Original Paper

Naringenin Regulates CFTR Activation and Expression in Airway Epithelial Cells

Rui Shi^{a,b,c} Zi-Ting Xiao^{a,b,c} Yi-Jun Zheng^c Yi-Lin Zhang^c Jia-Wen Xu^c
Jie-Hong Huang^c Wen-Liang Zhou^c Pei-Bo Li^{a,b,c} Wei-Wei Su^{a,b,c}

^aGuangdong Engineering & Technology Research Center for Quality and Efficacy Re-evaluation of Post-market Traditional Chinese Medicine, School of Life Sciences, Sun Yat-sen University, Guangzhou;

^bState Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou; ^cSchool of Life Sciences, Sun Yat-sen University, Guangzhou, China

Key Words

Naringenin • Airway epithelium • CFTR • Cl⁻ secretion • Traditional Chinese medicine

Abstract

Background/Aims: Sputum symptoms are commonly seen in the elderly. This study aimed to identify an efficacious expectorant treatment stratagem through evaluating the secretion-promoting activation and cystic fibrosis transmembrane conductance regulator (CFTR) expression of the bioactive herbal monomer naringenin. **Methods:** Vectorial Cl⁻ transport was determined by measuring short-circuit current (I_{sc}) in rat airway epithelium. cAMP content was measured by ELISA in primary cultured epithelial cells and Calu-3 cells. CFTR expression in Calu-3 cells was determined by qPCR. **Results:** Addition of naringenin to the basolateral side of the rat airway led to a concentration-dependent sustained increase in I_{sc} . The current was suppressed when exposed to Cl⁻-free solution or by bumetanide, BaCl₂, and DPC but not by DIDS and IBMX. Forskolin-induced I_{sc} increase and CFTRinh-172/MDL-12330A-induced I_{sc} inhibition were not altered by naringenin. Intracellular cAMP content was significantly increased by naringenin. With lipopolysaccharide stimulation, CFTR expression was significantly reduced, and naringenin dose-dependently enhanced CFTR mRNA expression. **Conclusion:** These results demonstrate that naringenin has the ability to stimulate Cl⁻ secretion, which is mediated by CFTR through a signaling pathway by increasing cAMP content. Moreover, naringenin can increase CFTR expression when organism CFTR expression is seriously hampered. Our data suggest a potentially effective treatment strategy for sputum.

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Introduction

It is commonly known that airway surface liquid (ASL) comprises a mucus layer (ML) and a periciliary fluid layer (PCL). The superficial ML overlies the PCL, which contacts airway surface epithelial cells [1]. The ML consists of a large amount of water, salt and proteins

Wei-Wei Su
and Wen-Liang Zhou

School of Life Sciences, Sun Yat-sen University
No. 135, Xingangxi Street, Guangzhou (China)
Tel. +86 20 84110808; Tel. +86 20 39386076, E-Mail lsswwhk@gmail.com

secreted by submucosal gland serum cells and surface epithelial goblet cells. It determines sputum viscosity to trap dust in the airway. The PCL, secreted by epithelial cells and submucosal glands, has lower viscosity than the ML due to higher water content and acts as the medium for cilia clearing dust and germs from the respiratory tract. The depth, composition and viscosity of ASL are important determinants of mucociliary clearance, bactericidal activity, and the functions of epithelial and immune cells [1]. Ion channels, transporters and pumps are located on apical and basolateral membranes in certain strategies that determine the absorption and secretion of electrolytes and the fluid content in epithelial cells. Particularly, airway epithelial Cl^- channels, such as cystic fibrosis transmembrane conductance regulator (CFTR), are crucial factors in the regulation of electrolytes and fluid secretion across the respiratory system [2]. CFTR abnormality is considered to be the main cause of cystic fibrosis (CF), which is an inherited disorder widespread among the white race [3]. A lack of CFTR leads to weak electrolyte and fluid secretion, thus leading to a decreased PCL [4, 5]. Therefore, the epithelial cilia cannot swing to expel sputum from the airway, resulting in sputum increase. Hence, drugs with regulatory effects on CFTR-mediated Cl^- secretion and channel expression may relieve the symptoms of the disease to some extent.

Naringenin is a type of dihydroflavone found in our regular diet and is mainly derived from *Exocarpium Citri grandis* (Huajuhong in Chinese, the dried unripe fruit peel of *Citrus grandis* 'Tomentosa' or *Citrus grandis* (L.) Osbeck), which has been a well-known traditional Chinese medicine to treat cough and sputum for thousands of years [6]. As an additive, naringenin can be incorporated into foods or beverages to inhibit ACAT activity, inhibit macrophage-lipid complex accumulation, and prevent or treat hepatic diseases [7]. It has been demonstrated that naringenin exhibits antioxidative, anti-inflammatory, anti-asthmatic and expectorant effects both *in vitro* and *in vivo* [6, 8, 9]. A previous study demonstrated that naringenin could significantly attenuate human neutrophil elastase (HNE)-induced MU-C5AC secretion in HBE16 human airway epithelial cells by inhibiting the activity of NF- κ B via EGFR-PI3K-Akt/ERK MAPKinase signaling pathways [10] and dose dependently enhancing mucociliary velocity and phenol red secretion into the airway [6]. This evidence showed that naringenin could decrease the viscosity of mucus and enhance mucociliary clearance by stimulating the volume of airway secretions. Nonetheless, whether naringenin improves ASL volume secretion in airway epithelial cells mediated by CFTR remains unclear. Therefore, to find curative potential for medication of sputum and other analogous diseases, the current study is designed to investigate the regulatory mechanism of naringenin.

Materials and Methods

Animal preparation

After one week of adjustment to the new environment, 100-200 g male and female Sprague-Dawley rats were killed via CO_2 asphyxiation and used to measure transepithelial I_{sc} , culture primary epithelial cells and quantify cyclic adenosine monophosphate (cAMP) content. The rats were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China), housed in a temperature- and humidity-controlled room in a 12 hour light/dark cycle and fed a standard laboratory diet and fresh water. All experimental procedures were based on the guiding principles for the care and use of animals approved by the Animal Care and Use Committee of the School of Life Sciences, Sun Yat-sen University, PR China. Adequate measures were taken to minimize the pain of the animals.

Cell culture

Calu-3 cells (a human lung adenocarcinoma epithelial cell line) were gifts from Dr. Wing-Hung Ko at The Chinese University of Hong Kong, HK, China. Cells were cultured with Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, Australian), 1% MEM NEAA (Gibco, USA), and 100 U/ml penicillin-streptomycin solution (HyClone, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Primary rat airway epithelial cells were digested for 16 hours from acutely dissociated rat airway using trypsin-EDTA (0.25%) and maintained in Keratinocyte Serum Free Medium (K-SFM) (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, Australian) and 100 U/ml penicillin-streptomycin solution (HyClone, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Chemicals

Lipopolysaccharides (LPS), naringenin, diphenylamine-2-carboxylic acid (DPC), 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS), 4-[[[4-Oxo-2-thioxo-3-[3-trifluoromethyl]phenyl]-5-thiazolidinylidene]methyl]benzoic acid (CFTRinh-172), N-(cis-2-phenyl-cyclopentyl)azacyclotridecan-2-imine-hydrochloride (MDL-12330A) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the above reagents were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%, which had no significant effects on the I_{sc} or CFTR expression.

I_{sc} measurements

The airway segment (7-8 mm in length) used for I_{sc} measurement and cell culture was isolated from the main trachea, starting from a location 1 mm under the thyroid. To avoid epithelial damage, blunt separation rather than hard tearing was applied. After removing the blood vessels and connective tissue, the tracheas were cut into 3 mm segments, and the ventral side of the trachea was cut to expose the lumen. Airway tissue was dipped in Kerbs-Henseleit (K-H) solution containing (in mM) 117 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 1.2 MgSO₄, 2.56 CaCl₂, and 11.1 glucose (pH 7.4) warmed to 37°C and ventilated in 95% O₂ and 5% CO₂. In ion substitution experiments, HCO₃⁻ was substituted by HEPES, and Cl⁻ was substituted by gluconate. HCO₃⁻ free solution was ventilated in 100% O₂. To control the solution effects, fluid offsets were used to adjust the fluid resistance to 0 [11]. When short-circuit current measurements were taken in different ionic composition solutions, new tissue was used each time, and the bathing solution was not changed with the same tissue. The fluid resistance of the different solutions was adjusted to 0 before the tissue was mounted. Therefore, fluid resistance did not affect the accuracy of the experiment. After adjusting the fluid resistance to 0, tissue samples were mounted on a Ussing chamber with a 0.03 cm² circular internal area, using vertical force to avoid friction with the epithelium and affecting the function. The diameter of Ussing chamber's aperture was 1.96 mm, which was greater than the space enclosed by the adjacent cartilage rings, and thus, the cartilage rings were seen in the mounted segment. The airway epithelial cells located on the surface of the cartilage ring segment showed no functional differences from that on the surface of the muscle segment. Thus, the cartilage rings presented in the mounted segment did not influence I_{sc} measurement. Calu-3 cells grown on transwell inserts (0.4 μm pore size, 12 mm diameter) were incubated with DMEM/F12 for a fortnight. A confluent Calu-3 cell monolayer was used to measure I_{sc} . After the transepithelial potential difference (PD) was locked at 0 mV, the I_{sc} was recorded by a voltage-current clamp amplifier (VCC MC6, Physiologic instrument, San Diego, CA) and then exhibited by a BL-420E signal collection and analysis system (Chengdu Techman Software Co. Ltd., China). Change in the current per unit area (μA/cm²) normalized the I_{sc} variation. When the current flowed from the mucosal to serosal side, the I_{sc} value was expressed as positive [12].

Immunofluorescence

Rat primary airway epithelial cells grown on coverslips were incubated with K-SFM for 3 days. After being fixed with 4% paraformaldehyde, the cells were permeated with 0.3% Triton and 5% BSA mixed solution for 1 hour at 25°C. The cells were incubated with smooth muscle antibody (SMA) and keratin antibody for 2 hours at room temperature. Following three washes with phosphate-buffered saline (PBS), the cells were incubated with fluorescein-conjugated antibody to mark target cells and visualized using microscopy.

Measurement of intracellular cAMP concentrations

Measurement of the cAMP concentration in airway tissue might include the cAMP of other cells at the same time, which affects the accuracy of the experiment. Therefore, we used primary rat airway epithelial cells to measure cAMP content and Calu-3 cells to further verify the results.

The cytosolic cAMP content was measured via enzyme linked immunosorbent assay (ELISA). After primary epithelial cells were cultured in six-well plates for 3 days, the degree of confluence was approximately one hundred percent [13]. The cells were treated with DMSO, naringenin, IBMX, IBMX with naringenin, or

IBMX with forskolin, for 20 min. Calu-3 cells were treated in the same way. The cAMP content was assayed using a Mouse/Rat cAMP Assay Kit (R&D systems, USA). The protein content was determined with a BCA Protein Assay Kit (Beyotime, China). The concentration of cytosolic cAMP is expressed in picomoles per milligram of protein.

RNA isolation and reverse transcription

Calu-3 cells grown in six-well plates were induced with or without 10 µg/ml LPS and treated with 0.1% DMSO (control group) or naringenin (25 µM, 50 µM and 100 µM) for 8 hours to test CFTR mRNA expression. In advance of the experiment, an MTT assay had been performed to certify that the drugs had no effect on Calu-3 cell viability within 8 hours.

Total RNA was extracted from Calu-3 cells using RNAiso Plus (TaKaRa, Japan), and reverse transcribed with a GoScript™ Reverse Transcription System (Promega, USA). Samples were quantified with a NanoDrop 2000 (Thermo, USA).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantitatively determine the mRNA level of CFTR. The gene-specific primers used for qRT-PCR analysis were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Gene-specific primers: CFTR forward 5'-AAG CTG TCA AGC CGT GTT CT-3' and reverse 5'-GAT TAG CCC CAT GAG GAG TG-3'; β-actin forward 5'-CCT GTA CGC CAA CAC AGT GC-3' and reverse 5'-ATA CTC CTG CTT GCT GAT CC-3'. The reaction was performed using GoTaq® qPCR Master Mix (Promega, USA), with the following cycles: 10 min at 95°C, 45 cycles of 10 s at 95°C, 20 s at 60°C, 20 s at 72°C, one cycle of 5 s at 95°C, 60 s at 65°C, 30 s at 97°C, and finally, 30 s at 40°C. A Roche LightCycler 480 System (Roche, Mannheim, Germany) was used to run the reactions. Measurements were performed in triplicate for one sample and approved if the discrepancy between the threshold value (Ct) was less than 1. To implement a relative expression method, β-actin was used as an internal control to normalize the data, and the final results are expressed as a percentage of the control group.

Data analysis and statistics

The data are presented as the mean ± SEM (n is the number of tissue preparations or experiment replications). Statistical significance was determined by Student's t-test. A value of $P < 0.05$ was considered statistically significant.

Results

Naringenin-induced I_{sc} response

A change in I_{sc} was defined as the maximal rise in I_{sc} following drug stimulation, and it was normalized to current change per unit area of the airway (µA/cm²). The basal I_{sc} in isolated rat airway tissue was 8.86 ± 0.72 µA/cm² (n = 4). Naringenin (100 µM) applied to the apical side of rat airway tissue could not cause a change in I_{sc} (Fig. 1A). However, basolateral application of naringenin at a concentration of 100 µM caused an obvious I_{sc} increase, and then, the I_{sc} remained stable (Fig. 1B). Furthermore, the augmentation was concentration-dependent (Fig. 1C), with an EC₅₀ of approximately 71.49 ± 10.76 µM. There was no significant difference in the osmotic pressure before and after naringenin addition. Therefore, the concentration-dependent results shown in Fig. 1C were not caused by a change in the permeability but by the pharmacodynamic effects of naringenin.

Ionic basis of the naringenin-induced I_{sc} response

To investigate the ion species mediating the naringenin-induced I_{sc} , ion substitution experiments (Fig. 2) and Cl⁻ channel blocker tests (Fig. 4) were designed.

To remove HCO₃⁻ from the K-H solution, HCO₃⁻ was substituted with 10 mM HEPES. The solution only slightly suppressed the naringenin-evoked I_{sc} by 5.12% (n = 4) (Fig. 2A), while solution without Cl⁻ (the representative salts of gluconate acted as substitutes) restrained the I_{sc} enhancement by 91.53% (n = 4, $P < 0.001$, Fig. 2B). Furthermore, when extracellular

Fig. 1. Effect of naringenin on anion transport across the rat airway. Naringenin (100 μM) applied to the apical side of rat airway tissue could not cause a change in I_{sc} . (A). Naringenin (100 μM) applied to the basolateral side of rat airway tissue resulted in an increase in I_{sc} . (B). Different concentrations of naringenin (5 μM - 1000 μM) stimulated a concentration-dependent I_{sc} response on the basolateral side (C). Each data point represents a mean $\pm$ SEM ($n = 4$).

Cl^- and HCO_3^- were removed, the naringenin-evoked I_{sc} was decreased by 98.21%, ($n = 4$, $P < 0.001$, Fig. 2C). To confirm that the results of the gluconate substitution experiments were reliable, different Ca^{2+} concentrations were added to the Cl^- free K-H solution. The results showed that naringenin could not obviously increase the I_{sc} in different Ca^{2+} concentrations of Cl^- free K-H solution ($n = 4$, $P < 0.001$, Fig. 3). These results indicated that the response of naringenin to Cl^- substitution was irrelevant to the lowering of free calcium in the bathing solution.

The Cl^- channel blocker DPC (1 mM) (Fig. 4A) and CFTR inhibitor CFTRinh-172 (10 μM) both strongly inhibited the naringenin-induced I_{sc} increase on the mucosal side, whereas subsequent application of ATP (100 μM) also caused an obvious increase at the same sample (Fig. 4B). The naringenin-induced I_{sc} could not be changed by DIDS (100 μM), whereas it was inhibited by subsequent application of CFTRinh-172 (Fig. 4C). The above results indicate that the change in I_{sc} induced by naringenin could be influenced, to a large extent, by CFTR-dependent Cl^- secretion.

To test what influenced the naringenin-induced I_{sc} on the basolateral side, 100 μM bumetanide, a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter inhibitor, was added to the basolateral side and abolished the naringenin-induced I_{sc} increase (Fig. 5A). Further, the response to naringenin was strongly affected by basolateral addition of BaCl_2 , a K^+ channel inhibitor (Fig. 5B). The naringenin-induced I_{sc} could be abolished by adding bumetanide (97.01%) and BaCl_2 (97.96%) (Fig. 5C). The above results suggest that the I_{sc} response induced by naringenin is attributed to K^+ absorption on the basolateral side. The basolateral membrane $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and K^+ channel play important roles in maintaining the driving force for apical CFTR-mediated chloride secretion.

Involvement of cAMP in mediating the effect of naringenin

It is well known that CFTR activated by cAMP induces a long-lasting stimulatory effect on I_{sc} . The naringenin-induced I_{sc} is similar to a short circuit current reaction mediated

Fig. 2. Replacement of extracellular anions resulted in different I_{sc} values induced by naringenin. Representative I_{sc} recording with arrows indicating naringenin (100 μM) added basolaterally in different K-H solutions. HCO₃⁻ free K-H (A); Cl⁻ free K-H (B); Cl⁻/HCO₃⁻ free K-H (C); Comparison of the naringenin-induced total charges transferred in various bathing solutions (D). Values are the mean ± SEM (n = 4, *** P<0.001 vs. control).

by cAMP. After treatment with forskolin, an adenylate cyclase activator, was used to consume the intracellular adenylate cyclase, intracellular cAMP cannot be further elevated upon subsequent addition of cAMP-elevating agents. IBMX, an inhibitor of phosphodiesterase, is used to inhibit degradation of intracellular cAMP. Pretreatment with forskolin abolished the I_{sc} increase induced by naringenin (Fig. 6A). Meanwhile, the naringenin-induced I_{sc} was attenuated by pretreatment with MDL-12330A (Fig. 6B), an inhibitor of adenyl cyclase, whereas subsequent application of ATP (100 μM) caused an obvious increase. However, in the IBMX-induced I_{sc} , naringenin could further obviously increase the I_{sc} (Fig. 6C). Statistical analysis showed that the naringenin-induced I_{sc} was absolutely abrogated by pretreatment with forskolin (90.23%) and MDL-12330A (97.77%), but IBMX did not affect the I_{sc} induced by naringenin (Fig. 6D). In addition, in the naringenin-induced I_{sc} response, forskolin (Fig. 7A) and IBMX (Fig. 7C) obviously increased the I_{sc} . After naringenin pretreatment, the I_{sc} increase induced by forskolin

Fig. 3. Different concentrations of Ca²⁺ in Cl⁻ free K-H solutions resulted in the same I_{sc} induced by naringenin (100 μM). Comparison of the naringenin-induced total charges transferred in various bathing solutions. The values are the mean ± SEM (n = 4, *** P<0.001 vs. control).

Fig. 4. Effect of different Cl⁻ channel blockers and ATP on the naringenin-evoked I_{sc} in the rat airway. Pretreatment of tissues with DPC (1 mM) on the apical side, naringenin (100 μM) could not induce an I_{sc} increase (A). CFTRinh-172 (10 μM) applied to the apical side of airway tissue inhibited the naringenin-evoked I_{sc} , which was aroused by apical application of 100 μM ATP (B). The naringenin-evoked I_{sc} increases were not affected by pretreatment with DIDS (100 μM) on the tissue apical side, but they were completely inhibited by 10 μM CFTRinh-172 (C). Comparison of different Cl⁻ channel blockers inhibiting the naringenin-induced I_{sc} increases on the apical side (D). The values are the mean ± SEM (n = 4, *** P < 0.001 vs. control).

showed a significant decrease compared with the use of forskolin alone (Fig. 7B), while the IBMX-induced I_{sc} increase showed no significant difference with itself (Fig. 7D). The above results suggest that cAMP is of critical importance in mediating chloride secretion and regulating CFTR on the apical side. Naringenin modulates the activity of CFTR by increasing adenylyl cyclase activity rather than by directly inhibiting phosphodiesterase activity.

To rule out the possibility that naringenin acts on sub-epithelial interstitial cells of the airway to release a paracrine agent that alters the I_{sc} response of the airway epithelial cells, we supplemented the I_{sc} responses of Calu-3 cells with naringenin and forskolin addition (Fig. 8). The results showed that both naringenin and forskolin could increase I_{sc} , and the results were identical to the airway tissue experiment results. In the naringenin-induced I_{sc} response, forskolin obviously increased the I_{sc} , while on the basis of the forskolin-induced I_{sc} increase, naringenin could not further induce a change in the I_{sc} . These experiments suggested that the I_{sc} response of the airway to naringenin did not act on sub-epithelial interstitial cells of the airway but directly acted on epithelial cells to stimulate Cl⁻ secretion.

Before ELISAs, the primary cultured cells were authenticated as epithelial cells (Fig. 9). The data (Fig. 10) showed that the cAMP dependent pathway played a notable role in mediating the naringenin-induced response to regulate Cl⁻ secretion. Under basal conditions (0.1% DMSO), the intracellular cAMP content was 5.29 ± 3.74 pmol/mg protein (n = 3) in primary airway epithelial cells and 15.09 ± 3.12 pmol/mg protein (n = 3) in Calu-3 cells. After adding naringenin at a concentration of 100 μM, the cAMP levels were 34.55 ± 7.49 pmol/mg protein (n = 3) in primary airway epithelial cells and 45.56 ± 1.85 pmol/mg protein (n = 3) in Calu-3 cells. IBMX, a phosphodiesterase inhibitor, was used to assess whether cAMP degradation contributed to the increase in cAMP concentration. It caused an enhancement

Fig. 5. Effect of bumetanide and BaCl₂ on the naringenin-evoked I_{sc} in rat airway. After pretreating tissues with bumetanide (100 μM) on the basolateral side, naringenin (100 μM) could not induce I_{sc} increases (A). BaCl₂ (100 μM) applied to the basolateral side of the airway tissue inhibited the naringenin-evoked I_{sc} (B). Comparison of the naringenin-induced I_{sc} increases on the basolateral side inhibited by bumetanide and BaCl₂ (C). The values are the mean ± SEM (n = 4, *** P<0.001 vs. control).

Fig. 6. Effect of forskolin, MDL-12330A and IBMX on the naringenin-evoked I_{sc} in rat airway tissue. Pretreatment with forskolin (10 μM) reduced the naringenin-induced I_{sc} by 90.23% (A and D). Pretreatment with MDL-12330A (10 μM) reduced the naringenin-induced I_{sc} by 97.77% (B and D). Pretreatment with IBMX (100 μM) could not reduce the naringenin-induced I_{sc} increase, the increase of which showed no significant difference with using naringenin alone (C and D). The values are the mean ± SEM (n = 4, *** P<0.001 vs. control).

Fig. 7. I_{sc} response to naringenin followed by forskolin and IBMX addition on rat airway tissue. After pretreating tissues with naringenin (100 μ M), forskolin (10 μ M) could also induce I_{sc} increases (A). IBMX (100 μ M) applied to the airway tissue could not inhibit the naringenin-evoked I_{sc} (C). The comparison of I_{sc} increases between adding forskolin (10 μ M) (B), IBMX (100 μ M) (D) alone and naringenin (100 μ M) pretreatment. The values are the mean $\pm$ SEM ($n = 4$).

Fig. 8. Effect of naringenin and forskolin on I_{sc} response in Calu-3 cells. After cells were pretreated with naringenin (100 μ M), forskolin (10 μ M) could also induce I_{sc} increases (A). Pretreatment with forskolin reduced the naringenin-induced I_{sc} (B). The comparison of I_{sc} increases between naringenin (100 μ M) pretreatment and forskolin (10 μ M) pretreatment (C). The values are the mean $\pm$ SEM ($n = 4$).

Fig. 9. Labeling primary cultured cells with anti-smooth muscle antibody (SMA) (A and B) and anti-keratin antibody (C and D) confirmed the primary airway epithelial cells.

Fig. 10. Effect of naringenin on the endocellular cAMP level of rat primary airway epithelial cells (A) and Calu-3 cells (B). Contrast of the cAMP increase induced by naringenin (100 μ M), IBMX (100 μ M), and forskolin (10 μ M) with control. The values are the mean $\pm$ SEM ($n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. control).

in cAMP levels to 56.74 ± 4.31 pmol/mg protein ($n = 3$) in primary airway epithelial cells and 63.44 ± 7.97 pmol/mg protein ($n = 3$) in Calu-3 cells. In addition, incubation with naringenin (100 μ M) and IBMX (100 μ M) led to an obvious increase in cAMP levels to 84.65 ± 14.33 pmol/mg protein ($n = 3$, primary airway epithelial cells) and 81.28 ± 6.09 pmol/mg protein ($n = 3$, Calu-3 cells). Furthermore, IBMX (100 μ M) and forskolin (10 μ M) induced the highest increase in cAMP levels in primary airway epithelial cells (198.80 ± 66.47 pmol/mg protein ($n = 3$)) and Calu-3 cells (100.25 ± 4.78 pmol/mg protein ($n = 3$)), to reference the naringenin contribution.

Fig. 11. Calu-3 cell CFTR mRNA expression in the presence of naringenin. Transcription of CFTR was determined by RT-qPCR, using β -actin as a housekeeper gene. Calu-3 cells were treated without (A) or with (B) 10 μ g/ml LPS for 8 hours ($n = 3$, * $P < 0.05$, ** $P < 0.01$ vs. control group (A)/LPS-treated group (B)). Without LPS stimulation, the relative expression of CFTR mRNA was not changed. However, CFTR mRNA expression was down-regulated when the sample was treated with LPS and significantly up-regulated by naringenin. The data are presented as the mean $\pm$ SEM.

Effect of naringenin on CFTR mRNA expression

To evaluate differences in CFTR mRNA expression in response to concentration changes of naringenin (25 μ M, 50 μ M and 100 μ M), Calu-3 cells were treated for 8 hours. Compared with control cells, the qRT-PCR assay showed that naringenin incubation did not induce a significant change (Fig. 11A) at any concentration ($n = 3$, $p > 0.05$). In addition, after CFTR expression in Calu-3 cells was decreased by LPS stimulation, various concentrations of naringenin (25 μ M, 50 μ M and 100 μ M) were added to the cell medium, and the cells were incubated for 8 hours to detect the effect of naringenin on CFTR mRNA expression (Fig. 11B). When cells were treated with LPS (10 μ g/ml) for 8 hours, CFTR mRNA expression was decreased by 1.9-fold compared with the control ($n = 3$, $p < 0.01$). All concentrations of naringenin had positive effects on the mRNA expression of CFTR after cells were stimulated by LPS, but only 50 μ M and 100 μ M naringenin ($n = 3$, $p < 0.05$) had a significant difference in the LPS treatment group. Forskolin did not change the decrease in CFTR mRNA expression induced by LPS. MDL12330A did not inhibit the naringenin recovery of CFTR mRNA expression in the presence of LPS.

Discussion

The present study aimed to analyze the CFTR activation and expression regulated by naringenin and to develop an effective and comprehensive treatment strategy for sputum. CFTR, a potential primary pathway that regulates periciliary fluid balance [2] and inflammatory signaling [14, 15, 16] in airway epithelium, could potentially be used as a therapeutic target for sputum clinical therapies. Interest in the discovery of CFTR activators as potential small molecular targets has risen in recent years. Naringenin, a small molecule derived from plant-based compounds, has been generally used in respiratory diseases for relieving cough, expectoration and reducing lung infection [6, 17]. A previous study found that naringenin, a major metabolite of naringin, was able to stimulate cAMP-dependent Cl^- secretion across human colonic epithelia [12]. Apigenin, having a common 2-phenyl-g-benzopyron structure with naringenin, could activate CFTR on Calu-3 cells owing to its specific structural components [18]. Nobiletin, a citrus flavonoid, can stimulate CFTR-mediated Cl^- secretion by adenylate cyclase- and cAMP/PKA-dependent pathways across bronchial epithelia

[19]. Trimethoxyflavone and other flavones can also active CFTR both *in vitro* and *in vivo* [20]. It has been suggested that naringenin may regulate Cl⁻ secretion and fluid transport in cAMP-mediated CFTR function in airway epithelia. The current study initially proves that naringenin has the ability to trigger cAMP-dependent Cl⁻ secretion via CFTR transportation in airway epithelia and regulate CFTR expression in an LPS-induced CFTR down-regulation model *in vivo*, ultimately producing an expectorant effect.

To test whether naringenin has the ability to regulate CFTR activation, normal rat airway epithelium was used in the I_{sc} test. The present study showed that naringenin could dose-dependently stimulate a sustained increase in I_{sc} (Fig. 1), generating cAMP-dependent CFTR-mediated Cl⁻ secretion across rat airway epithelia. We used 100 μ M naringenin in subsequent experiments because it was the most suitable value that was close to the naringenin EC₅₀ value of $71.49 \pm 10.76 \mu$ M. At this concentration, naringenin significantly stimulated an increase in the short-circuit current value, thus facilitating the records and measurements of follow-up experiments. The Hill coefficient was 1.84, which was significantly greater than 1. This suggested that there was a positive cooperativity between naringenin molecules. After binding to the receptor, naringenin may improve the receptor binding affinity to other naringenin molecules. When Cl⁻ was eliminated from the bathing K-H solution, the naringenin-induced I_{sc} was greatly attenuated (Fig. 2), which was due largely to transmembrane Cl⁻ secretion from the apical side rather than HCO₃⁻ secretion and electrogenic Na⁺ absorption. Using a Cl⁻ channel blocker to screen apical Cl⁻ channels that were activated by naringenin showed that both the Cl⁻ channel blocker DPC and the CFTR inhibitor CFTRinh-172 could interdict the naringenin-induced I_{sc} , but the calcium-activated chloride channel (CaCC) blocker DIDS had no effect on it (Fig. 4). These results clearly established that naringenin has the ability to induce a continuous I_{sc} response by stimulating CFTR-mediated Cl⁻ secretion on the apical side.

Previous studies have shown that the Na⁺-K⁺-2Cl⁻ cotransporter provides the driving force for apical chloride secretion across epithelia. In addition, K⁺ channels also play an important role in this process to expel K⁺, which is transported by the Na⁺-K⁺-2Cl⁻ cotransporter from the cells to preserve K⁺ concentration stability [21]. The K⁺ channel blocker BaCl₂ and the Na⁺-K⁺-2Cl⁻ cotransporter inhibitor bumetanide were used to detect the naringenin-induced current on the basolateral membrane. As predicted, the increase of current caused by naringenin was sensitive to bumetanide and BaCl₂ (Fig. 5). These results indicate that the effect of naringenin on CFTR function is supported by Na⁺-K⁺-2Cl⁻ cotransporters and K⁺ channels.

It has been confirmed that the cAMP signaling pathway can regulate Cl⁻ transport across a wide range of tight epithelia. Cytosolic cAMP, a second messenger, regulates CFTR activation via protein kinase A (PKA) [21-24]. The adenylate cyclase activator forskolin stimulated the cAMP-dependent secretion, which generates a maximum current, while subsequent addition of naringenin could not further increase the current. In the naringenin-induced I_{sc} response, forskolin could also obviously increase the I_{sc} , while the increase was significantly lower than that observed when using forskolin alone. The difference between the increases was approximately equal to the value of the naringenin-induced I_{sc} . Pretreatment with an adenylate cyclase inhibitor (MDL12330A) had an inhibitory effect on the naringenin-induced I_{sc} , while pretreatment with a phosphodiesterase inhibitor (IBMX) did not affect the I_{sc} increase induced by naringenin. In addition, the I_{sc} increase of airway epithelia was not significantly different when adding IBMX before or after naringenin. These results suggest the possibility that the function of naringenin is similar to that of forskolin in the intracellular second messenger pathway, which can be impeded by MDL12330A, rather than the IBMX-associated pathway (Fig. 6 and 7). In addition, ELISA measurement demonstrated that cAMP production exhibited a noteworthy increase in primary rat airway epithelial cells and Calu-3 cells induced by naringenin. Simultaneously treating cells with naringenin and IBMX further enhanced the cAMP level when compared to naringenin-only treatment. Moreover, forskolin also further increased cAMP production compared with naringenin in the presence of IBMX (Fig. 10). These results showed that naringenin modulated the activity of CFTR by increas-

ing adenylate cyclase activity rather than by directly inhibiting phosphodiesterase activity. Naringenin could increase the cAMP content by promoting cAMP formation rather than by inhibiting cAMP degradation.

It is commonly known that Akt is a key mediator of signal transduction in CFTR synthesis [25, 26]. LPS can bind to Toll-like receptor 4 (TLR4) and phosphorylate Akt to activate the phosphoinositide3-kinase (PI3K)-Akt pathway, which results in down-regulation of CFTR expression [27, 28]. This regulation can be abrogated by LY294002 (a PI3K/Akt inhibitor) [28]. Calu-3 cells isolated from human airway adenocarcinoma have high expression of CFTR in the apical membrane and selectively secrete Cl^- to physiologically regulate serous secretion. Culturing Calu-3 cells with naringenin for 8 hours did not significantly increase the CFTR mRNA expression in any of the concentration tests. Compared with non-LPS treatment, an increase in CFTR expression was observed in the LPS-induced CFTR down-regulation model induced by naringenin stimulation. After LPS was incubated with Calu-3 cells for 8 hours, CFTR expression was significantly decreased, while naringenin dose-dependently enhanced the CFTR mRNA expression. Moreover, the middle and high dose of naringenin significantly increased CFTR expression. Relatively, forskolin had no effect on LPS-induced CFTR down-regulation in Calu-3 cells. Furthermore, MDL12330A could not inhibit the naringenin recovery of CFTR mRNA expression in the presence of LPS. (Fig. 11). The results suggest that naringenin exerts its expectorant function by increasing CFTR expression while organismal CFTR expression was seriously disrupted, but it has no effect on normal cells. However, the intracellular cAMP content did not influence the regulation of CFTR mRNA by naringenin. In the CFTR mRNA expression process, expression regulation is affected by many factors, such as the abundance of miR-138 (a type of microRNA) [29], activation of NF- κ B [30], activation of JAK2 via the JAK/STAT pathway [31] and activation of vasopressin [32]. In our study, naringenin increased the intracellular cAMP content, which was what forskolin did. However, separate administration of naringenin and forskolin failed to affect the expression of CFTR mRNA, indicating that the elevation of the cAMP content could not regulate the expression of CFTR mRNA. We speculated that naringenin suppressed LPS-induced down-regulation of CFTR mRNA through other signaling pathways, although the underlying mechanism needs further investigation. In brief, naringenin can both modulate the activity of CFTR via the cAMP pathway and rehabilitate CFTR expression when CFTR expression in the organism is suppressed.

The bioavailability of naringenin in rats was 39.8%. When rats were orally administered naringenin at a dose of 350 mg/kg, the plasma concentration of the drug reached a maximum value of 45200 ng/ml (166 μM) after 4 hours [33]. It was not the minimum effective concentration of naringenin used for *in vivo* experiments. In our *in vivo* expectorant experiments, naringenin could enhance tracheal phenol red output when rats were orally administered naringenin above a dose of 30 mg/kg [6]. Correspondingly, according to previous experimental results, the maximum plasma concentration value was 14.23 μM . The dose-response curve showed that naringenin stimulated Cl^- secretion when the concentration was higher than 20 μM , which was in close proximity to 14.23 μM . This result confirmed the reliability of the *in vivo* experiment. However, the current study cannot provide a distribution ratio of naringenin in airway tissue, and therefore, a more accurate oral dosage cannot be calculated. Moreover, the above dosage is calculated on the basis of the maximum value, which neglects the maintenance period of the concentration in airway tissue, and thus, this only serves as a reference. In subsequent studies, we will analyze the drug efficacy curve and pharmacodynamic data to make a more reasonable clinical dose target for airway tissue.

Conclusion

In conclusion, this study indicated that naringenin has a potent effect on CFTR expression in an LPS-induced CFTR down-regulation model, which is supported by Na^+ - K^+ -2 Cl^- co-transporters and K^+ channels on the basolateral membrane and simultaneously regulated

by intracellular cAMP. The results indicate the mechanism by which naringenin provides a potential treatment strategy for sputum.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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