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Luteolin inhibited the gene expression, production and secretion of MUC5AC mucin via regulation of nuclear factor kappa B signaling pathway in human airway epithelial cells

Hyun Jae Lee [1], Hyo-Seok Seo [1], Jiho Ryu, Yong Pill Yoon, Su Hyun Park, Choong Jae Lee*

Department of Pharmacology, School of Medicine, Chungnam National University, Daejeon, Republic of Korea

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ABSTRACT

Luteolin, a flavonoidal compound derived from *Lonicera japonica* Thunb. and *Chrysanthemum indicum* L., has been reported to show anti-inflammatory, anti-oxidative and anti-carcinogenic effects. In this study, we investigated whether luteolin significantly affects the secretion, production and gene expression of airway mucin. Confluent NCI-H292 cells were pretreated with luteolin for 30 min and then stimulated with EGF (epidermal growth factor) or PMA (phorbol 12-myristate 13-acetate) for 24 h or the indicated periods. The MUC5AC mucin gene expression was measured by RT-PCR. Production and secretion of MUC5AC mucin protein were measured by ELISA. To elucidate the action mechanism of luteolin, effect of luteolin on PMA-induced NF-κB signaling pathway was investigated by western blot analysis. The results were as follows: (1) Luteolin inhibited the secretion of MUC5AC mucin protein induced by EGF or PMA; (2) Luteolin inhibited the production of MUC5AC mucin protein and the expression of MUC5AC mucin gene induced by EGF or PMA; (3) Luteolin inhibited PMA-induced phosphorylation and degradation of inhibitory kappa Bα (IκBα); (4) Luteolin inhibited PMA-induced phosphorylation and nuclear translocation of nuclear factor kappa B (NF-κB) p65. This result suggests that luteolin can regulate the secretion, production and gene expression of mucin by acting on airway epithelial cells via regulation of NF-kB signaling pathway.

1. Introduction

Airway mucus plays pivotal role in defense against invading pathogenic microorganisms, chemicals and particles. The protective function of airway mucus is attributed to the viscoelasticity of mucins. Mucins are multimillion dalton glycoproteins present in the airway mucus and produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, any abnormality in the quality or quantity of mucins not only cause altered airway physiology but may also impair host defenses often leading to severe airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis [1]. Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excessive mucin secretion (production) by the compounds derived from various medicinal plants. We have tried to investigate the possible activities of some natural products on mucin secretion from cultured airway epithelial cells. As a result of our trial, we previously reported that several natural products affected mucin secretion and/or production from airway epithelial cells [2–6]. According to many reports, luteolin (Fig. 1) [7], a flavonoidal compound derived from *Lonicera japonica* Thunb. and *Chrysanthemum indicum* L., has been reported to show anti-inflammatory, anti-oxidative and anti-carcinogenic effects [7–10]. However, to the best of our knowledge, there are no reports about the potential effects of luteolin on gene expression, production and secretion of mucin from airway epithelial cells. Therefore, we examined the effect of luteolin on EGF- or PMA-induced MUC5AC mucin gene expression, production and secretion from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating intracellular signaling pathways involved in airway mucin production and gene

Abbreviations: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; RT-PCR, reverse transcription - polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa B; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IκBα, inhibitory kappa B alpha; IKK, inhibitory kappa B kinase.

* Corresponding author. Department of Pharmacology, School of Medicine, Chungnam National University, 6 Munhwa-Dong, Joong-Gu, Daejeon, Republic of Korea. Tel.: +82 42 580 8255; fax: +82 42 585 6627.
E-mail address: LCJ123@cnu.ac.kr (C.J. Lee).

[1] These two authors equally contributed to this work.

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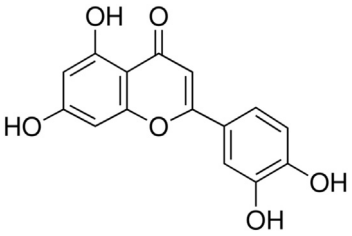

Fig. 1. Chemical structure of luteolin.

expression [11–13]. Also, in order to elucidate the action mechanism of luteolin, we checked whether luteolin affects PMA-induced NF-κB signaling pathway in NCI-H292 cells.

2. Materials and methods

2.1. Materials

All the chemicals and reagents used in this experiment including luteolin (purity: 95.0%) were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified. Anti–NF–κB p65, anti-IkBα, anti-actin and anti-p84 antibodies were purchased from Santacruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho–specific anti-p65 (serine 536) and phospho-specific anti-IκBα (serine 32/36) antibodies were purchased from Cell signaling Technology Inc. (Danvers, MA, U.S.A.).

2.2. NCI-H292 cell culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100 μg/mL) and HEPES (25 mM) at 37 °C in a humidified, 5% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% fetal bovine serum for 24 h.

2.3. Treatment of cells with luteolin

After 24 h of serum deprivation, cells were pretreated with varying concentrations of luteolin for 30 min and treated with EGF (epidermal growth factor) (25 ng/mL) or PMA (phorbol 12-myristate 13-acetate) (10 ng/mL) for 24 h in serum-free RPMI 1640. Luteolin was dissolved in dimethylsulfoxide and treated in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulfoxide did not affect mucin gene expression, production and secretion from NCI-H292 cells. After 24 h, the spent media were collected to measure the secretion of MUC5AC protein and 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, U.S.A.) and collected to measure the production of MUC5AC protein (in 24-well culture plate). The total RNA was extracted for measuring the expression of MUC5AC gene (in 6-well culture plate) by using RT-PCR.

2.4. MUC5AC mucin analysis

MUC5AC airway mucin production and secretion were measured by ELISA. Spent media and cell lysates were prepared with PBS at 1:10 dilution, and 100 μL of each sample was incubated at 42 °C in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, U.S.A.), 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 (1:3000) 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.

2.5. Total RNA isolation and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc. Kyung-gi-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μL (RT reaction). 2 μL of RT reaction product was PCR amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, U.S.A.). 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′. As quantitative controls, primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used. 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.

2.6. Protein extraction and western blot analysis

NCI-H292 cells (confluent in 150 mm culture dish) were pretreated for 24 h at 37 °C with 20 μM of luteolin and then stimulated with PMA (50 ng/mL) for the indicated periods. After the treatment of the cells with luteolin, the cells were harvested using 3× trypsin-EDTA solution and then centrifuged in a microcentrifuge (1200 rpm, 3 min, 4 °C). The supernatant was discarded and the cell pellet was washed by suspending in PBS. The cytoplasmic and nuclear protein fraction were extracted using NE-PER® nuclear and cytoplasmic extraction reagent (Thermo-Pierce Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions. Both extracts were stored at −20 °C. For obtaining the whole cell lysates, after the treatment of the cells with luteolin, media were aspirated and the cells were washed with cold PBS. The cells were collected by scraping and centrifuged at 3000 rpm for 5 min. The supernatant was discarded. The cells were mixed with RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min with continuous agitation. The lysate was centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. The supernatant was used or immediately stored at −80 °C. Protein content in extract was determined by Bradford method. Cytosolic, nuclear and whole cell extracts containing proteins (each 50 μg as protein) were subjected to 10% SDS-PAGE (polyacrylamide gel electrophoresis) and then transferred onto the PVDF membrane. The blots were blocked using 5% skim milk in Tris-buffered saline/Tween 20 (TBS-T) and probed with appropriate primary antibodies in blocking buffer overnight at 4 °C. The membrane was washed with TBS-T and then probed for 1 h with the secondary antibodies conjugated with horseradish peroxidase (Calbiochem, CA, U.S.A.). After four intensive washes with TBS-T,