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Resistin upregulates *MUC5AC/B* mucin gene expression in human airway epithelial cells

Soyoung Kwak [a], Yong-Dae Kim [b, c], Hyung Gyun Na [b], Chang Hoon Bae [b], Si-Youn Song [b], Yoon Seok Choi [b, *]

[a] Department of Medical Science, College of Medicine, Graduate School of Yeungnam University, Daegu, Republic of Korea
[b] Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Yeungnam University, Daegu, Republic of Korea
[c] Regional Center for Respiratory Diseases, Yeungnam University Medical Center, Daegu, Republic of Korea

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ABSTRACT

Adipokines, a group of proteins including leptin, visfatin, resistin, and adiponectin, are produced by adipocytes. Among adipokines, resistin is implicated in insulin resistance and inflammatory response modulation. Mucus hypersecretion has been greatly linked to airway diseases, such as asthma, chronic obstructive pulmonary disease, and rhinosinusitis. Increasing evidence has indicated that adipokines, such as leptin and visfatin, play important regulatory roles in various biological processes involved in mucus secretion. However, the effects of resistin on mucin expression in human airway epithelial cells, as well as the underlying mechanisms, have not been investigated yet. We showed that resistin affected mucin expression in human airway epithelial cells *via* the mitogen-activated protein kinase/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Resistin increased MUC5AC and MUC5B expression in NCI-H292 and primary human nasal epithelial cells. Additionally, it significantly increased the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and NF-κB. ERK1/2 and p38 specific inhibitors significantly attenuated resistin-induced MUC5AC/5B expression; however, NF-κB inhibitor reduced resistin-induced MUC5AC, but not MUC5B, expression. Knockdown of ERK1, ERK2, and p38 by ERK1, ERK2, and p38 small interfering RNA (siRNA), respectively, significantly blocked resistin-induced MUC5AC and MUC5B mRNA expression. In addition, NF-κB siRNA attenuated resistin-induced MUC5AC, but not MUC5B, expression. These results suggested that resistin induced MUC5AC and MUC5B expression *via* activation of different signaling pathways in human airway epithelial cells.

1. Introduction

Airway mucus is mainly composed of water, ions, lipids, and various macromolecules. Among these macromolecules, mucins and other proteins are present as a thin layer of fluids covering the luminal surface of airways to protect the respiratory epithelium [1]. This airway surface liquid (ASL) plays a pivotal role in human airway defense mechanisms. However, mucus dysfunction occurs in almost all respiratory diseases. Mucus hypersecretion is related to chronic airway diseases, such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis, chronic rhinosinusitis, and cigarette smoke-related airway inflammation [2,3]. Mucins, the major contributors to the viscoelastic property of mucus, play important roles in the maintenance of mucociliary clearance that defends the epithelium against infection, dehydration, and environmental challenges. To date, 21 human mucin genes have been identified; the expression of 14 of them has been verified in the airways. Among these airway-associated mucins, MUC5AC and MUC5B glycoproteins have been linked to gel formation. Abnormal mucin gene expression contributes to airway obstruction [4,5]. In an ongoing clinical trial, a mucin regulator is being examined as a potential therapeutic option [6,7].

Obesity is strongly linked to respiratory diseases, particularly asthma and COPD [8]. Obese patients are more susceptible to respiratory diseases; moreover, obesity can exacerbate respiratory diseases [9]. Previous *in vitro* studies have provided important

* Corresponding author. Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu, 42415, Republic of Korea.
E-mail address: choiys@ynu.ac.kr (Y.S. Choi).

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insights into the link between asthma and obesity [10,11]. Obesity is associated with airway hyperresponsiveness, which is a major pathophysiological feature of asthma. Adipokines, including leptin, visfatin, resistin, omentin, and adiponectin, are hormones secreted by the adipose tissue, particularly in obesity. They are involved in the pathophysiology of respiratory diseases *via* various signaling pathways. Leptin and adiponectin are well-known proinflammatory and anti-inflammatory adipokines, respectively. Patients with asthma tend to exhibit high serum leptin and low serum adiponectin levels [12]. Moreover, our previous study showed that leptin and visfatin increased mucin gene expression in human airway epithelial cells *via* the mitogen-activated protein kinase (MAPK) signaling pathway [13,14]. Resistin, known as adipose tissue-specific secretory factor, is implicated in insulin resistance and inflammatory responses [15]. In addition, resistin is used as a severity marker of asthma [12].

Therefore, in this study, we aimed to investigate the effects of resistin on the regulation of mucin secretion in human airway epithelial cells. In particular, the effects of resistin on MUC5AC and MUC5B expression and the underlying signaling pathways in human airway epithelial cells were investigated.

2. Materials and methods

2.1. Materials

Recombinant human resistin was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). NCI-H292 cells and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Human nasal epithelial cells and airway epithelial cell growth medium were purchased from PromoCell (Heidelberg, Germany).

The specific inhibitors U0126, SB203580, and BAY 11-7085 were purchased from Calbiochem (San Diego, CA, USA), BIOMOL (Plymouth Meeting, PA, USA), and Sigma-Aldrich (St. Louis, MO, USA), respectively. For transfection with small interfering RNA (siRNA), control siRNA and predesigned siRNA (extracellular signal-regulated kinase [ERK]1, ERK2, p38, and nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB]) were obtained. This study was approved by the Institutional Review Board for human studies at the Yeungnam University Medical Center, and written informed consents were obtained from all patients (YUMC 2016-05-040).

2.2. Cell culture and treatment

NCI-H292 cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin G/streptomycin) at 37 °C in 5% CO_2-fully humidified air. When the cell cultures reached 80–90% confluency, cells were incubated in a serum-starved medium for 24 h. They were then rinsed with phosphate-buffered saline (PBS) and exposed to the indicated concentrations of recombinant human resistin. Human nasal epithelial cells were cultured in airway epithelial cell growth medium at 37 °C in 5% CO2-fully humidified air and subcultured, according to the manufacturer's instructions. To investigate the signaling pathways involved in MUC5AC and MUC5B expression, cultured cells were pretreated with a specific inhibitor for 1 h before exposure to resistin, whereas control cells were incubated with the culture medium for 1 h.

2.3. Cell viability assay

Water-soluble tetrazolium salt-1 (WST-1) was used as an indicator of cell viability and proliferation. Cells were grown in 96-well plates at a density of 1×10^4 cells/well. After 24 h, cells were washed with a fresh medium and treated with resistin. After incubation for 48 h, cells were washed, and 10 μL of EZ-Cytox cell viability assay kit (Daeil Lab, Seoul, Korea) was added, followed by incubation for 4 h. The amount of formazan salt was determined by measuring the absorbance at 450 nm (reference wavelength 600–650 nm) using a microplate reader (Tecan Austria GmbH, Austria).

2.4. RT-PCR analysis

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. GeneAmp RNA PCR core kit (Thermo Fisher Scientific, Foster City, CA, USA) was used for the detection and analysis of RNA gene expression. We performed two-step polymerase chain reaction (PCR) experiments. Briefly, murine leukemia virus (MuLV) reverse transcriptase was used for reverse transcription of RNA to cDNA, and Amplitaq DNA polymerase was employed for subsequent PCR amplification. Results were presented as previously described [13,16]. PCR products were quantified and normalized to the expression of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Subsequently, PCR products were detected using 2% agarose gel and visualized *via* staining with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and transillumination using a UV light source.

2.5. Real-time PCR analysis

Real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA)\, according to the manufacturer's protocol. Briefly, 1 ng/μL of total RNA was reverse transcribed. A maximum of 2 μL of each cDNA sample was used per 20 μL of PCR mixture. PCR reactions were carried out using a CFX96 real-time PCR system C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) up to triplicate wells and 40 cycles. The primer sequences and conditions were adopted from previous studies [13].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were extracted from NCI-H292. Samples were diluted with PBS, transferred to a F96 Cert. Maxisorp Nunc-Immuno plate (Fisher scientific, Lenexa, KS, USA), and incubated at 4 °C overnight. Subsequently, samples were blocked with 2% bovine serum albumin (BSA) for 1 h and incubated with the following primary antibodies: rabbit anti-MUC5AC (H-160) (sc-20118; Santa Cruz Biotechnology, USA; 1:200 dilution) and rabbit anti-MUC5B (H-300) (sc-20119; Santa Cruz Biotechnology, USA; 1:200 dilution), in PBS containing 0.05% Tween 20 for 1 h. Further, they were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. After 1 h, color was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution, and the reaction was stopped using 2 N H_2SO_4. Optical density was measured using an EL800 ELISA reader at 450 nm (BIO-TEK Instruments, Winooski, VT, USA). Results were expressed as fold increase from the baseline control.

2.7. Western blot analysis

NCI-H292 cells were seeded in a 6-well plate, and treated with the indicated concentrations of resistin. Cells were harvested in 200 μL of radioimmunoprecipitation assay (RIPA) buffer (Thermo scientific, Rockford, lL, USA) with phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and incubated for 20 min at 4 °C.

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