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Glycopyrronium bromide inhibits lung inflammation and small airway remodeling induced by subchronic cigarette smoke exposure in mice

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ABSTRACT

The effects of long-acting muscarinic receptor antagonists (LAMAs) have not been evaluated in a model with simultaneous lung inflammation and small airway remodeling induced by cigarette smoke (CS). We exposed the mice to CS for four weeks with daily treatment with a LAMA (glycopyrronium bromide, NVA237) or its vehicle. Human bronchial epithelial cells (PBECs) and lung fibroblasts were exposed to CS extract (CSE) or acetylcholine with or without NVA237 treatment. We found that NVA237, but not its vehicle, suppressed elevations in inflammatory score, epithelial thickness, and peribronchial collagen deposition in CS-exposed mice. NVA237 alleviated CS-induced increased levels of chemokines, inflammatory cells, and total protein in the bronchoalveolar lavage fluid. NVA237 suppressed acetylcholine- or CSE-induced elevations in IL-8 production in PBECs and elevations in proliferation and collagen production in lung fibroblasts. These phenomena were also prevented by a p44/42 MAPK inhibitor. In conclusion, NVA237 exerted a potent suppressive effect on lung inflammation and small airway remodeling induced by subchronic CS exposure.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by lung inflammation and small airway remodeling, which are mainly caused by chronic cigarette smoke (CS) exposure (Chung and Adcock, 2008; Hogg et al., 2004; Rennard et al., 2006). Initially, CS directly stimulates lung epithelial cells to release cytokines and chemotactic factors, followed by recruitment of inflammatory cells and induction of lung inflammation (Chung and Adcock, 2008; Tang et al., 2011; Wu et al., 2014; Ko et al., 2015). Persistent CS exposure results in the development of small airway (diameter < 2 mm in adult human) remodeling, especially peribronchial fibrosis, because of inadequate control of fibroblast repair, extracellular matrix accumulation, and fibrillar collagen production (Hogg et al., 2004; Rennard et al., 2006). Small airways become narrowed, causing airflow obstruction and other clinical manifestations of COPD (Hogg et al., 2004; Rennard et al., 2006). Thus, CS-induced lung inflammation and small airway remodeling markedly contribute to the development of COPD (Chung and Adcock, 2008; Hogg et al., 2004; Rennard et al., 2006). Although COPD management has attracted much interest, an optimal therapeutic strategy has not been established.

Long-acting muscarinic receptor antagonist (LAMA) has been applied as a bronchodilator in COPD management, because this class of drugs can block the neuronal cholinergic function (Rodrigo et al., 2017; Karakiulakis and Roth, 2012; Kistemaker and Gosens, 2015). Recent studies suggest that a non-neuronal cholinergic system in various types of lung cells may play important roles in inducing inflammation and remodeling, thereby contributing to the development of COPD (Karakiulakis and Roth, 2012; Kistemaker and Gosens, 2015). These observations suggest that LAMA has beneficial effects in the treatment

Abbreviations: BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, cigarette smoke extract; LAMA, long-acting muscarinic receptor antagonists; MIP-2, macrophage inflammatory protein-2; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; PBECs, primary human bronchial epithelial cells; qPCR, quantitative polymerase chain reaction

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of COPD in addition to the bronchodilator action by blocking the non-neuronal cholinergic function (Karakiulakis and Roth, 2012; Kistemaker and Gosens, 2015). This implication is supported by *in vitro* findings reporting that LAMA suppressed inflammatory or fibrotic responses to acetylcholine (Buhling et al., 2007; Pieper et al., 2007; Profita et al., 2009; Gosens et al., 2009) or CS extract (CSE) (Gosens et al., 2009; Cortijo et al., 2011; Profita et al., 2011; Milara et al., 2013) in various types of lung cells. This concept is also supported by *in vivo* findings on the anti-inflammatory and anti-remodeling effects of LAMA in animal models with CS exposure. Several studies have focused on the effect of LAMA on acute lung inflammation induced by CS exposure for four days in mice (Wollin and Pieper, 2010; Shen et al., 2014; Zhang et al., 2015). One investigation concentrated on the effect of LAMA on small airway remodeling and lung emphysema induced by CS exposure for 24 weeks in guinea pigs (Dominguez-Fandos et al., 2014). Acute lung inflammation and lung emphysema occur at the early and late phases of the COPD development, respectively (Chung and Adcock, 2008; Rennard et al., 2006). Thus, the beneficial effects of LAMA have not been thoroughly evaluated in a transitional stage with lung inflammation and small airway remodeling.

This study was performed to explore the therapeutic effects of glycopyrronium bromide (NVA237; a LAMA) on lung inflammation and small airway remodeling induced by subchronic CS exposure for four weeks using a well-established murine model (Tang et al., 2011; Wu et al., 2014; Ko et al., 2015). We also investigated the suppressive effects of NVA237 on the responses of primary human bronchial epithelial cells (PBECs) and lung fibroblasts to CSE or acetylcholine using *in vitro* models (Perng et al., 2003; Lee et al., 2011).

2. Materials and methods

2.1. Ethic statement

All experiments involving the use of animals were approved by the Animal Care and Use Committee of the National Yang-Ming University. All experiments involving the use of human lung tissues were approved by the Institutional Review Board of Taipei Veterans General Hospital, and written informed consent was obtained from subjects.

2.2. Murine model of subchronic CS exposure

The murine model of subchronic CS exposure has been previously described in detail (Tang et al., 2011; Wu et al., 2014). Male C57BL/6J mice at eight weeks of age (National Laboratory Animal Center, Taipei, Taiwan) were randomly divided into four groups (n = 5 mice/group) for 4-week exposure to CS or air. Two of the CS-exposed groups received daily treatment with NVA237 (Novartis® Pharmaceuticals, Taiwan; 3 mg/kg) or saline vehicle (NS) by intraperitoneal (i.p.) injection during the 4-week exposure. The dose of NVA237 was suggested by a previous study (Baker et al., 2017). Thus, the four groups of mice were Air, CS, CS + NS, and CS + NVA237. Animals were given *ad libitum* access to food and water, and the averaged body weights did not vary among the study groups after the 4-week exposure. For each CS exposure, the mice were placed in an exposure chamber, and 750 mL of fresh CS generated from 1.5 cigarettes (Marlboro Red Label; 10.8 mg of nicotine and 10.0 mg of tar per cigarette) was delivered to the chamber. The CS passed out of the chamber via four exhaust holes (1 cm) on the side panels. During exposure, the mice were conscious and breathed spontaneously in the chamber for 10 min. After exposure, the mice were transferred to a new cage and allowed to normally inspire air. The mice were exposed at 10:00 AM and 4:00 PM each day for four weeks. The control animals underwent identical procedures in another chamber but were only exposed to air. For each CS exposure, the initial particle concentration inside the exposure chamber was approximately 625 mg/m^3, which decreased over time because the CS passed out of the chamber via the exhaust holes (Wu et al., 2014). The HbCO levels immediately after the 10-min exposure protocol for air-exposed and CS-exposed mice were 0.4% and 32%, respectively (Wu et al., 2014).

2.3. Preparation of bronchoalveolar lavage fluid (BALF) and lung tissues

The mice were euthanized with CO_2 at the end of each experiment, and a middle thoracotomy was performed. The left lung was ligated, and the right lung was lavaged four times with warm PBS (0.6 mL) containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The BALF samples were centrifuged at 350*g* for 5 min at 4 °C, and the supernatant of the first lavage fluid was stored at −80 °C for subsequent analysis of total protein using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2; a human IL-8 homolog) using ELISA kits (R&D Systems, Minneapolis, MN, USA). The cell pellets of the BALF samples were resuspended in PBS for cell counting. The right lung was then stored at −80 °C for subsequent analysis. The left lung was fixed with 4% paraformaldehyde and embedded in paraffin.

2.4. Histological assessment

Formalin-fixed, paraffin-embedded tissue blocks were cut into 8-μm sections. The sections were deparaffinized, rehydrated, and then covered with 3% H_2O_2 for 10 min. The slides were counterstained with hematoxylin and eosin (H&E) for cellular infiltration and epithelial thickening and with Masson's trichrome for peribronchial collagen deposition. Then, the cells were examined under a microscope (Motic TYPE 102 M, Xiamen, China). Histological assessments were conducted by a pathologist who was blinded to the treatment. Each histological characteristic was scored on a scale of 0 (normal) to 5 (maximal). The lung inflammatory score was categorized according to the average of the score for number of infiltration cells and damage level, including thickening of alveolar walls and epithelium, as well as increases in peribronchial and perivascular cuff area. The thickness of the epithelium and peribronchial collagen area was also measured using the image analysis software Motic Images Plus 2.0 (Motic China Group, Xiamen, China). The small airway in small animals was defined as lumen perimeter below the median value of the airways (Dominguez-Fandos et al., 2014).

2.5. Modified air–liquid interface culture for PBECs

Preparation of the modified air-liquid interface culture for PBECs has been previously described in detail (Perng et al., 2003). The human bronchus was obtained from surgical lobectomy for lung cancer. The bronchus was rinsed several times with Leibovitz's L-15 medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). The tissue was cut into 1–2 mm^2 pieces and 3–4 pieces of tissue were planted with the epithelium side facing down onto 6-well culture inserts (growth area of membrane, 4.2 cm^2; pore size, 0.4 μm) coated with type IV collagen (50 μg/cm^2). PBEC culture medium (2 mL) [containing antibiotics/antimycotic, insulin (2.5 μg/mL), transferrin (2.5 μg/mL), hydrocortisone (1 μg/mL), 2 mM glutamine and 0.1% FBS in RPMI 1640, and Medium 199 (v/v 1:1)] was added to the basal chamber, and 100 μL was added to the insert. Culture medium in the basal chamber was changed every 48 h to 72 h, and no medium was added to the insert. PBECs were grown on a porous membrane, and they formed a continuous epithelial sheet with the basal aspect exposed to the medium and the apical surface exposed to air.

2.6. Human primary lung fibroblast culture

Preparation of human primary lung fibroblasts has been previously described in detail (Lee et al., 2011). Lung parenchyma, obtained from