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Bombesin receptor-activated protein regulates neutrophil elastase-induced mucin5AC hypersecretion in human bronchial epithelial cells

Qing Xu[a], Ling-Xiu Chen[a], Dan-Hua Ran[b], Wen-Yue Xie[a], Qi Li[c], Xiang-Dong Zhou[a,c,*]

[a] Division of Respiratory Medicine, Second Affiliated Hospital, Chongqing Medical University, No. 74, Linjiang Road, Yuzhong District, Chongqing 400010, China

[b] Department of Respiratory and Geriatrics Medicine, Chongqing Public Health Medical Center, No. 2, Huangjiaowan Road, Xiaolongkan Street, Shapingba District, Chongqing 400010, China

[c] Department of Respiratory Medicine, First Affiliated Hospital, Hainan Medical University, No. 31, Longhua Road, Haikou 570102, Hainan, China

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ABSTRACT

Bombesin receptor-activated protein (BRAP) is highly expressed in human bronchial epithelial cells. Recent studies have shown that BRAP reduces oxidative stress, inhibits airway inflammation and suppresses nuclear factor kappaB (NF-κB) activity. Mucus overproduction is an important feature in patients with chronic inflammatory airway diseases. Neutrophil elastase (NE) is a potent inducer of mucin5AC (MUC5AC), which is considered the predominant mucin secreted by human airway epithelial cells. Here, we hypothesize that BRAP may regulate NE-induced MUC5AC hypersecretion in a bronchial epithelial cell line (HBE16). We also investigated the underlying mechanism involved in the process. In this study, we found that BRAP was present in HBE16 human bronchial epithelial cells and was significantly increased by NE. Next, we found that the up-regulation of BRAP by pEGFP-N1-BRAP caused a significant decrease in the increased levels of MUC5AC expression, NF-κB activity, and the phosphorylation of extracellular signal-regulated kinases (ERK) and epidermal growth factor receptor (EGFR) induced by NE. Meanwhile, there was a significant decrease in ROS, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels when BRAP was up-regulated by pEGFP-N1-BRAP. Moreover, when cells were transfected with pEGFP-N1-BRAP and pretreated with NF-κB, ERK or EGFR inhibitors before the NE stimulation, there were further decreased in MUC5AC expression, NF-κB activity, and the phosphorylation of ERK and EGFR. These results suggest that BRAP plays an important role in airway inflammation and its overexpression may regulate NE-induced MUC5AC hypersecretion in HBE16 cells via the EGFR/ERK/NF-κB signaling pathway.

1. Introduction

Mucus secretion is the first line of defense against the barrage of irritants in the respiratory tract [1,2]. However, mucus hypersecretion contributes to the pathophysiology of many patients with chronic inflammatory airway diseases, such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and bronchitis [3,4]. The accumulation of excessively secreted mucus in the airway can aggravate airway narrowing and serve as a hotbed for bacterial growth, contributing to recurrent infections, clinical deterioration and even death [5–7]. Mucin5AC (MUC5AC) is considered the predominant mucin secreted by human airway epithelial cells [8–10]. MUC5AC synthesis and gene expression are up-regulated by a variety of stimulators such as cytokines, lipopolysaccharide and cigarette smoke [11–14]. It has been demonstrated that the expression of MUC5AC is regulated by multiple signaling pathways, including the epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), and signal transducers and activators of transcription pathways [15–17]. Neutrophil elastase (NE), a serine protease secreted by neutrophils that exists in high concentrations in the airway secretions of these patients, is one of the most potent inducers for mucous overproduction [18–20]. Previous studies have revealed that the activation of EGFR, extracellular signal-regulated kinases (ERK) and nuclear factor kappaB (NF-κB) are involved in MUC5AC expression in human airway epithelial cells after stimulation with NE or cigarette smoke [12,21–23].

Bombesin receptor-activated protein (BRAP), which was identified in a bacterial two-hybrid screen for proteins interacting with the

Abbreviations: BRAP, bombesin receptor-activated protein; ROS, reactive oxygen species; NE, neutrophil elastase

* Corresponding author.
E-mail addresses: xq770816@163.com (Q. Xu), zxd999@263.net (X.-D. Zhou).

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orphan G protein-coupled bombesin receptor subtype −3, is encoded by *C6ORF89* [24,25]. Full-length BRAP cDNA is 6751 bp, and the gene is located at 6p21.2 (accession number: NM_152734). The ORF length is 1065 bp and encodes a protein with 354 amino acids [25,26]. Studies have shown that BRAP is widely expressed in human airway epithelial cells and vascular endothelial cells [26,27]. BRAP mRNA and protein levels were significantly increased in lung tissue after exposure to ozone [24]. The up-regulation of BRAP promotes cell repair, enhances the proliferation of human bronchial epithelial cells, reduces reactive oxidative stress and maintains airway homeostasis [25]. It has been suggested that BRAP may play an important role during the response to airway inflammation and airway hyper-responsiveness [24]. Additionally, a recent study has shown that BRAP negatively regulates NF-κB activity [27].

Based on these findings, we speculated that BRAP may play an important role in MUC5AC hypersecretion in chronic inflammatory airway disease. To test this hypothesis, the goals of this study were the following: (1) confirm the expression of BRAP with or without NE stimulation in HBE16 human bronchial epithelial cells; (2) explore the role of NE-mediated activation of BRAP in the enhanced expression of MUC5AC; and (3) investigate the role of EGFR/MAPK/NF-κB in the underlying mechanisms of BRAP-mediated NE-induced MUC5AC overproduction.

2. Materials and methods

2.1. Reagents

The reagents used in these experiments are as follows: The 16HBE human bronchial epithelial cell line was from the Experimental Medical Research Center of Guangzhou Medical College (Guangzhou, Guangdong, CHN); the ROS detection kit was from GenMed (Boston, MA, USA); NE, anti-β-actin, anti-p -ERK, anti-p-EGFR, anti-p-p38, anti-p-c-Jun N-terminal kinase (p-JNK), anti-ERK, anti-EGFR, anti-p38, anti-JNK and anti-BRAP antibodies were from Abcam (Cambridge, MA, USA); the horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jianqiao Biotech (Beijing, CHN); TRIzol, the NF-κB (PDTC), ERK (U0126) and EGFR (AG1478) inhibitors, pNF-κBLuc luciferase reporter vector and double luciferase reporter gene assay kit were obtained from BioTime Technology (Beijing, China); pRL-TK Renilla luciferase reporter vector was purchased from Promega (Madison, WI, USA); the Western blot reagents, MTT assay were from Beyotime Biotechnology (Beijing, CHN); all recombined plasmids and small interfering RNAs were from Hanbio Biotechnology (Shanghai, CHN).

2.2. Cell culture

HBE16 cells were seeded into a six-well plate and cultured in 3 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified incubator. The culture media was renewed every 2–3 days. The cells were passaged at 80–90% confluency and used for experiments at this time.

2.3. Plasmids and siRNAs transfection

To investigate whether NE-induced MUC5AC expression was mediated by BRAP, we overexpressed BRAP by transfecting recombined plasmid (pEGFP-N1-BRAP) and downexpressed BRAP by transfecting BRAP-small interfering RNA (siBRAP). 16HBE cells were plated at a density of 1×10^6 -2×10^6 cells/ml at 60–80% confluence. All plasmids and siRNAs were respectively transfected using Lipofectamine 2000 prior to NE stimulation in accordance with the manufacturer's instructions. pEGFP-N1 empty vector and negative control siRNA (siNT)-transfected cells were respectively used as the control group.

2.4. Cell viability assay

Cell viability was assessed by the MTT assay. Briefly, cells (5×10^4 cells/well) were seeded in 96-well plates and cultured in 200 μl of RPMI 1640 medium at different treatment conditions for the indicated time periods. Next, 20 μl of MTT was added and the plates were incubated for an additional 4 h at 37 °C. After incubation, 150 μl of dimethyl sulfoxide (DMSO) was added to each well and allowed to incubate for 10 min at room temperature. Subsequently, the absorbance was measured at 570 nm with the plate reader.

2.5. ROS detection

The ROS (including superoxide radical, hydrogen peroxide, hydroxyl radical, and single oxygen) generated in each group were measured using an ROS colorimetric quantity assay kit according to the manufacturer's protocol. Briefly, when the cells cultured in the 24-well plates reached 70% confluence, the medium was removed and fresh culture medium containing staining solution (tetrazolium compound, 1000:1) was added to each well. After incubation at 37 °C for 30 min, the cells were washed twice before the addition of the dissolving solution and further incubation for 5 min at room temperature. The absorbance in each well was read at 650 nm (Sunrise remote; Tecan Austria GmbH, Grodig, Austria).

2.6. Real-time PCR

Total RNA was extracted from the HBE16 cells in each group using TRIzol reagent. The extraction was verified by electrophoresis on a 1.0% agarose gel and an absorbance (A260/280) value of 1.8–2.0. Reverse transcription for complementary DNA was performed using an RT-PCR kit. All primers were from Sangon Biotech (Shanghai, CHN). The PCR primers were as follows: MUC5AC, forward-5'-AACTGCAGCTGGACAGTGTG-3', reverse-5'-TGCAGATCTGGGTCTCACAG-3'; BRAP, forward-5'-GCTGCTGGAAAAGAATGAACC-3', reverse-5'-TGGCAATGGGCAAGGACA-3'; TNF-α, forward-5'-AGGACTCAGCTTCGACACCA-3', reverse-5'-CGTCCACAGACTTCCCATTC-3'; IL-1β, forward -5'-CAGGATGAGGACATGAGCACC-3', reverse-5'-CTCTGCAGACTCAAACTCCAC-3'; GAPDH, forward-5'-AGTGGATATTGTTGCCATCA-3', reverse-5'-GAAGATGGTGATGGGATTTC-3'. The PCR conditions were as follows: denaturation at 94 °C for 10 min; 30 cycles of 94 °C for 30 s, 57 °C for 45 s, 70 °C for 45 s; 72 °C for 7 min. The results were normalized to GAPDH as an internal control.

2.7. ELISA

After various treatments, the production of MUC5AC protein was measured using a specific ELISA kit (Yuanye, Shanghai, China) according to the manufacturer's instructions. Cell lysates were prepared with phosphate buffered saline (PBS) at multiple dilutions. Fifty microliters of each sample were incubated overnight with bicarbonate-carbonate buffer (50 μl) at 40 °C in a 96-well plate. The plates were washed 3 times with PBS and blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. Then, the samples were incubated with 50 μl of the primary antibody (1:200 for mouse for TNF-α and IL-1β; 1:200 for mouse for MUC5AC antibody) for 1 h at 37 °C, followed by incubation with 100 μl of HRP-conjugated goat anti-mouse IgG (1:1000) at room temperature for 1 h. A color reaction was developed with 3,3',5,5'-tetramethylbenzidine peroxidase solution and quenched with 1 mol/L H_2SO_4. The absorbance was read at 450 nm using a microplate reader (Sunrise Remote; Tecan). The protein concentrations were calculated by comparison with standard samples(μg/ml).

2.8. Western blotting

Before harvesting cells and washing thrice with PBS, the super-