE, we performed western blot analyses using two rabbit monoclonal antibodies against pERK1/2 and against pIkBα (Cell Signaling Technology, Beverly, MA) respectively, and an anti-β-actin antibody (Sigma St. Louis, MO). Additionally, we evaluated the ERK1/2 and

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