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Tilianin Inhibits MUC5AC Expression Mediated Via Down-Regulation of EGFR-MEK-ERK-Sp1 Signaling Pathway in NCI-H292 Human Airway Cells S

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In the human airway, mucus exists to protect the respiratory system as a primary barrier of the innate immune system. However, hyperexpressed mucus limits airflow, resulting in a decrease of lung function. Among more than 20 mucin family members, MUC5AC and MUC5B are major glycoproteins in human airway mucus. The epidermal growth factor receptor (EGFR) signaling pathway is one of the mechanisms of these mucins expression and specificity protein-1 (Sp1) transcription factor is the downstream signal of this pathway, playing pivotal roles in mucin expression. Even though there are some drugs for treating mucus hypersecretion, no drug has proven effects on humans. We found that the flavonoid tilianin regulated MUC5AC expression and also inhibited Sp1 phosphorylation. In this study, we investigated how tilianin would modulate EGFR signaling and regulate mucin production. In conclusion, tilianin inhibited MUC5AC expression mediated via modulating the EGFR-MEK-ERK-Sp1 signaling pathway in NCI-H292 human airway epithelial cells. This study may provide the basis for the novel treatment of mucus hypersecretion.

Keywords: Specificity protein-1, tilianin, mucin 5AC, chronic obstructive pulmonary disease, epidermal growth factor

Introduction

According to the World Health Organization, more than 3 million people died of chronic obstructive pulmonary disease (COPD) in 2012 [1]. To date, smoking is known as the primary cause of COPD, but indoor and outdoor air pollutions are also considered as causes of COPD. Against smoking and air pollutants, airway mucus plays a key role in the protective innate immunity of the respiratory system. However, mucus hypersecretion is frequently observed in asthma and COPD patients, and this hypersecreted mucus limits airflow and suppresses lung function [2]. Moreover, this symptom is closely related with disease mortality and morbidity [2, 3]. The main component of secreted mucus is mucin glycoprotein. MUC5AC and MUC5B are major secreted mucin proteins in the human airway and are targets of hypersecreted mucus treatment [2]. The epidermal growth factor receptor (EGFR) signaling pathway is one of the regulatory mechanism of mucin production, and EGFR activates downstream signals MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) after stimulation by EGFR-ligand [4–6]. Then, activated ERK phosphorylates Sp1 transcription factor to initiate mucin gene expression [7, 8]. Thus, the EGFR-MEK-ERK-Sp1 signaling pathway plays a pivotal role in mucin gene expression. Several drugs have been developed and available for the treatment of mucus hypersecretion by targeting this signaling pathway. However, there is no evidence of these drugs in humans [2]. In nature, some plants are known for their beneficial effects on human health and these plants have been used as herbal medicinal products [9–11]. Flavonoids are a type of plant metabolites and have components with

therapeutic properties. Flavonoids have been reported for anti-inflammatory, antiviral, antioxidant, and even anticancer effects [12–15].

Agastache rugosa Kuntze (Korean mint) is a perennial herb and well known as Bangah in the southern region of Korea [16]. Tilianin is a major constituent of *A. rugosa* and has been associated with numerous pharmacologic effects, such as antioxidant, antiatherogenic, antitumor, and anti-inflammatory activities [17, 18]. This plant contains flavonoids, including acacetin, tilianin, and rosmarinic acid, which contribute to these pharmacological activities [17, 19]. In this study, as part of an ongoing search for new anti-COPD agents, tilianin was isolated from *A. rugosa* leaves.

For studying mucin expression in vitro, NCI-H292 and A549 cells were used. A549 cells can produce MUC5AC without stimulation [20] and have a K-Ras mutation (G12S) which keeps activating K-Ras signals [21]. However, NCI-H292 cells have wild-type K-Ras and do not produce MUC5AC without stimulation. In this study, we investigated whether the flavonoid tilianin would have modulating effects on mucin production in wild-type K-Ras NCI-H292 and K-Ras-mutated A549 cells.

Materials and Methods

Extraction and Isolation of Tilianin from *Agastache rugosa*

The *Agastache rugosa* leaves were collected at Gonyang in Sacheon, South Korea in December 2014. The plant was authenticated by Dr. Joong Ku Lee (Korea Research Institute of Bioscience & Biotechnology (KRIBB)), and a voucher specimen (KRIB0059129) was deposited at the herbarium of the Plant Extract Bank of KRIBB in Daejeon, Korea. Dried powder of *A. rugosa* (49.0 g) was extracted three times with 3 volumes of absolute (99.8%) methanol at room temperature. The extracts were combined and concentrated in vacuo at 40°C to produce a dried extract (8.9 g, 18.1%). The air-dried *A. rugosa* was chopped and extracted with methanol (10 L × 3) at room temperature for 3 days. The combined filtrate was concentrated in vacuo to yield a dark brown gum. The MeOH extract (1.2 g) was performed by prep-HPLC using an Atlantis T3 column (H_2O: ACN, 20 → 50% (50 min); 50→100% (10 min); flow rate: 14 ml/min) to yield tilianin (7.5 mg) and acacetin (5.2 mg). Purities (>98%) were confirmed by UPLC-PDA-QTOF-MS. Purified compounds were identified by comparing ^{1}H and ^{13}C NMR, MS, MS/MS, and HRESIMS data with literature values (Supplementary data).

Cell Culture

NCI-H292 and A549 cells were cultured in RPMI-1640 medium (WELGENE, South Korea), supplemented with 10% FBS (WELGENE) and 1% penicillin and streptomycin (WELGENE). Cells were serum-starved for 24 h after reaching confluence. Cells were pre-treated with tilianin for 30 min after starvation and then stimulated with epidermal growth factor (25 ng/ml; Millipore Sigma, Germany) for indicated time periods.

Western Blot Analysis

Cells were collected by Trypsin-EDTA and lysed in NP-40 lysis buffer that contained 150 mM NaCl, 1% NP-40, and 50 mM Tris-HCl (pH 8.0) supplemented with complete protease inhibitor cocktail EDTA-Free (Roche Diagnostics, Switzerland) and PhosSTOP (Roche Diagnostics). In order to isolate Sp1 and other nuclear proteins, NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) were used after trypsinization. The Pierce BCA Protein Assay kit (Thermo Fisher Scientific) was used to determine protein concentration. The 3× SDS sample buffer contained 187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, and 0.03% (w/v) bromophenol blue in lysates, and an equal volume and equal amounts of proteins were loaded in SDS-PAGE gels. After SDS-PAGE, the proteins were transferred to Immobilon-P PVDF membranes (Merek Milipore, Germany). The membranes were blocked with 1× Blocking Solution (BioFACT, South Korea) at room temperature for 1 h. Specific proteins were detected with respective primary antibodies. Phospho-MEK, MEK, phospho-ERK1/2, phospho-p65, p65, PARP, beta-actin, and phospho-threonine antibodies were purchased from Cell Signaling Technology (USA). ERK, phospho-Akt, Akt, and Sp1 antibodies were from Santa Cruz Biotechnology (USA). The membranes were incubated with the primary antibodies at 4°C overnight and then with secondary antibodies at room temperature for 1–2 h. After primary or secondary antibody probing, the membranes were washed in PBS-T (0.1% Tween-20) three times. The results were captured by using Western Bright ECL HRP substrate (Advansta, USA) and EZ-Capture MG (ATTO, Japan).

Immunoprecipitaion Assay

For the immunoprecipitation assay, cells were serum-starved for 24 h and treated with tilianin for 30 min. The cells were then lysed in NP-40 lysis buffer. An equal amount of protein lysate was incubated with anti-Sp1 antibodies (Santa Cruz Biotechnology) at 4°C overnight and then incubated with ProA/G agarose beads (AMICOGEN, South Korea) at 4°C overnight. After washing with PBS three times, proteins were eluted by boiling. Eluted proteins were analyzed by western blot assay and probed by anti-phospho threonine antibodies.

Real-Time PCR Analysis

For analysis of mRNA expression of MUC5AC, cells were seeded in 12-well plates at 5×10^6 cells/well. The cells were pre-treated with tilianin for 30 min after serum starvation for 24 h, and then stimulated with EGF for 24 h. After dissociation with Trypsin-EDTA, the cells were lysed and RNA was isolated and purified by Ribospin (GeneAll Biotechnology, South Korea) following the manufacturer's instruction manual. cDNA synthesis was performed by using the HyperScript First Strand Synthesis

kit (GeneAll Biotechnology). The PCR was performed with BioFACT 2× H-Star Taq PCR Pre-Mix (BioFACT) and specific primers. Real-time quantitative PCR analyses were performed with the SensiFAST SYBR No-ROX Kit (Bioline, USA). Expression levels were normalized to that of H2A histone family member Z (H2A.Z), calculated by the delta-delta Ct method.

Dot-Blot Assay

NCI-H292 cells were cultured in a 12-well plate. After EGF stimulation for 24 h, the cultured media were collected. Each medium (2 μl) was loaded onto nitrocellulose membranes. After 1 h of blocking with 5% skim milk, the membranes were incubated with anti MUC5AC antibodies (clone 45M1; Neomarkers, USA) for overnight at 4°C. After washing in PBS-T three times, the membranes were incubated with secondary antibodies for 1 h at room temperature. The results were captured by using Western Bright ECL HRP substrate (Advansta) and EZ-Capture MG (ATTO).

Statistics

Data were analyzed by using the Student's *t*-test to evaluate significant differences between experimental and control groups. A *p*-value of <0.05 was considered statistically significant. The data were presented as the mean (SD) of three independent experiments

Results

Tilianin Inhibited EGFR Downstream Signals in NCI-H292 Cells, but Not in A549 Cells

We used two different human airway cell-lines, NCI-H292 and A549. To investigate whether tilianin has cytotoxicity in these cell-lines or not, we performed a CCK-8 assay (Dojindo Molecular Technologies, USA). We also used acacetin, which is a flavonoid similar to tilianin, in order to compare the effects (Fig. 1A). As already reported, acacetin showed cytotoxic effect in both cell lines [22], whereas tilianin did not show significant cytotoxic effects (Fig. 1B). As the EGFR signaling pathway is mainly involved in MUC5AC expression, we used EGF for inducing mucin expression. In order to elucidate whether tilianin would modulate the EGFR signaling pathway, we pre-treated cells with tilianin before EGF treatment. After EGF stimulation in NCI-H292 cells, the EGFR downstream signals MEK/ERK were reduced by tilianin at 30 min (Fig. 2A). Phospho-p38 levels were also reduced from 15 min (Fig. 2A). Akt signaling, which has been reported to be involved in MUC5AC expression [23], was also down-regulated by tilianin (Fig. 2B). Next, we examined the EGF-stimulated

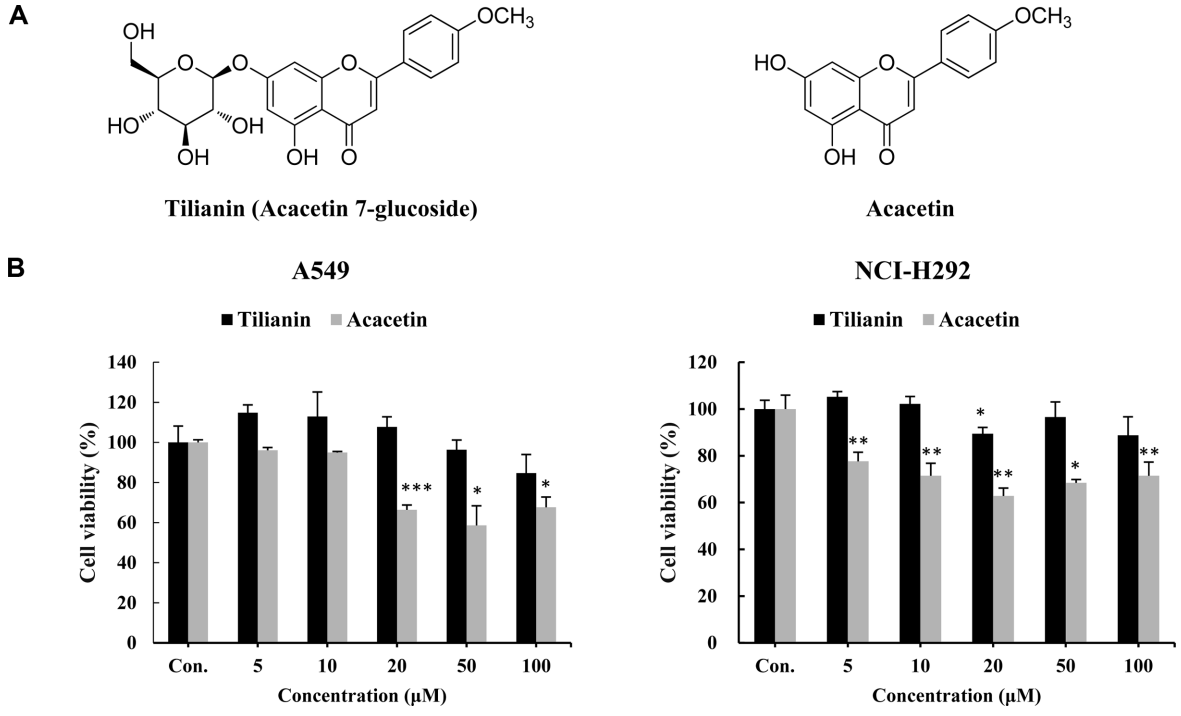

Fig. 1. Structure and cytotoxic effect of tilianin.

(**A**) Structure of tilianin and acacetin. (**B**) In a 96-well plate, 5×10^3 cells per well were seeded. After 24 h, tilianin and acacetin were treated for another 24 h with serum-free medium. Viability was measured by CCK-8 assay. Asterisks (*) indicate significant results compared with the non-treated control. *Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$.

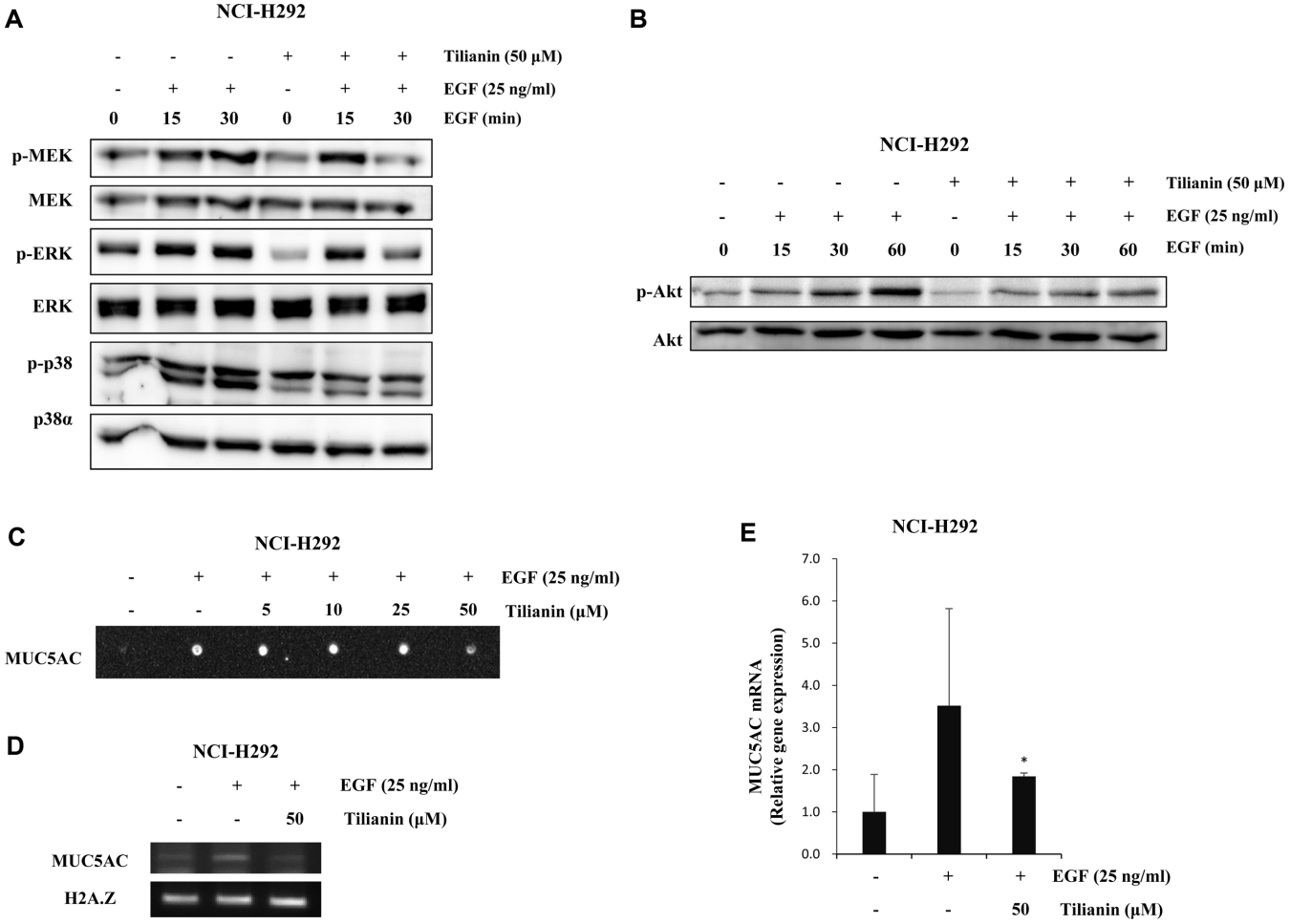

Fig. 2. Tilianin inhibited MEK/ERK, p38, and Akt signals in NCI-H292 cells, resulting in inhibition of MUC5AC expression.
(**A**, **B**) Cells were treated with tilianin for 30 min followed by EGF stimulation for indicated time periods, and protein signals were analyzed by western blotting. Each control was detected on the same membrane after the stripping step. (**C**) Secreted MUC5AC proteins were measured by dot-blot assay. Cells were treated with tilianin for 30 min and then EGF stimulation for 24 h. Cultured media were applied onto nitrocellulose membranes. After the blocking step, the membranes were probed by anti-MUC5AC antibodies. (**D**, **E**) MUC5AC mRNA levels were compared by using reverse-transcription polymerase chain reaction and real-time quantitative polymerase chain reaction methods. After tilianin pre-treatment for 30 min, cells were EGF-stimulated for 24 h. cDNA was synthesized from isolated total RNA. With specific primers, the PCR was performed. Asterisks (*) indicate significant results ($p < 0.05$) compared with EGF-treated samples.

MUC5AC protein and mRNA levels. As shown in Fig. 2, EGF treatment enhanced the MUC5AC protein and mRNA levels. However, when cells were treated with tilianin before EGF treatment, the MUC5AC protein and mRNA expression levels were reduced (Figs. 2C–2E).

Furthermore, we also analyzed EGFR downstream signaling in A549 K-Ras-mutated cells, to confirm whether tilianin would inhibit the MEK/ERK and p38 pathway like NCI-H292 cells. In this case, we could not see the inhibitory effect of tilianin on MEK/ERK and p38 signaling in A549 cells (Fig. 3A). In addition, Akt phosphorylation was not altered in A549 cells (Fig. 3B). We performed reverse-transcription polymerase chain reaction to confirm the effect of tilianin on MUC5AC mRNA expression. Interestingly, we could not see any difference, even in EGF-treated A549 cells. Consequently, mRNA expression of MUC5AC in A549 cells was not altered by tilianin (Fig. 3C).

Sp1 Phosphorylation Was Inhibited by Tilianin

Mucin production is mediated via Sp1 and NF-kB transcription factors. [7, 8, 24]. As tilianin reduced MUC5AC mRNA expression in NCI-H292 cells, we assumed tilianin has a modulating effect on transcription factors such as Sp1 or NK-kB. It was previously reported that NF-kB is

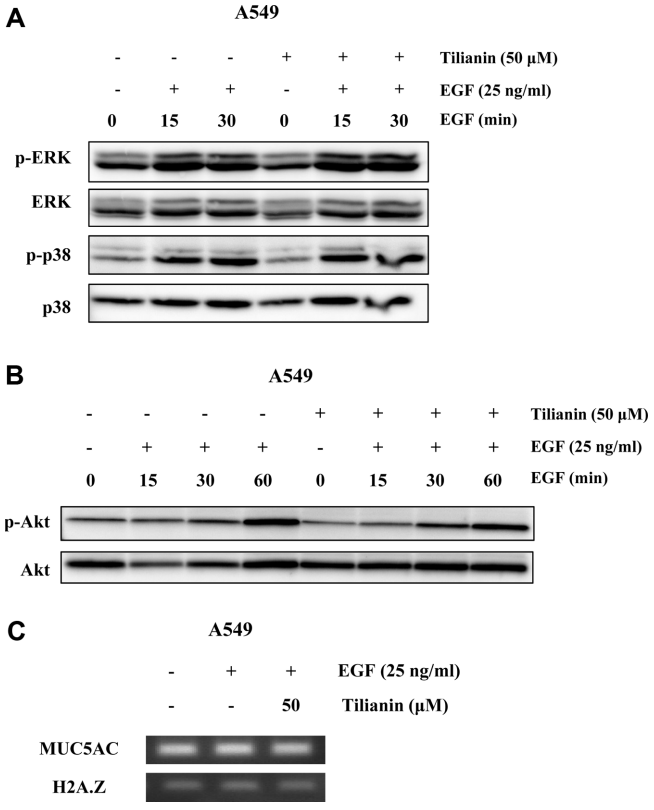

Fig. 3. Tilianin did not affect MEK/ERK, p38, and Akt signals in A549 cells.

(**A**, **B**) A549 cells were treated with tilianin for 30 min followed by EGF stimulation for indicated time periods and then analyzed by western blotting. Each control was detected on the same membrane after the stripping step. (**C**) MUC5AC mRNA levels were compared by the using reverse-transcription polymerase chain reaction. After tilianin pre-treatment for 30 min, cells were EGF-stimulated for 24 h. cDNA was synthesized from isolated total RNA. With specific primers, the PCR was performed.

involved in MUC5AC expression [24], and tilianin has an inhibitory effect on NF-kB activation in hyperlipidemic mice [25]. To evaluate the effect of tilianin on NF-kB in NCI-H292 cells, we performed western blot analysis. The membranes were probed by anti-p65 and anti-phospho-p65 antibodies. As shown in Fig. 4A, p65 and phospho-p65 levels were not altered by tilianin treatment whereas Sp1 was decreased and appeared as two bands (105 and 95 kDa) in NCI-H292 cells. Surprisingly, also in A549 cells, Sp1 was decreased and appeared as two bands (105 and 95 kDa) in the presence of tilianin regardless of EGF stimulation (Fig. 4B). As we found that phosphorylation of ERK was down-regulated by tilianin (Fig. 2A) and ERK has been reported to phosphorylate Sp1 [26, 27], we performed

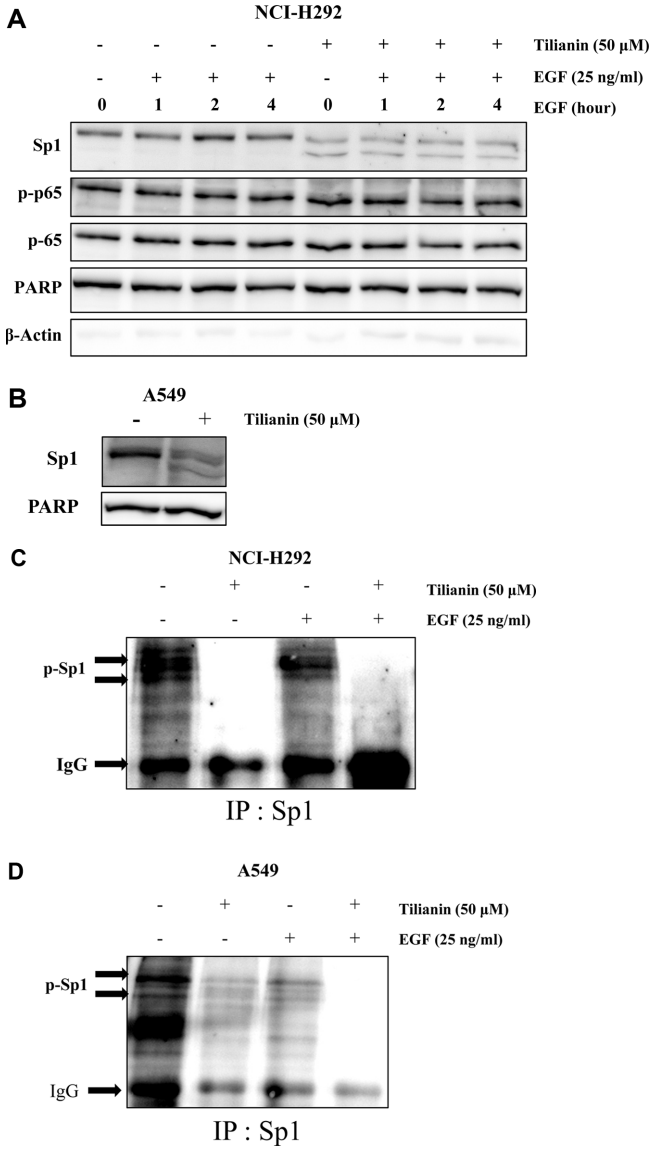

Fig. 4. Sp1 phosphorylation was decreased by tilianin in both NCI-H292 cells and A549 cells.

(**A**) After serum starvation for 24 h, NCI-H292 cells were treated with tilianin for 30 min followed by EGF stimulation for indicated time periods. Nuclear proteins were isolated and detected by specific antibodies. Controls were detected in the same membrane after the stripping step. (**B**) In A549 cells, without EGF stimulation, Sp1 was detected. After serum starvation for 24 h, tilianin was treated for 30 min. Nuclear proteins were isolated and Sp1 was detected by specific Sp1 antibodies by western blotting. (**C**, **D**) Prior to immunoprecipitation, cells were serum starved for 24 h. Samples pre-treated with tilianin for 30 min were marked as positive. EGF-positive samples were stimulated by EGF for 2 h. Both positive samples were tilianin pre-treated for 30 min and then EGF-stimulated for 2 h. After immunoprecipitation of Sp1, anti-phospho threonine antibodies were used for detecting phospho-Sp1. Arrows indicate phophos-Sp1 (105 and 95 kDa) and IgG (25 kDa).

immunoprecipitation assays in order to verify that Sp1 phosphorylation was modulated by tilianin treatment. Sp1 was pull-downed by anti-Sp1 antibodies and then phospho-threonine antibodies were used to detect phospho-Sp1. As shown in Figs. 4C–4D, we found tilianin inhibited Sp1 phosphorylation in not only NCI-H292 cells but also A549 cells.

Discussion

Mucins are closely related with lung pathogenesis and its mortality and morbidity [2, 3]. In asthma and other COPD patients, mucus hypersecretion is commonly observed. This symptom obstructs airflow in small airways, and limits and decreases lung function [1–3]. As MUC5AC is a major mucin protein in human airway mucus, it has been investigated to find the way to control MUC5AC expression [2, 3, 7, 28].

In this study, we investigated whether tilianin would have an inhibitory effect on mucin production in human airway epithelial cell lines, A549 and NCI-H292. These two different cells have been studied for in vitro mucin production. A549 cells produce MUC5AC not only with stimulators but also just itself [20], as A549 cells have a K-Ras mutation (G12S) [29] that keeps activating K-Ras signals and it is closely related with mucin expression [21, 30]. It has been previously reported that K-Ras-mutated cancer cell lines have resistance against MEK inhibitors [31, 32]. Our results indicating that EGF-induced ERK and AKT activities were not affected by tilianin (Fig. 3A) and EGF and tilianin did not affect MUC5AC gene expression (Fig. 3C) in K-Ras-mutated A549 cells, might be due to this K-Ras mutation (Fig. 3).

However, NCI-H292 cells, which have wild-type K-Ras [33], cannot produce MUC5AC without stimulators. NCI-H292 cells can produce MUC5AC with stimulators such as EGF, phorbol 12-myristate 13-acetate, and tumor necrosis factor alpha [4, 5, 7, 8, 34]. Our study showed that tilianin inhibited the EGF-induced MEK/ERK and p38 pathways (Fig. 2A) and decreased MUC5AC expression in NCI-H292 cells (Fig. 2C), whereas there was no effect on A549 cells (Fig. 3).

Sp1 transcription factor plays a pivotal role in mucin production [7, 8]. Sp1 activity is regulated by post-translational modification such as phosphorylation, glycosylation, sumoylation, and ubiquitination [27, 35]. Phosphorylation of Sp1 by ERK [26] triggers endoproteolytic cleavage of the inhibitory domain in the N-terminus of Sp1 and activates Sp1-dependent-transcription [35–37]. After cleavage, Sp1 is highly unstable, and then degraded by proteasome [35, 36]. We used EGF for activating the MEK/ERK signaling pathway and inducing phosphorylation of Sp1. In K-Ras wild-type NCI-H292 cells, tilianin inhibited expression of MUC5AC (Fig. 2C) and EGF-related downstream signals, MEK/ERK/Sp1 (Figs. 2A, 4A, and 4C). By immunoprecipitation assay, we showed that phospho-Sp1 was decreased in the presence of tilianin in NCI-H292 cells (Fig. 4C). In addition, Sp1 was decreased and appeared as two bands (105 and 95 kDa) in tilianin pre-treated NCI-H292 cells (Fig. 4A). Interestingly, in A549 cells, Sp1 was decreased and appeared as two bands (Fig. 4B) and phosphorylation was also decreased like NCI-H292 cells regardless of ERK phosphorylation and EGF treatment (Fig. 4D).

As the Sp1 N-terminus cleaved part has 25% of serine and threonine phosphorylation sites [35], 105 kDa Sp1 protein is phosphorylated more than 95 kDa Sp1 [35, 38]. As described above, tilianin inhibited phosphorylation of Sp1 by decreasing 105 kDa Sp1 in both A549 and NCI-H292 cells (Figs. 4A and 4B). By using anti-phospho-threonine antibodies, immunoprecipitation assay confirmed that tilianin reduced phosphorylation of both Sp1 105 kDa and 95 kDa in both cell lines (Figs. 4C and 4D). These results suggest that tilianin can inhibit phosphorylation of Sp1 in A549 and NCI-H292 cells regardless of ERK phosphorylation and EGF treatment. However, even Sp1 phosphorylation was inhibited in A549 cells, where MU5AC expression was not reduced (Fig. 3A). We assumed that genetic differences, like K-Ras mutation, may keep expressing MUC5AC despite the inhibitory effects of tilianin on Sp1 phosphorylation in A549 cells (Figs. 4B and 4D).

In summary, we demonstrated that tilianin inhibited MUC5AC in NCI-H292 cells. This effect was mediated through the Ras/Raf/MEK/ERK and p38 pathways. Additionally, tilianin can inhibit Sp1 phosphorylation regardless of ERK phosphorylation and EGF stimulation. Based on these results, tilianin can be a potential modulator of asthma and other COPD to reduce mucus hypersecretion.

Acknowledgments

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