BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 28(5), 431-436 (2020)

Diclofenac Inhibits Phorbol Ester-Induced Gene Expression and Production of MUC5AC Mucin via Affecting Degradation of IkBα and Translocation of NF-kB p65 in NCI-H292 Cells

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Abstract

In this study, diclofenac, a non-steroidal anti-inflammatory drug, was investigated for its potential effect on the gene expression and production of airway MUC5AC mucin. The human respiratory epithelial NCI-H292 cells were pretreated with diclofenac for 30 min and stimulated with phorbol 12-myristate 13-acetate (PMA), for the following 24 h. The effect of diclofenac on PMA-induced nuclear factor kappa B (NF-kB) signaling pathway was also investigated. Diclofenac suppressed the production and gene expression of MUC5AC mucins, induced by PMA through the inhibition of degradation of inhibitory kappa Bα (IkBα) and NF-kB p65 nuclear translocation. These results suggest diclofenac regulates the gene expression and production of mucin through regulation of NF-kB signaling pathway, in human airway epithelial cells.

Key Words: MUC5AC, Pulmonary mucin, Diclofenac

INTRODUCTION

Pulmonary mucus containing mucins (mucous glycoproteins), the major macromolecular component that gives mucus the viscoelasticity, protects the respiratory system from inhaled noxious factors and maintains its normal function. However, pathological changes in the quantity or quality of mucins as exemplified in the hypersecretion and/or hyperproduction of pulmonary mucus disrupt the normal defensive mechanism of respiratory system and contribute to the pathogenesis of various respiratory diseases such as chronic bronchitis, bronchiectasis, asthma, and cystic fibrosis (Voynow and Rubin, 2009). In order to remove the mucus from the respiratory system, lysed mucus can be aspirated after applying the mucolytic drugs. The other option is to suppress the production and/or secretion of mucus by a pharmacological agent. Clinically, aspiration of mucus using mucolytics might irritate the luminal wall of airway and provoke the rebound hypersecretion of mucus (Rogers, 2007). Thus, the development of a pharmacological agent, affecting the biosynthesis, to control production and/or secretion of mucin can be an important strategy for regulating the pathological hypersecretion of airway mucus. Although glucocorticoids have been reported to suppress the hypersecretion and/or hyperproduction of airway mucins, they showed a multitude of adverse effects in the course of pharmacotherapy (Sprenger *et al*., 2011). Therefore, in this study, we tried to develop a novel candidate for controlling the production and/or secretion of airway mucus, by examining a potential activity of diclofenac on production and gene expression of airway MUC5AC mucin, as a trial based on drug repositioning. Of the many subtypes of human mucins, MUC5AC subtype of mucin consists of the major type of human airway mucin (Rogers and Barnes, 2006; Voynow and Rubin, 2009). Diclofenac is an anti-inflammatory agent manifesting analgesic and antipyretic effects. It inhibits the production of prostaglandins by affecting cycloxygenase-1 and cycloxygenase-2. It is usually used for controlling the pain and inflammation in musculoskeletal diseases such as osteoarthritis, rheumatoid arthritis, temporomandibular joint pain, and ankylosing spondylitis. Diclofenac was also reported to suppress the synthesis of DNA of bacteria, thereby exerting the bacteriostatic activity (Dastidar *et al*., 2000). Thus, we suggest it is promising

Open Access **https://doi.org/10.4062/biomolther.2020.090**

Received May 20, 2020 **Revised** Jul 13, 2020 **Accepted** Jul 13, 2020
Published Online Aug 5, 2020

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to check the potential activity of an anti-inflammatory agent, diclofenac, on the production and gene expression of airway MUC5AC mucin in inflammatory status of airway. It has been reported that phorbol 12-myristate 13-acetate (PMA) induces airway MUC5AC mucin gene expression and production, and nuclear factor kappa B (NF-kB) signaling is involved into the activity in airway epithelial cells (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012; Choi *et al*., 2018). On the basis of this information, effect of diclofenac on the expression of MUC5AC mucin gene and production of MUC5AC mucin proteins was examined in NCI-H292 cells, a human airway epithelial cell line (Li *et al*., 1997; Takeyama *et al*., 1999; Shao *et al*., 2003).

MATERIALS AND METHODS

Materials

All the chemicals used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Anti-NF-κB p65 (sc-8008), anti-inhibitory kappa Bα (IkBα) (sc-371), and anti-β-actin (sc-8432) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nuclear matrix protein p84 (ab-487) antibody was purchased from Abcam (Cambridge, MA, USA). Phospho-specific anti-IκBα (serine 32/36, #9246) and anti-phospho-inhibitory kappa B kinase (IKK) α/β (Ser176/180, #2687) antibodies were purchased from Cell Signaling Technology Inc (Danvers, MA, USA). Either Goat Anti-rabbit IgG (#401315) or Goat Anti-mouse IgG (#401215) was used as the secondary antibody and purchased from Calbiochem (Carlsbad, CA, USA).

NCI-H292 cell culture

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 penicillin (100 units/mL), streptomycin (100 μg/mL) and HEPES (25 mM) at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% fetal bovine serum for 24 h.

Treatment of cells with diclofenac

After 24 h of serum deprivation, cells were pretreated with varying concentrations of diclofenac (Fig. 1) for 30 min and then treated with PMA (10 ng/mL) for 24 h in serum-free RPMI 1640. Diclofenac was dissolved in dimethyl sulfoxide and treated in culture medium (final concentrations of dimethyl sulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethyl sulfoxide did not affect mucin gene expression, mucin production, and expression and activity of molecules involved in NF-kB signaling pathway, in NCI-H292 cells. After 24 h, 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 proteins (in a 24-well culture plate). The total RNA was extracted in order to measure the expression of MUC5AC gene (in a 6-well culture plate) using RT-PCR. For the western blot analysis, cells were treated with diclofenac for 24 h and then with PMA for 30 min.

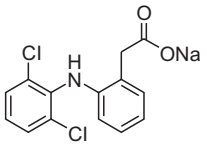

Fig. 1. Chemical structure of diclofenac.

Quantitation of MUC5AC mucin contents

MUC5AC airway mucin production was measured using ELISA. Cell lysates were prepared with PBS at 1:10 dilution, and 100 μL of each sample was incubated at 42°C in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) (fraction V) for 1 h at room temperature. Plates were washed another three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, USA), which was 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 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well. After 1 h, plates were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H_2SO_4. Absorbance was read at 450 nm.

Total RNA isolation and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Gyeonggi, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. Two μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μL (RT reaction). Two μL of RT reaction product was PCR-amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). Primers for MUC5AC were (forward) 5′-TGA TCA TCC AGC AGG GCT-3′ and (reverse) 5′-CCG AGC TCA GAG GAC ATA TGG G-3′. Primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used as quantitative controls. Primers for Rig/S15 were (forward) 5′-TTC CGC AAG TTC ACC TAC C-3′ and (reverse) 5′-CGG GCC GGC CAT GCT TTA CG-3′. The PCR mixture was denatured at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. After PCR, 5 μL of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

Preparation of whole cell extract

NCI-H292 cells (confluent in 150 mm culture dish) were pretreated for 24 h at 37°C with 1, 5, 10 or 20 μM of diclofenac, and then stimulated with PMA (50 ng/mL) for 30 min, in serum-free RPMI 1640. After the treatment of the cells with diclofenac, media were aspirated, and the cells washed with cold PBS. The cells were collected by scraping and were centrifuged at 3,000 rpm for 5 min. The supernatant was discarded. The cells were 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 in a microcentrifuge at 14,000 rpm for 15 min at 4°C. The supernatant was either used, or was immediately stored at –80°C. Protein content in extract was determined by Bradford method.

Preparation of nuclear and cytosolic extracts

After the treatment with diclofenac as outlined, the cells were harvested using Trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). The supernatant was discarded, and the cell pellet was washed by suspending in PBS. The cytoplasmic and nuclear protein fractions were extracted using NE-PER® nuclear and cytoplasmic extraction reagent (Thermo-Pierce Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Both extracts were stored at –20°C. Protein content in extracts was determined by Bradford method.

Detection of proteins by western blot analysis

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

Statistics

The means of individual groups were converted to percent control and expressed as mean ± SEM. The difference between groups was assessed using a one-way ANOVA and the Holm-Sidak test post-hoc. A p-value of <0.05 was considered significantly different.

RESULTS

Effect of diclofenac on PMA-induced MUC5AC mucin production and gene expression

Diclofenac inhibited PMA-induced MUC5AC mucin production, dose-dependently. The amounts of MUC5AC mucin in the cells of diclofenac-treated cultures were 100 ± 3% (control), 580 ± 4% (10 ng/mL of PMA alone), 593 ± 5% (PMA plus diclofenac 1 μM), 498 ± 16% (PMA plus diclofenac 5 μM), 417 ± 4% (PMA plus diclofenac 10 μM) and 294 ± 3% (PMA plus diclofenac 20 μM), respectively (Fig. 2). MUC5AC gene expression induced by PMA was also inhibited by pretreatment with 10 μM and 20 μM of diclofenac (Fig. 3). Cell viability was checked by sulforhodamine B (SRB) assay and there was no cytotoxic effect of diclofenac, at 1, 5, 10 or 20 μM concentration (data were not shown).

Effect of diclofenac on PMA-induced phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα

In order for NF-κB to be activated, PMA provokes the phosphorylation of IKK and this phosphorylated IKK, in turn, phosphorylates the IκBα. The phosphorylated IκBα dissociates from NF-κB and degraded. Thus, we checked whether diclofenac affects the phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα, provoked by PMA. As can be seen in Fig. 4, diclofenac mitigated PMA-stimulated phosphoryaltion of IKKα/β, by controlling the phosphorylation of the serine 176/180 moiety of IKKα/β. PMA stimulated the phosphorylation of IκBα, whereas diclofenac inhibited its phosphorylation. Also, PMA provoked the degradation of IκBα, whereas diclofenac inhibited the IκBα degradation.

Effect of diclofenac on PMA-induced phosphorylation and nuclear translocation of NF-κB p65

The activated NF-κB translocates from the cytosol to the nucleus and then connects to the specific site of DNA. This

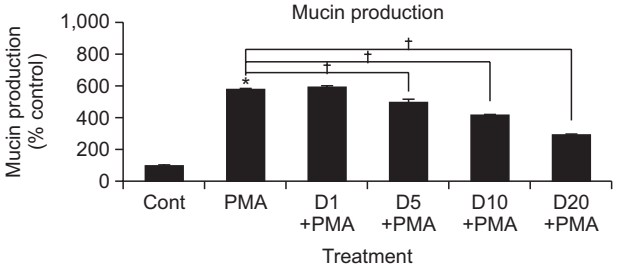

Fig. 2. Effect of diclofenac on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of diclofenac for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Each bar represents a mean ± SEM. of 3 culture wells compared to the control set at 100%. Three independent experiments were performed, and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; concentration unit is μM).

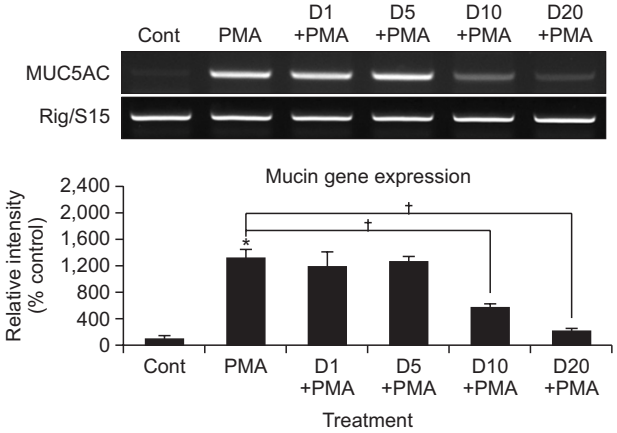

Fig. 3. Effect of diclofenac on PMA-induced MUC5AC mucin gene expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of diclofenac for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC gene expression using RT-PCR. Three independent experiments were performed, and the representative data were shown. *Significantly different from control (p< 0.05). †Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; concentration unit is μM).

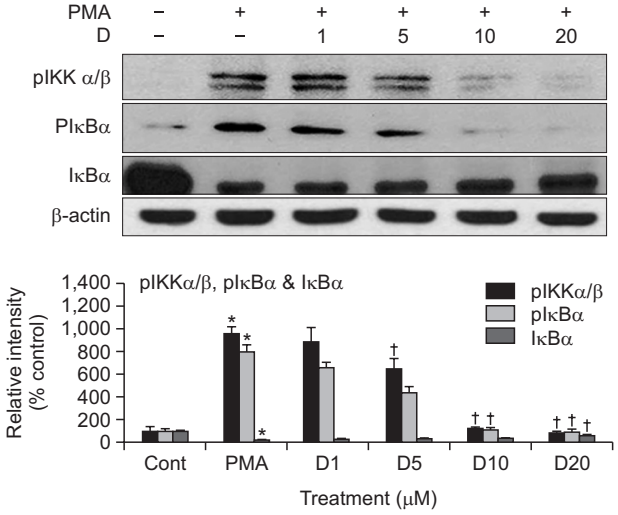

Fig. 4. Effect of diclofenac on PMA-induced IKK phosphorylation, IkBα phosphorylation, and IkBα degradation, in NCI-H292 cells. NCI-H292 cells were incubated with varying concentrations of diclofenac for 24 h and treated with 50 ng/mL PMA for 30 min. Cytoplasmic extracts were fractionated and then subjected to western blot analysis using phospho-specific IκBα (Ser 32/36) antibody or antibody against anti-IκBα. Whole cell lysates were prepared and then subjected to western blot analysis using phospho-specific IKKα/β (Ser 176/180) antibody. Equal protein loading was evaluated by β-actin levels. *Significantly different from control (p<0.05). †Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; IkBa, inhibitory kappa Bα, IKK, inhibitory kappa B kinase; concentration unit is μM).

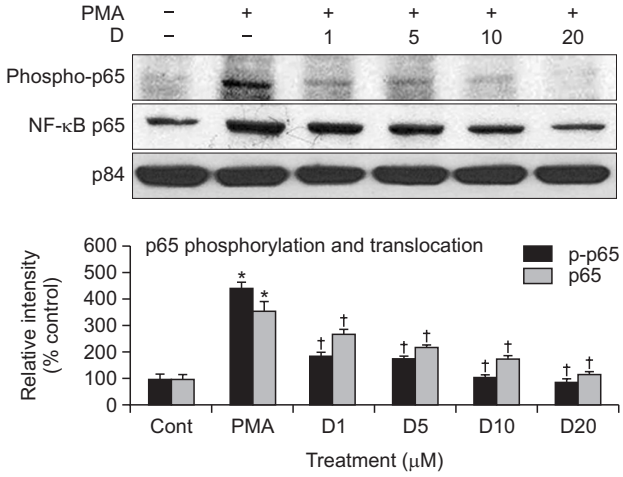

Fig. 5. Effect of diclofenac on PMA-induced phosphorylation and translocation of NF-kB p65, in NCI-H292 cells. Nuclear protein extracts were prepared and subjected to western blot analysis using phospho-specific p65 (Ser 536) antibody and antibody against p65. As a loading control, p84 levels were analyzed. The results shown are the representative of three independent experiments. *Significantly different from control (p<0.05). †Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; concentration unit is μM).

complex of DNA/NF-κB recruits the RNA polymerase and then the resulting mRNA is translated into the specific proteins, including MUC5AC mucins. Also, the transcriptional activity of NF-κB p65 has been known to be dependent upon its phosphorylation. As can be seen in Fig. 5, PMA stimulated the phosphorylation of p65, whereas diclofenac suppressed its phosphorylation. Finally, diclofenac blocked the nuclear translocation of NF-κB p65, provoked by PMA.

DISCUSSION

For the conventional pharmacotherapy of pulmonary diseases showing airway mucus hypersecretion, 2-mercaptoethane sulfonate sodium (MESNA), ambroxol, azithromycin, bromhexine, erdosteine, glucocorticoids, glyceryl guaiacolate, hypertonic saline solution, letocysteine, mannitol dry powder, myrtol, N-acetyl L-cysteine (NAC), dornase alfa, S-carboxymethyl cysteine, sobrerol, and thymosin β-4 have been used. However, these drugs failed to show the remarkable clinical efficacy in controlling such diseases and provoked the various side effects (Li *et al*., 2020). To control the diverse inflammatory pulmonary diseases effectively, the regulation of inflammatory response can be the first goal. For the development of the novel candidate to control the production and/or secretion of airway MUC5AC mucin, we have tried to examine the potential effect of diverse natural products and reported that a multitude of natural products affected the expression of MUC5AC mucin gene and production of mucin proteins, during the last two decades (Heo *et al*., 2007, 2009; Lee *et al*., 2011; Kim *et al*., 2012; Ryu *et al*., 2013, 2014; Seo *et al*, 2014; Sikder *et al*., 2014; Lee *et al*., 2015; Kim *et al*., 2016; Choi *et al*., 2018, 2019). However, the effective concentration of these natural products is generally high, and the druggability and pharmacokinetic profile of each agent are inadequate in general (Li *et al*., 2020).

Here, we adopted the drug repositioning strategy for finding the promising candidate. The definition of drug repositioning is the development of novel therapeutic uses of existing pharmaceutics. For example, a teratogenic antiemetics, thalidomide, has been examined to be utilized for the regulation of multiple myeloma and leprosy, and an antifungal agent, ketoconazole, has been investigated as a potential therapeutics for trypanosomal infection. Through drug repositioning, the novel mechanism of action for old pharmaceutics and new class of drugs can be discovered. In addition to that, the cost and time for the pharmaceutics to be marketed successfully can be diminished through the decreased number of phases in clinical trial (Kingsmore *et al*., 2020).

As shown in results, diclofenac significantly inhibited the expression of MUC5AC mucin mRNA and production of MUC5AC mucin proteins, through directly affecting NCI-H292 cells (Fig. 2, 3). This is the first report, as far as we perceive, on the effect of diclofenac, a non-steroidal anti-inflammatory drug, on MUC5AC mucin gene expression and production from airway epithelial NCI-H292 cells. Various research groups reported that diclofenac suppressed the NF-κB intracellular signaling pathway (Takada *et al*., 2004; Karakawa *et al*., 2009; Fredriksson *et al*., 2011). Fredriksson and his colleagues reported that diclofenac mitigated the nuclear translocation and transcriptional activity of NF-κB via suppression of the phosphorylation of IκBα and activity of IKK, in hepatocytes (Fredriksson *et al*., 2011). In osteoclasts, diclofenac suppressed the degradation of IκBα and nuclear translocation of NF-κB (Karakawa *et al*., 2009). In our results, diclofenac inhibited the phosphorylation

and nuclear translocation of NF-κB p65 through acting on the steps of the phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα, in human airway epithelial cells (Fig. 4, 5). Therefore, the pharmacological effect of diclofenac on MUC5AC production and gene expression might be manifested through the degradation of IkBa and nuclear translocation of NF-kB p65. Of course, diclofenac might inhibit MUC5AC gene expression and production via another intracellular signaling pathway rather than NF-kB signaling. In fact, we examined whether diclofenac suppresses MUC5AC gene expression and production via EGFR-MEK-MAPK-Sp1 signaling (unpublished data). However, diclofenac did not affect EGFR-MEK-MAPK-Sp1 signaling pathway. At the same time, many articles have reported that MUC5AC mucin gene expression and production can be increased by the inflammatory mediators which activate the transcription factors including NF-κB (Fujisawa *et al*., 2009; Kurakula *et al*., 2015; Garvin *et al*., 2016). As we stated above, it has been reported that PMA induces airway MUC5AC mucin gene expression and production, and nuclear factor kappa B (NF-kB) signaling is involved into the activity in airway epithelial cells (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012; Choi *et al*., 2018). Also, various research groups reported that diclofenac suppressed the NF-κB intracellular signaling pathway (Takada *et al*., 2004; Karakawa *et al*., 2009; Fredriksson *et al*., 2011). Based on these articles published and the present experimental results of our own, we cautiously suggest it is reasonable to conclude that the pharmacological effect of diclofenac on PMA-induced MUC5AC production and gene expression might be mediated, at least in part, via affecting the degradation of IkBa and nuclear translocation of NF-kB p65.

To be summarized, we suggest that it is promising to find a novel candidate agent that has a suppressive effect on MUC5AC production and gene expression, from the viewpoint of both the clinical and the basic sciences, through drug repositioning. These results suggest a potential of utilizing diclofenac as an efficacious mucoactive drug for pulmonary diseases. Possibly, any adverse effect of diclofenac might be problematic. For example, the patients suffering from bronchial asthma may be endangered to severe bronchospasm which can be provoked by diclofenac, a non-steroidal anti-inflammatory drug, likewise by acetylsalicylic acid (aspirin). However, as we stated in the above section, there is no specific pharmacological agent that can regulate the production and/or secretion of airway MUC5AC mucin in pulmonary mucus. Thus, it is very urgent to develop such a specific agent through preclinical and clinical study, in order to control the hypersecretion and/or hyperproduction of sticky, pathologically-transformed mucus in the airway of pulmonary diseases. This study exists in the very early stage of novel drug development. Therefore, potential toxicity and/or the other problems should be resolved through the diverse developing steps of the new drug. Findings in this study are just a clue in the long journey to the successful development of novel drug. It is ideal to modify and optimize the chemical structure of diclofenac using the research tools of medicinal chemistry, so as to manifest the strongest regulatory effect on the production and/or secretion of mucus to suggest the clinical efficacy.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by NRF-2014R1A6A1029617 and NRF-2017R1C1B1005126, Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education.

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