Diclofenac Inhibits Phorbol Ester-Induced Gene Expression and Production of MUC5AC Mucin via Affecting Degradation of IkBα and Translocation of NF-κB 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-κB) 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-κB p65 nuclear translocation. These results suggest diclofenac regulates the gene expression and production of mucin through regulation of NF-κB 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 reppositioning. 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 cyclooxygenase-1 and cyclooxygenase-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...
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., 2003; 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 B (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) c/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% CO2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640. After the treatment of the cells with diclofenac for 24 h and then with PMA for 30 min.

**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 were 0.5%). The final pH values of these solutions were adjusted 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., 2003; 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).

![Fig. 1. Chemical structure of diclofenac.](https://doi.org/10.4062/biomolther.2020.090)
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 Student’s t-test for groups with equal variance (p<0.05). The activated NF-κB translocates from the cytosol to the nucleus and then connects to the specific site of DNA. This

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 IKKa/β, phosphorylation of IxBα, and degradation of IxBα
In order for NF-κB to be activated, PMA provokes the phosphorylation of IKK and this phosphorylated IKK, in turn, phosphorylates the IxBα. The phosphorylated IxBα dissociates from NF-κB and degraded. Thus, we checked whether diclofenac affects the phosphorylation of IKKα/β, phosphorylation of IxBα, and degradation of IxBα, provoked by PMA. As can be seen in Fig. 4, diclofenac mitigated PMA-stimulated phosphorylation of IKKα/β, by controlling the phosphorylation of the serine 176/180 moiety of IKKα/β. PMA stimulated the phosphorylation of IxBα, whereas diclofenac inhibited its phosphorylation. Also, PMA provoked the degradation of IxBα, whereas diclofenac inhibited the IxBα 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
then subjected to western blot analysis using phospho-specific \( \kappa \) 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 \( \kappa \)B\( \alpha \) antibody or antibody against anti-\( \kappa \)B\( \alpha \). Whole cell lysates were prepared and then subjected to western blot analysis using phospho-specific IKK\( \beta \) (Ser 176/180) antibody. Equal protein loading was evaluated by \( \beta \)-actin levels. *Significantly different from control (p<0.05). **Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; \( \kappa \)B\( \alpha \), inhibitory kappa B \( \alpha \); IKK, inhibitory kappa B kinase; concentration unit is \( \mu \)M).

**Fig. 4.** Effect of diclofenac on PMA-induced IKK phosphorylation, \( \kappa \)B\( \alpha \) phosphorylation, and \( \kappa \)B\( \alpha \) 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 \( \kappa \)B\( \alpha \) (Ser 32/36) antibody or antibody against anti-\( \kappa \)B\( \alpha \). Whole cell lysates were prepared and then subjected to western blot analysis using phospho-specific IKK\( \beta \) (Ser 176/180) antibody. Equal protein loading was evaluated by \( \beta \)-actin levels. *Significantly different from control (p<0.05). **Significantly different from PMA alone (p<0.05) (cont, control; D, diclofenac; \( \kappa \)B\( \alpha \), inhibitory kappa B \( \alpha \); IKK, inhibitory kappa B kinase; concentration unit is \( \mu \)M).

Complex of DNA/NF-\( \kappa \)B recruits the RNA polymerase and then the resulting mRNA is translated into the specific proteins, including MUC5AC mucins. Also, the transcriptional activity of NF-\( \kappa \)B 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-\( \kappa \)B p65, provoked by PMA.

**DISCUSSION**

For the conventional pharmacotherapy of pulmonary diseases showing airway mucus hypersecretion, 2-mercaptoethane sulfonate sodium (MESNA), amboxrol, azithromycin, bromhexine, erdosteine, glucocorticoids, glyceryl guaiacolate, hypertonic saline solution, letocysteine, mannitol dry powder, myrtol, N-acetyl L-cysteine (NAC), dornase alfa, S-carboxymethyl cysteine, soberol, and thymosin \( \beta \)-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 pharmacuticals. 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 pharmacuticals and new class of drugs can be discovered. In addition to that, the cost and time for the pharmacuticals 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-\( \kappa \)B 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-\( \kappa \)B via suppression of the phosphorylation of \( \kappa \)B\( \alpha \) and activity of IKK, in hepatocytes (Fredriksson et al., 2011). In osteoclasts, diclofenac suppressed the degradation of \( \kappa \)B\( \alpha \) and nuclear translocation of NF-\( \kappa \)B (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 IκKα/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 IκBα and nuclear translocation of NF-κB p65. Of course, diclofenac might inhibit MUC5AC gene expression and production via another intracellular signaling pathway rather than NF-κB 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; Kurakawa 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-κB) 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 IκBα and nuclear translocation of NF-κB 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.

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