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Cigarette smoke augments MUC5AC production via the TLR3-EGFR pathway in airway epithelial cells

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ABSTRACT

Background: Viral infections are a major cause of chronic obstructive pulmonary disease (COPD) exacerbations. Toll-like receptor 3 (TLR3) reacts with double-stranded RNA (dsRNA) and participates in the immune response after viral infection. In the present study, we examined whether cigarette smoke, which is involved in the pathogenesis of COPD, enhances mucin production via the TLR3-epidermal growth factor receptor (EGFR) pathway in airway epithelial cells.

Methods: We studied the effects of cigarette smoke extract (CSE) on signal transduction and the production of mucin 5AC (MUC5AC) in NCI-H292 cells and differentiated primary human bronchial epithelial cells stimulated with a synthetic dsRNA analogue, poly-inosinic-polycytidylic acid [poly(I:C)], used as a TLR3 ligand.

Results: CSE significantly potentiated the production of MUC5AC in epithelial cells stimulated with poly(I:C). Antibodies to EGFR or EGFR ligands inhibited CSE-augmented MUC5AC release in poly(I:C)-treated cells. Treatment with poly(I:C) or CSE alone increased the phosphorylation of EGFR and extracellular signal-regulated kinase (ERK). However, after poly(I:C) stimulation, CSE did not enhance EGFR phosphorylation, but did augment ERK phosphorylation. EGFR inhibitors and an ERK inhibitor inhibited the augmented release of MUC5AC. In addition, treatment with *N*-acetylcysteine, an antioxidant, inhibited the CSE-augmented phosphorylation of ERK and MUC5AC.

Conclusions: These data show that cigarette smoke increases TLR3-stimulated MUC5AC production in airway epithelial cells, mainly via ERK signaling. The effect might be mediated in part by oxidative stress. Modulation of this pathway might be a therapeutic target for viral-induced mucin overproduction in COPD exacerbation.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death and a major cause of chronic morbidity and mortality worldwide [1]. COPD, a chronic inflammatory disease characterized by a specific pattern of inflammation involving neutrophils, macrophages, and CD8 T lymphocytes, is caused by long-term exposure to noxious gasses such as cigarette smoke [1,2]. Goblet cell hyperplasia and mucus hypersecretion are common features of the disease, especially during exacerbation. Mucociliary clearance is a potent, innate defense system against exogenous insults, including bacteria and viruses. However, excessive mucus production causes airway obstruction, which can lead to exacerbation of COPD. It is also associated with disease morbidity and mortality [3–6]. However, the mechanism for enhanced mucus production has yet not been fully elucidated.

At least 19 different mucin genes have been identified in humans. Mucin 5AC (MUC5AC) is a major component of the mucus produced by airway epithelial cells [7]. Various factors induce airway mucus production, including proinflammatory cytokines [8,9] and bacterial exoproducts [10,11]. Oxidative stress and cigarette smoke, which contains many particles [12] and oxidants, including hydrogen peroxide [13], enhance airway mucin production [14–16], mainly via the epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) (including extracellular signal-regulated kinase [ERK]) signaling pathways [15,17].

Viral infection is a major cause of COPD exacerbations [18,19]. Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns, have a key role in the innate immune system [20]. TLR3, which reacts with virus-derived double-stranded RNA, is thought to play a key role in virus-induced immune reactions [21]. TLR3 is mainly detected on endosomes within airway epithelial cells, dendritic cells, and macrophages [20–22]; its activation leads to the release of proinflammatory mediators and type I interferons [20]. Viral infection and virus-derived dsRNA also induce airway mucus production and enhance *MUC5AC* expression via TLR3 and the activation of EGFR and ERK [23,24]. Previously, we and others have shown that oxidative stress and cigarette smoke augment the TLR3-mediated response in airway epithelial cells [25,26]. Cigarette smoke also reportedly enhances MUC5AC induction in response to proinflammatory stimuli [27]. However, the effect of cigarette smoke on the induction of mucin production by viral-derived dsRNA via the TLR3-EGFR pathway in airway epithelial cells remains unclear.

Here, we used a synthetic dsRNA analogue, polyinosinic-polycytidylic acid [poly(I:C)], as a TLR3 ligand to mimic viral infection in order to determine whether (a) cigarette smoke affects poly(I:C)-induced MUC5AC production in airway epithelial cells and (b) cigarette smoke modulates poly(I:C)-induced TLR3-EGFR signaling.

2. Materials and methods

Detailed methods are provided in the online Supplementary data.

2.1. Materials

Bafilomycin was purchased from Alexis Biochemicals (Lausen, Switzerland). Poly(I:C) (polyinosinic acid/polycytidylic acid, sodium salt, double-stranded), AG1478, U0126, BIBX1382, TAPI-1, GM6001, and cycloheximide were from Calbiochem (La Jolla, CA). The mouse anti-EGFR neutralizing antibody was purchased from Calbiochem (La Jolla, CA); other neutralizing antibodies (anti-transforming growth factor [TGF]-α, anti-amphiregulin) were from R&D Systems, Inc. (Minneapolis, MN). Lipopolysaccharide (LPS), imiquimod, and ssRNA40 were from InvivoGen (San Diego, CA). *N*-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of epithelial cells

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were grown to 80% confluence in 6-well, 24-well, or 96-well plates and were maintained in FBS-free medium for 24 h before stimulation. Primary normal human bronchial epithelial cells (HBECs) from three different donors were purchased from Lonza (Walkersville, MD) and ScienCell Research Laboratories (Carlsbad, CA). Air–liquid culture of human primary bronchial epithelial cells was performed using Clonetics® B-ALI™ Air–Liquid Interface (ALI) medium. The ALI state was maintained for 7–10 days; previous studies have shown this duration is required for mucociliary differentiation [28,29].

To investigate the effect of poly(I:C) on the cells, supernatants were harvested 24 h (unless otherwise indicated) after treatment with poly(I:C). Compounds or neutralizing

Abbreviations: ALI, air–liquid interface; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBEC, human bronchial epithelial cell; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MUC5AC, mucin 5AC; NAC, *N*-acetylcysteine; poly(I:C), polyinosinic-polycytidylic acid; ROS, reactive oxygen species; siRNA, small interfering RNA; TACE, TNF-α-converting enzyme; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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