INTRODUCTION

A thin layer of gel called mucus is found on the luminal surface of the respiratory tract. Mucus contains several molecules, water, and ions that exert antimicrobial and antioxidative effects. Most macromolecules that impart viscoelasticity to mucus are mucins. Mucins maintain the normal function of the pulmonary system and protect it from inhaled noxious factors, including various particles, bacteria, viruses, and irritating gases. However, qualitative and/or quantitative changes in mucins, such as over-secretion and/or overproduction of respiratory mucus, interfere with the normal defensive action of the pulmonary system, thereby contributing to the pathogenesis of bronchiectasis, chronic bronchitis, cystic fibrosis, and asthma. Among the various subtypes of human mucins, MUC5AC mucin is reported to be the major type of gel-forming pulmonary mucin (Adler and Li, 2001; Lillehoj and Kim, 2002; Rose and Voynow, 2006; Voynow and Rubin, 2009).

To efficiently control mucus in the pulmonary system, S-carboxymethyl cysteine, ambroxol, 2-mercaptoethane sulfonate sodium, erdosteine, azithromycin, bromhexine, hypertonic saline solution, glyceryl guaiacolate, dornase alfa, letocysteine, myrtol, mannitol dry powder, thymosin β-4, N-acetyl L-cysteine (NAC), and sobrerol have been used, despite their potential to induce diverse side effects, including rebound oversecretion of mucus and irritation of the luminal wall of the respiratory tract (Rogers, 2007; Li et al., 2020). Therefore, the development of a novel agent that regulates the biosynthesis and/or degradation of mucin to control its production and/or secretion is warranted. Such development might be pivotal for controlling the oversecretion of pulmonary mucus. Although corticosteroids have been used to decrease the secretion and/or production of respiratory mucus, they have been demonstrated to exhibit various pharmacological adverse effects (Voynow and Rubin, 2009).

The current study aimed to reveal the potential effect of meclofenamate, a nonsteroidal anti-inflammatory drug, on the gene expression of airway MUC5AC mucin. Human pulmonary mucoepidermoid NCI-H292 cells were pretreated with meclofenamate for 30 min and stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h. Thereafter, the effect of meclofenamate on the PMA-induced nuclear factor kappa B (NF-κB) signaling pathway was assessed. Meclofenamate inhibited glycoprotein production and mRNA expression of MUC5AC mucins induced by PMA by inhibiting the degradation of inhibitory kappa B α (IkBα) and NF-κB p65 nuclear translocation. These results suggest meclofenamate suppresses mucin gene expression by regulating NF-κB signaling pathway in human pulmonary epithelial cells.

Key Words: MUC5AC, Pulmonary mucin, Meclofenamate

Meclofenamate Suppresses MUC5AC Mucin Gene Expression by Regulating the NF-κB Signaling Pathway in Human Pulmonary Mucoepidermoid NCI-H292 Cells

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Abstract

The current study aimed to reveal the potential effect of meclofenamate, a nonsteroidal anti-inflammatory drug, on the gene expression of airway MUC5AC mucin. Human pulmonary mucoepidermoid NCI-H292 cells were pretreated with meclofenamate for 30 min and stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h. Thereafter, the effect of meclofenamate on the PMA-induced nuclear factor kappa B (NF-κB) signaling pathway was assessed. Meclofenamate inhibited glycoprotein production and mRNA expression of MUC5AC mucins induced by PMA by inhibiting the degradation of inhibitory kappa B α (IkBα) and NF-κB p65 nuclear translocation. These results suggest meclofenamate suppresses mucin gene expression by regulating NF-κB signaling pathway in human pulmonary epithelial cells.

Keywords: MUC5AC, Pulmonary mucin, Meclofenamate
activity of phorbol ester in airway epithelial cells (Ishinaga et al., 1997; Takeyama et al., 1999; Shao et al., 2003). Phorbol ester induces MUC5AC mucin gene expression, and intracellular signaling pathways involving the production of pulmonary mucin (mucous glycoprotein) owing to their gene expression (Kim et al., 2012; Seo et al., 2014; Choi et al., 2019; Li et al., 2020). This finding might be the rationale for the present study as these previous findings could indicate a potential link between inflammation and mucin production and/or secretion.

Meclofenamate is a non-steroidal anti-inflammatory drug (NSAID) that is clinically used to regulate mild to moderate pain, dysmenorrhea, menorrhagia, osteoarthritis, and rheumatoid arthritis (Conroy et al., 1991). Meclofenamate was previously utilized to ameliorate psoriatic arthritis (Ellis et al., 1986), treat a specific type of severe nephrotic syndrome (Velosa et al., 1985), and regulate parasitic infection by hookworm (Cho et al., 2011). Meclofenamate can be used as an anticancer agent for small cell lung carcinoma and prostate cancer (Chen et al., 2020). Therefore, to elucidate the mechanism of action of meclofenamate, we sought to determine whether meclofenamate regulates the activation of the NF-κB signaling pathway stimulated by phorbol ester in NCI-H292 cells.

MATERIALS AND METHODS

Materials

Phospho-specific anti-IκBα (serine 32/36, #9246) and anti-p65 (serine 536, #3036S) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-β-actin (sc-8432), anti-NF-κB p65 (sc-8008), and anti-inhibitory kappa Bα (IkBα) (sc-371) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nuclear matrix protein p84 (ab-487) was purchased from Abcam (Cambridge, MA, USA). Goat Anti-mouse IgG (#401215) or Goat Anti-rabbit IgG (#401315) was purchased from AbD Serotec (Oxford, UK). Goat Anti-mouse IgG (#401215) or Goat Anti-rabbit IgG (#401315) was purchased from AbD Serotec (Oxford, UK). Other chemicals used in the current experiment, including meclofenamate, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Culture of NCI-H292 cells

NCI-H292 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of pen-strep mixture (penicillin (100 units/mL), streptomycin (100 μg/mL)) and HEPES (25 mM) at 37°C in a humidified, 5% CO2/95% air water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and then cultured in RPMI 1640 with 0.2% FBS for 24 h.

Treatment of cells with meclofenamate

After serum deprivation, the cells were pretreated with varying concentrations of meclofenamate for 30 min followed by phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) in serum-free RPMI 1640 for 24 h. Meclofenamate was dissolved in dimethyl sulfoxide and then culture medium (final concentration of dimethyl sulfoxide was 0.5%). The final pH values of the solutions were between 7.0 and 7.4. The culture medium and 0.5% dimethyl sulfoxide did not affect mucin gene expression or activity and the expression of molecules involved in NF-κB signaling pathway in NCI-H292 cells. After 24 h, the cells were lysed with buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, and protease inhibitor cocktail (Roche Diagnostics, IN, USA) and collected to measure the production of MUC5AC glycoproteins (in a 24-well culture plate). Total RNA was extracted to determine the expression of the MUC5AC gene (in a 6-well culture plate) using RT-PCR. For western blot analysis, the cells were treated with meclofenamate for 24 h and then with PMA for 30 min.

Quantitative analysis of MUC5AC mucin

Airway MUC5AC mucin production was measured using enzyme-linked immunosorbent assay (ELISA). Cell lysates were prepared with PBS at a 1:10 dilution, and 100 μL of each sample was incubated at 42°C in a 96-well plate until dry. The plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) (fraction V) for 1 h at room temperature. The plates were then washed three times with PBS and incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, USA) diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 h, the wells were washed three times with PBS and incubated with 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000). After 1 h, the plates were washed three times with PBS. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H2SO4. The absorbance was measured at a wavelength of 450 nm.

Isolation of total RNA and RT-PCR

Total RNA was isolated using an Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Seongnam, Korea) and reverse transcribed using AccuPower RT Premix (Bioneer Corporation, Daejeon, Korea) according to the manufacturer’s instructions. Two μg of total RNA was primed with 1 μg oligo (dT) in a final volume of 50 μL (RT reaction). Two microliters of the reaction product were PCR-amplified in a 25 μL volume using Thermoprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). The primers for MUC5AC were (forward) 5'-TGA
tein in the extracts was quantified using the Bradford method.

Whole cell extract preparation
NCI-H292 cells (confluent in a 100 mm culture dish) were pretreated with 1, 5, 10, or 20 μM meclofenamate for 24 h at 37°C, and then stimulated with PMA (50 ng/mL) in serum-free RPMI 1640 for 30 min. After cells were treated with meclofenamate, the media were aspirated, and the cells were washed with cold PBS. For cell collection, the cells were scraped and centrifuged at 3,000 rpm for 5 min. After the supernatant was discarded, the cell pellet was mixed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min with continuous agitation. The lysate was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was either used or immediately stored at −80°C. The amount of protein in the extract was quantified using the Bradford method.

Nuclear and cytosolic extract preparation
After treatment with meclofenamate, the cells (confluent in 150 mm culture dish) were harvested using trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). After the supernatant was discarded, the cell pellet was washed via suspension in PBS. The cytoplasmic and nuclear protein fractions were extracted using NE-PER® nuclear and cytoplasmic extraction reagents (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Both extracts were stored at −20°C. The amount of protein in the extracts was quantified using the Bradford method.

Western blotting for the detection of proteins
Whole cell, cytosolic, and nuclear extracts containing proteins (each 50 μg as proteins) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% skim milk and probed with appropriate primary antibody in blocking buffer overnight at 4°C. The membrane was washed with PBS and probed with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce ECL western blotting Substrate, Thermo Scientific).

Statistical analysis
The means of individual groups were converted to percent control and are expressed as mean ± SEM. Differences between groups were assessed using one-way ANOVA and the Holm-Sidak test as a post-hoc test. Statistical significance was set at p<0.05.
κB against anti-IκB extracts were fractionated and subjected to western blot analysis 24 h and then treated with 50 ng/mL PMA for 30 min. Cytoplasmic were incubated with varying concentrations of meclofenamate for 30 min, and the nuclear fractions then stimulated with PMA.

MUC5AC mRNA expression in NCI-H292 cells. NCI-H292 cells were pre-treated with varying concentrations of meclofenamate for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected to measure MUC5AC gene expression using RT-PCR. Three independent experiments were performed, and representative data are shown. *Significantly different from the control (p<0.05). †Significantly different from that with PMA alone (p<0.05), cont: control, M: meclofenamate, concentration units are μM.

**DISCUSSION**

As mentioned in the Introduction, no specific compound efficiently controls the secretion and/or production of pulmonary mucus, despite the use of several clinical medicines to exert such pharmacological activity. Controlling the inflammatory response might be the ultimate approach to efficiently regulate different inflammatory airway diseases. We determined the potential effects of various natural compounds. Notably, diverse natural anti-inflammatory compounds have a regulatory effect on the gene expression of MUC5AC mucin. Accordingly, we are attempting to develop a novel candidate that alters the production and/or secretion of airway MUC5AC mucin (Kim et al., 2012; Ryu et al., 2013, 2014; Seo et al., 2015; Sikder et al., 2012; Ryu et al., 2014; Lee et al., 2015; Kim et al., 2016). However, the effective concentrations of these natural compounds are usually high, and the pharmacokinetic profile and druggability of each compound are generally not satisfactory (Li et al., 2020).

Here, we used drug repurposing as a strategy to search for promising candidates for new drugs. Drug repurposing, also known as drug repositioning, is the process of identifying novel therapeutic uses for drugs approved and used clinically for human diseases. Instead of developing new drugs from the initial stage, drug repurposing involves identifying new uses for drugs that have already undergone extensive testing and have a well-established safety profile. Drug repurposing can be a cost-effective approach to drug development as it avoids many of the risks and costs associated with developing new drugs from the initial stage. Additionally, drug repurposing can decrease the time taken to deliver new therapies to patients as the safety and pharmacokinetic profiles of the drug have already been established.

As revealed by our results, meclofenamate significantly suppressed the production of MUC5AC mucin proteins and...
expression of MUC5AC mucin mRNA (Fig. 1, 2). To our knowledge, this is the first study to reveal the effect of meclofenamate on MUC5AC mucin gene expression in airway epithelial cells. Meclofenamate was also found to suppress the phosphorylation and nuclear translocation of NF-κB p65 by affecting the phosphorylation and degradation of IkBa in NCI-H292 cells (Fig. 3, 4). Thus, the pharmacological effect of meclofenamate on the gene expression of MUC5AC may be manifested via the degradation of IkBa and nuclear translocation of NF-κB p65. Additionally, meclofenamate might inhibit MUC5AC gene expression through a certain intracellular signaling pathway other than NF-κB signaling. We determined whether meclofenamate inhibits MUC5AC mucin expression through an intracellular signaling pathway mediated by the epidermal growth factor receptor (EGFR). Based on the results, meclofenamate did not affect the EGF-induced mitogen-activated protein kinase (MAPK) signaling pathway. Further, meclofenamate did not affect EGF-induced EGFR phosphorylation, p38 MAPK phosphorylation, extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, or nuclear translocation of specificity protein-1 (Sp1) (unpublished data). Various original studies have reported that the gene expression of MUC5AC mucin might be stimulated by inflammatory mediators that activate transcription factors, including NF-κB (Fujisawa et al., 2009; Kurakula et al., 2015; Garvin et al., 2016). PMA has been reported to stimulate MUC5AC mucin gene expression, and nuclear factor kappa B (NF-κB) signaling is involved in airway epithelial cell activity (Ishinaga et al., 2005; Laos et al., 2006; Wu et al., 2007; Kim et al., 2012; Choi et al., 2018). Based on these reports and the present experimental results, the pharmacological effect of meclofenamate on PMA-induced MUC5AC gene expression might be mediated, at least partly, by the degradation of IkBa and nuclear translocation of NF-κB p65.

Meclofenamate rapidly induced the onset of its pharmacological effect, and exhibited a desirable duration of action. Meclofenamate is also highly bioavailable when administered as a capsule, and is reported to be extensively metabolized. A specific type of metabolite displays a significant amount of activity compared to the parent drug (Conroy et al., 1991). The common adverse effects of meclofenamate as an NSAID, including disturbances in gastrointestinal tract function, might be problematic, especially for a specific group of patients. However, as previously mentioned, a pharmacological agent that specifically controls the production and/or secretion of airway mucus is unavailable. Therefore, it is crucial to search for specific compounds that will regulate the abnormal secretion and/or production of mucus in the respiratory tract of pulmonary diseases via preclinical/clinical studies. Notably, this study was conducted in the early stages of new drug development. Potential adverse effects should be resolved at each development step and stage of the novel drug. Using medicinal chemistry to modify and optimize the chemical structure of meclofenamate is a promising strategy to ensure it exerts an adequate controlling effect on the secretion and/or production of pulmonary mucus.

In conclusion, a drug repurposing strategy should be adopted to identify a new candidate compound that exhibits an inhibitory effect on the gene expression of MUC5AC. The results of the current study suggest the possibility of utilizing meclofenamate as an efficacious mucoregulatory agent for diverse respiratory diseases.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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