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Betulin, an Anti-Inflammatory Triterpenoid Compound, Regulates MUC5AC Mucin Gene Expression through NF-kB Signaling in Human Airway Epithelial Cells

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Abstract

Betulin is a triterpenoid natural product contained in several medicinal plants including Betulae Cortex. These medicinal plants have been used for controlling diverse inflammatory diseases in folk medicine and betulin showed anti-inflammatory, antioxidative, and anticancer activities. In this study, we tried to examine whether betulin exerts a regulative effect on the gene expression of MUC5AC mucin under the status simulating a pulmonary inflammation, in human airway epithelial cells. Confluent NCI-H292 cells were pretreated with betulin for 30 min and then stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h or the indicated periods. The MUC5AC mucin mRNA expression and mucin glycoprotein production were measured by reverse transcription - polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. To elucidate the action mechanism of betulin, effect of betulin on PMA-induced nuclear factor kappa B (NF-kB) signaling pathway was also investigated by western blot analysis. The results were as follows: 1) Betulin significantly suppressed the production of MUC5AC mucin glycoprotein and down-regulated MUC5AC mRNA expression induced by PMA in NCI-H292 cells. 2) Betulin inhibited NF-κB activation stimulated by PMA. Suppression of inhibitory kappa B kinase (IKK) by betulin led to the inhibition of the phosphorylation and degradation of inhibitory kappa B alpha (IκBα), and the nuclear translocation of NF-κB p65. This, in turn, led to the down-regulation of MUC5AC glycoprotein production in NCI-H292 cells. These results suggest betulin inhibits the gene expression of mucin through regulation of NF-kB signaling pathway, in human airway epithelial cells.

Key Words: Betulin, MUC5AC, Pulmonary mucin

INTRODUCTION

Pulmonary mucus is a thin layer of gels present in the luminal surface of airway. The various molecules, ions, water are the constituents of mucus and those molecules exert antioxidative and antimicrobial activities (Lillehoj and Kim, 2002). Mucins assigning mucus the viscoelasticity is the major macromolecular biochemical constituent of mucus. In the physiological conditions of the pulmonary system, mucus in the airway is well-known to play a critical role in a physical defense against damage of pulmonary epithelium, provoked by irritating gases, inhaled particles, and many viruses and bacteria (Adler and Li, 2001). However, the overproduction and/or oversecretion of airway mucus resulting from the changes in quantity or quality of mucins (biochemically, mucous glycoproteins) have been reported to compromise the host defense system and, eventually, provoke the increase of mortality and morbidity, in the pulmonary diseases like asthma, chronic obstructive pulmonary diseases (COPD), and cystic fibrosis (CF) (Rose and Voynow, 2006).

Although glucocorticoids, expectorants, and mucolytics have been utilized clinically to regulate the abnormal secretion of pulmonary mucus, these agents might provoke the irritation of luminal wall of airway, and various negative side

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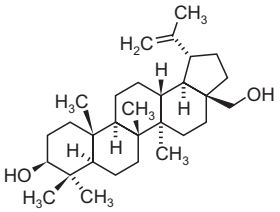

Fig. 1. Chemical structure of betulin.

effects like the rebounding hypersecretion of mucus (Li *et al*., 2020). Therefore, it is very promising to develop a novel agent controlling the production and/or secretion of mucins through affecting the degradation and/or biosynthesis of them.

In this context, we suggest it is encouraging to examine the potential activity of regulating the pathological production and/or secretion of mucins, using natural products isolated from a multitude of medicinal plants used empirically for controlling the inflammatory pulmonary diseases. As a result of our trial, we reported previously that diverse natural compounds modulated the production of airway mucous glycoprotein (mucin), resulting from its gene expression (Kim *et al*., 2012; Seo *et al*, 2014; Choi *et al*., 2019; Li *et al*., 2021).

In accordance with several previous reports, Betulin (Fig. 1) is a lupane type pentacyclic ring-structured triterpenoid natural product, contained in several medicinal plants including Betulae Cortex. These medicinal plants have been reported to be used for controlling diverse inflammatory diseases in folk medicine and betulin showed anti-inflammatory, antioxidative, and anticancer activities (Alakurtti *et al*., 2006; Nader and Baraka, 2012; Guo *et al*., 2015; Zhang *et al*., 2015; Bai *et al*., 2016; Zhao *et al*., 2016a, 2016b; Kamaraj *et al*., 2021). Betulin inhibited allergic inflammation of airway through modulating inflammatory cytokines, transglutaminase, and antioxidants, in ovalbumin-induced asthma model (Kamaraj *et al*., 2021).

Betulin suppressed renal injury and alcoholic liver injury via TLR4/NF-κB and SIRT1/AMPK intracellular signaling pathways (Bai *et al*., 2016; Zhao *et al*., 2016b). In lung inflammation model of rat induced by lipopolysaccharide, a derivative of betulin affects the recruitment of neutrophil and the expression of inflammatory mediators (Nader and Baraka, 2012). Betulin has been reported to mitigate injury of the lung in sepsis and inflammatory injury of mammary gland (Guo *et al*., 2015; Zhao *et al*., 2016a). In human cardiac cells, betulin mitigated the proinflammatory cytokines expression (Zhang *et al*., 2015). Also, Betulin was reported to be used a palliative therapy for chronic obstructive pulmonary disease (COPD) through P2X7 receptor in human bronchial epithelial cells (Jiao *et al*., 2022).

However, there is no study relating to the effect of betulin on the gene expression of mucin in airway epithelial cells. Of the many subtypes of human mucins, MUC5AC mucin is known to be the major type of secreted mucin from airway (Lillehoj and Kim, 2002). Here, based upon this information, we examined the effect of betulin on mRNA expression and glycoprotein production of MUC5AC mucin, stimulated by phorbol ester, in NCI-H292 cells. A human pulmonary mucoepidermoid cell line, NCI-H292 cells, is frequently used for specifying the signaling pathways involved into the gene expression of airway mucin (Li *et al*., 1997; Takeyama *et al*., 1999; Shao *et al*., 2003). Also, phorbol ester, PMA, stimulates the gene expression of airway MUC5AC mucin, and intracellular nuclear factor kappa B (NF-kB) signaling is involved into the activity of phorbol ester in airway epithelial cells, suggesting that PMA might develop a status simulating a pulmonary inflammation (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012; Choi *et al*., 2019; Jin *et al*., 2020). Thus, to elucidate an action mechanism of betulin, we investigated whether betulin affects the activation of NF-kB signaling pathway stimulated by phorbol ester, in NCI-H292 cells.

MATERIALS AND METHODS

Materials

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-phospho-inhibitory kappa B kinase (IKK) α/β (Ser176/180, #2687), phospho-specific anti-IκBα (serine 32/36, #9246), and phospho-specific anti-p65 (serine 536, #3036S) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-nuclear matrix protein p84 (ab-487) antibody was purchased from Abcam (Cambridge, MA, USA). Either Goat Anti-mouse IgG (#401215) or Goat Anti-rabbit IgG (#401315) was purchased from Calbiochem (Carlsbad, CA, USA) and used as the secondary antibody. The other chemicals, including betulin (purity: 98.0%), used in the current experiment 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) plus 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 then cultured in RPMI 1640 with 0.2% FBS, for 24 h.

Treatment of cells with betulin

After serum deprivation, cells were pretreated with varying concentrations of betulin for 30 min and then treated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) for 24 h in serum-free RPMI 1640. Betulin 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, and expression and activity of molecules involved into 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 glycoproteins (in a 24-well culture plate). The total RNA was extracted to check the expression of MUC5AC gene (in a 6-well culture plate) using RT-PCR. For the western blot analysis, cells were treated with betulin 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 1:10 dilution, and 100 μL of each sample was incubated at 42°C in a 96-well plate, until it would be 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.

Isolation of total RNA and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Seongnam, 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.

Whole cell extract preparation

NCI-H292 cells (confluent in 100 mm culture dish) were pretreated for 24 h at 37°C with 1, 5, 10 or 20 μM of betulin, and then stimulated with PMA (50 ng/mL) for 30 min, in serum-free RPMI 1640. After the treatment of the cells with betulin, media were aspirated, and the cells washed with cold PBS. For the 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 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. The amount of protein in extract was quantified by Bradford method.

Nuclear and cytosolic extracts preparation

After the treatment with betulin as stated, 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 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. The amount of protein in extracts was quantified by 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 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).

Statistical analysis

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 as a post-hoc test. A p-value of <0.05 was considered significantly different.

RESULTS

Effect of betulin on PMA-induced mRNA expression and glycoprotein production of MUC5AC mucin

Betulin suppressed PMA-induced MUC5AC mucin mRNA expression (Fig. 2). Also, betulin suppressed PMA-induced MUC5AC mucin glycoprotein production, dose-dependently. The amounts of MUC5AC mucin in the cells of betulin-treated cultures were 100 ± 6% (control), 562 ± 6% (10 ng/mL of PMA alone), 527 ± 5% (PMA plus betulin 1 μM), 454 ± 15% (PMA

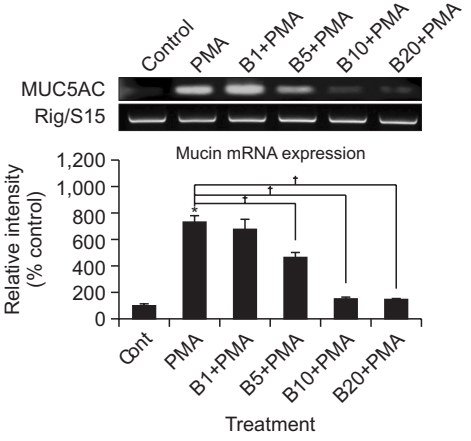

Fig. 2. Effect of betulin on PMA-induced MUC5AC mucin mRNA expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of betulin for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin mRNA expression using RT-PCR. Three independent experiments were performed, and the representative data were shown (cont: control, B: betulin, concentration unit is μM).

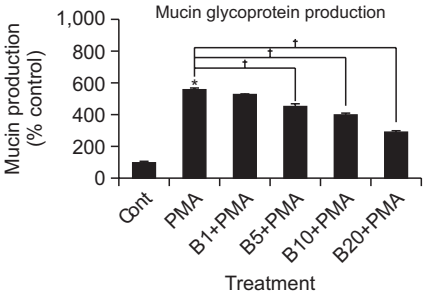

Fig. 3. Effect of betulin on PMA-induced MUC5AC mucin glycoprotein production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of betulin for 30 min and then stimulated with PMA (10 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin glycoprotein production by ELISA. Each bar represents a mean ± SEM. of three 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, B: betulin, concentration unit is μM).

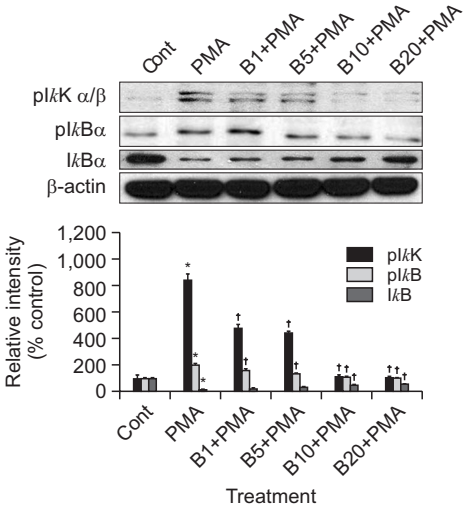

Fig. 4. Effect of betulin on PMA-induced IKK phosphorylation, IkBα phosphorylation, and IkBα degradation, in NCI-H292 cells. NCI-H292 cells were incubated with varying concentrations of betulin for 24 h and treated with 50 ng/mL PMA for 30 min. Cytoplasmic extracts were fractionated and then subjected 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, B: betulin, IkBa: inhibitory kappa Bα, IKK: inhibitory kappa B kinase, concentration unit is μM).

plus betulin 5 μM), 403 ± 4% (PMA plus betulin 10 μM) and 293 ± 5% (PMA plus betulin 20 μM), respectively (Fig. 3). Cell viability was checked by sulforhodamine B (SRB) assay and there was no cytotoxic effect of betulin, at 1, 5, 10 or 20 μM concentration (data were not shown).

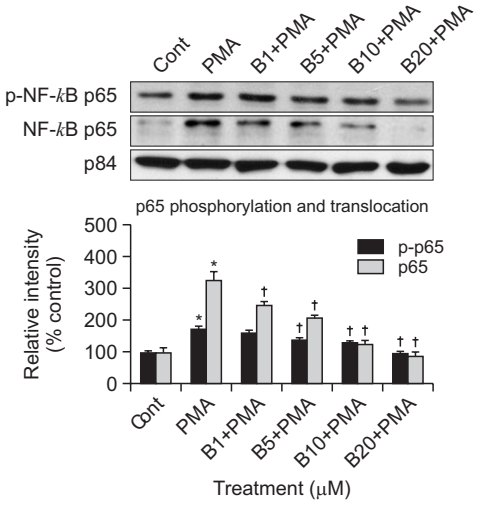

Fig. 5. Effect of betulin on PMA-induced phosphorylation and translocation of NF-kB p65, in NCI-H292 cells. Nuclear protein extracts were prepared and subjected to western blot analysis using phospho-specific p65 (Ser 536) antibody and antibody against p65. As a loading control, p84 levels were analyzed. 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, B: betulin, concentration unit is μM).

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

For the activation of NF-κB, PMA gives rise to the phosphorylation of IKK and this phosphorylated IKK sequentially phosphorylates the IκBα. The phosphorylated IκBα dissociates from NF-κB and degraded. Therefore, we tested whether betulin affects the phosphorylation of IKKα/β, phosphorylation of IκBα, and degradation of IκBα, induced by PMA. As can be seen in Fig. 4, betulin attenuated PMA-stimulated phosphoryaltion of IKKα/β, through controlling the phosphorylation of the serine 176/180 moiety of IKKα/β. PMA increased the phosphorylation of IκBα, whereas betulin suppressed its phosphorylation. Also, PMA increased the degradation of IκBα, whereas betulin suppressed its degradation.

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

After the activation of NF-κB, it translocates from the cytosol to the nucleus, followed by being combined to the specific site of DNA. This assembly of NF-κB /DNA recruits the RNA polymerase and then the resulting mRNA is translated into the specific proteins, including MUC5AC mucins. The transcriptional activity of NF-κB p65 is dependent on its phosphorylation. As shown in Fig. 5, PMA increased the phosphorylation of p65, whereas betulin suppressed its phosphorylation. Eventually, betulin decreased the nuclear translocation of NF-κB p65, stimulated by PMA.

DISCUSSION

Although a multitude of clinical medicine has been used for controlling the secretion and/or production of pulmonary

mucus, still there is no specific compound showing such a pharmacological action. Many compounds in a clinical use for controlling pulmonary diseases relating to the abnormal quality or quantity of airway mucus are not successful to manifest the eminent clinical efficacy in managing such diseases and elicited various side effects. These compounds are the expectorants and mucolytics, for example, N-acetyl L-cysteine (NAC), bromhexine, glucocorticoids, ambroxol, dornase-α, azithromycin, erdosteine, hypertonic saline solution, glyceryl guaiacolate, letocysteine, 2-mercaptoethane sulfonate sodium (MESNA), mannitol, S-carboxymethyl cysteine, myrtol, thymosin β-4, and sobrerol (Li *et al*., 2020).

Therefore, it is very promising to develop a novel agent controlling the production and/or secretion of pulmonary mucins, through affecting the degradation and/or biosynthesis of them. Simultaneously, the regulation of inflammatory response might be the first goal, in order to control the inflammatory pulmonary diseases efficiently. In this context, we suggest it is encouraging to examine the potential activity of regulating the pathological production and/or secretion of mucins, using natural products isolated from a multitude of medicinal plants used empirically for controlling the inflammatory pulmonary diseases.

In the current study, we showed that betulin, a triterpenoid compound with anti-inflammatory activity, decreased the expression of MUC5AC mucin mRNA and the production of MUC5AC mucin glycoprotein, stimulated by PMA (Fig. 2, 3). These results suggest that betulin might regulate the gene expression of pulmonary mucin, by directly acting on airway epithelial cells. As far as we know, this is the first report about the regulatory activity of betulin on the gene expression of pulmonary mucin, in the steps of transcription and translation.

It is well-known that triterpenoids including betulin are the natural product contained in various medicinal plants showing diverse pharmacological effects such as anticancer, anti-inflammatory, antimicrobial, and antioxidative activities. Especially, the molecular mechanism lying under the anti-inflammatory effect of triterpenoids might be exerted via regulating the activity of transcription factor like NF-kB (Ren *et al*., 2018; Verma *et al*., 2018; Kou *et al*., 2021).

As a multifunctional transcription regulator, NF-κB signaling commonly exists in eukaryotic cells. The activation of NF-κB occurs by preferentially provoking the phosphorylation of the IKKs. IKKs will then activate IκBα phosphorylation. Consequently, the dissociated form of phosphorylated IκBα is accumulated and degraded by the proteasome. NF-κB subunits (p65 and p50) are then released and entered into the nucleus, binding with DNA (Nie *et al*., 2012; Liu *et al*., 2020). NF-κB signaling pathway plays a crucial role in mediating the biological functions relating to immunity and inflammations (Huang *et al*., 2018; Lee *et al*., 2019; Shang *et al*., 2019). Under the pathological conditions of airway epithelial cells, NF-κB is involved in hypersecretion of mucin and regulation of activity of cytokine (Liu *et al*., 2020). Also, in patients suffering from asthma, activation of NF-κB can be abnormal (Huang *et al*., 2018). It has been reported that the gene expression of MUC5AC mucin might be evoked by the inflammatory mediators which induce the activity of the transcription factors including NF-κB (Fujisawa *et al*., 2009; Kurakula *et al*., 2015; Garvin *et al*., 2016). It has been well-known that PMA provokes the gene expression of MUC5AC mucin, and NF-kB signaling is reported to be involved into the effect of PMA (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012).

In the current study, as can be seen in results, betulin suppressed the phosphorylation and nuclear translocation of NF-κB p65 via affecting the steps of the phosphorylation and degradation of IκBα, in human airway epithelial cells (Fig. 4, 5). Thus, the pharmacological action of betulin on MUC5AC mucin gene expression might be exerted, at least partly, via mitigating IkBα degradation and NF-kB p65 nuclear translocation. This relationship among IκBα, NF-kB p65 and inflammation was identified in the recent report by Choi and colleagues (Choi *et al*., 2021). They suggested that certain chalcone derivatives suppressed the activation of the nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome, mediated by interrupting the interleukin-1receptor-associated kinase 4 (IRAK4)/IκBα/NF-κB signaling pathway, in Helicobacter pylori-infected THP-1 cells.

Collectively, the inhibitory activity of betulin on airway mucin gene expression might be mediated by controlling PMA-stimulated IkBα degradation and NF-kB p65 nuclear translocation. These results suggest a potential of utilizing betulin as a novel mucoregulator for inflammatory pulmonary diseases. It is desirable to modify the chemical structure of betulin, so as to show the optimal regulating effect on the secretion and/or production of pulmonary mucus, by the optimally modified compound.

CONFLICT OF INTEREST

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

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