European Journal of Pharmacology 690 (2012) 207–213

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

Naringin attenuates EGF-induced MUC5AC secretion in A549 cells by suppressing the cooperative activities of MAPKs-AP-1 and IKKs-IκB-NF-κB signaling pathways

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ARTICLE INFO

Article history:
Received 25 March 2012
Received in revised form
20 June 2012
Accepted 20 June 2012
Available online 2 July 2012

Keywords:
Naringin
MUC5AC
Epithelium growth factor
Epithelium growth factor receptor
Nuclear factor kappa B
Activator protein-1

ABSTRACT

Naringenin, the aglycone of naringin, has been reported to attenuate MUC5AC secretion by inhibiting activity of nuclear factor kappa B (NF-κB) via EGFR-PI3K-Akt/ERK MAPKinase signaling pathways. However, previous studies demonstrated that the MUC5AC promoter was located in two different regions: an activator protein-1 (AP-1) binding site and a NF-κB binding site. The current study comprehensively determined the involvement of MAPKs/AP-1 and IKKs/IκB/NF-κB in epidermal growth factor (EGF)-induced A549 cells, and sought to ascertain the signaling pathways of naringin imparted in suppression of EGF-induced MUC5AC secretion. The results showed that naringin of 100 μM not only significantly decreased EGF-induced overexpressions of both MUC5AC mucin and mRNA in A549 cells, but also suppressed the phosphorylation of EGF receptor, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK), as well as nucleus NF-κB p65 and AP-1. Moreover, any of three MAPKs inhibitors (PD98059, SB203580, and SP600125) significantly inhibited EGF-induced MUC5AC secretion. And as compared to MG132, the inhibitor κB (IκB) phosphorylation inhibitor of SN50 was more effective in reducing EGF-induced MUC5AC secretion because of suppression of nucleus AP-1. Meanwhile, as compared to naringin, both SP600125 and azithromycin were less effective in suppressing EGF-induced secretion of MUC5AC because of the unchanged nucleus NF-κB p65. These results indicated that naringin attenuates EGF-induced MUC5AC secretion in A549 cells by suppressing the cooperative activities of MAPKs/AP-1 and IKKs/IκB/NF-κB signaling pathways.

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1. Introduction

Naringin, a bioflavonoid derived from Chinese Herbal *Citrus grandis* 'Tomentosa', has been reported to possess anti-inflammatory (Liu et al., 2011; Kanno et al., 2006) and anti-oxidant effects (Jeon et al., 2001). Our previous studies showed that naringin not only dose-dependently decreased lipopolysaccharide-induced mucin secretion in cultured rat tracheal (Lin et al., 2008), but also blockaded activity of nuclear factor kappa B (NF-κB) p65 by inhibiting the phosphorylation of inhibitor κB-α (IκB-α), which then significantly decreased lipopolysaccharide-induced tumor necrosis factor-alpha (TNF-α) secretion (Liu et al., 2011). Moreover, it is reported that naringenin, the aglycone of naringin, attenuated human neutrophil elastase (HNE)-induced MUC5AC secretion by inhibiting activity of NF-κB via EGFR-PI3K-Akt/ERK MAPKinase signaling pathways (Yang et al., 2011). However, the existing results cannot fully reveal the mechanism of naringin on suppression of MUC5AC secretion. Previous studies demonstrated that the MUC5AC promoter was located in two different regions: an activator protein-1 (AP-1) binding site and a NF-κB binding site (Chen et al., 2004; Araki et al., 2010). Thus, it is necessary to investigate the role of naringin on regulation of NF-κB and AP-1 that imparted in expression of MUC5AC.

NF-κB is a transcription factor that plays a central role in the onset of inflammation and tumor progression (Lixuan et al., 2010; Shi et al., 2009). It is kept as an inactive form in the cytoplasm through interaction with IκB proteins (IκB-α, -β, and -ε) that causes its localization to the cytoplasm and prevents its association with DNA. The activation of NF-κB occurs mainly through the phosphorylation of IκB by inhibitor κB kinases (IKKs) and degradation by proteasome, which leading to the NF-κB p65:p50 heterodimer translocation to the nucleus and bind DNA (Kida et al., 2005).

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http://dx.doi.org/10.1016/j.ejphar.2012.06.040

On the other hand, AP-1 is another transcriptional activator of MUC5AC, which is composed of homo- and heterodimers of c-Jun and c-Fos proteins (Shen et al., 2008). Previous studies showed that AP-1 was activated by principal MAPK family members, such as extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), via enhancing their downstream transcription factors including Elk-1, c-Jun, ATF2 and CREB, which in turn regulated the expression of c-Fos and c-Jun, which were the components of the AP-1 complex (Su et al., 2011).

Epidermal growth factor (EGF) receptor has a C-terminal tail that contains specific tyrosine containing sequences, which was phosphorylated by tyrosine kinase after ligand such as EGF binding to the EGF receptor dimerization (Nadel and Burgel, 2001). Previous studies have revealed that three MAPKs such as ERK1/2, p38 MAPK, and JNK, as well as PI3K-Akt were the downstream signaling cascades of EGF receptor, which activated the expression of MUC5AC would through the cooperativity between NF-κB and AP-1(Grandis and Sok, 2004; Xu and Shu, 2007; Hurd and Rozengurt, 2001; Shen et al., 2010).

In the present study, we comprehensively determined the involvement of MAPK/AP-1 and IKK/IκB/NF-κB in EGF-induced A549 cells, and sought to ascertain the signaling pathways of naringin imparted in suppression of EGF-induced MUC5AC secretion.

2. Materials and methods

2.1. Drugs and solutions

Naringin was extracted by our laboratory (extracted from *Citrus grandis* 'Tomentosa' by water, deposited in ethanol, with concentrated filtrate obtained after one to ten times of recrystallization, purity $>98.3\%$, determined by peak area normalization). EGF, PD98059, SP600125, SB203580, SN50, MG132, azithromycin, β-actin, HRP-anti-rabbit monoclonal antibody, and HRP-anti-mouse monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against EGF receptor (Ab-1092), P-EGF receptor (Phospho-Tyr1092), ERK1/2 (Ab-202), P-ERK1/2 (Phospho-Thr202), JNK (Ab-183), P-JNK (Phospho-Thr183) were purchased from SAB (Pearland, TX, USA). Antibodies against P-p38 MAPK (Phospho-Tyr182), p38 MAPK (Ab-Tyr182), AP-1, and NF-κB p65 were obtained from Cell Signaling (Beverly, MA, USA).

2.2. Cell culture

A549 cells (human lung adenocarcinoma cell line) were friendly provided by Sun Yat-sen University Cell Center, which were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with RPMI-1640 medium (Gibco BRL, Gland Island, NY) which supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad CA, USA), 100 μg/ml streptomycin (Invitrogen, Carlsbad CA, USA), and 100 U/ml penicillin (Invitrogen, Carlsbad CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell viability assay

Cell viability was evaluated by a conventional 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipenyl tetrazolium bromide (MTT) reduction assay. Briefly, A549 cells were plated at a density of 10^6 cells/ml in 96-well plates for 24 h, and then exposed to various concentrations of naringin, PD98059, SB203580, SP600125, SN50, MG132, or azithromycin, which were dissolved into serum-free RPMI-1640 medium. After incubation for predetermined times up to 96 h, the cells were incubated in MTT solution (0.5 mg/ml) for an additional 4 h at 37 °C. After washing each well with PBS for 3 times, the colored formazan was dissolved in 100 μl of DMSO. The absorption values were read at 570 nm in a microplate reader (Bio-Rad, Hercules CA, USA), and the cell viability of the DMSO vehicle control group was set at 100%. Each assay was performed in triplicate.

2.4. ELISA for MUC5AC mucin expression in A549 cells

A549 cells were plated at a density of 10^6 cells/ml in 25 cm^2 flasks and proliferated for 48 h with daily replacement of fresh standard medium, and then pretreated with various concentrations of naringin, PD98059, SB203580, SP600125, SN50, MG132, or azithromycin solutions (3 ml, dissolved in serum-free RPMI 1640 medium). 1 h later, A549 cells were dealt with EGF of 10 ng/ml for predetermined times up to 24 h. Then content of MUC5AC in cell culture supernatant and cell lysate were measured by ELISA according to the protocol of Human MUC5AC ELISA kit (USCNK, Wuhan, China). The protein in cell lysate and nucleus was collected followed the method described previously (Su et al., 2011).

2.5. Real-time rt-PCR for MUC5AC mRNA expression in A549 cells

Total cellular RNA from A549 cells was extracted using Trizol kit (Invitrogen, Carlsbad CA, USA), quantified by Thermo BioMate3 and diluted into 1 mg/ml. cDNA was then synthesized using an oligo (dT) 15 primer and SuperScript™ II reverse transcriptase reagents (TaKaRa, Dalian, China) according to the manufacturer's protocol. Primers for MUC5AC (forward: 5′-CTG AGG GTC TCA GGA ATG ACG C-3′; reverse: 5′-TTT ATG CAA CAG ATT GGC CGT G-3′) were based on published mRNA sequences (GenBank Data Library accession numbers: AJ001402-MUC5AC), and β-actin (forward: 5′-CCT GTA CGC CAA CAC AGT GC-3′; reverse: 5′-ATA CTC CTG CTT GCT GAT CC-3′). The primers were synthesized by Invitrogen (Guangzhou, China), which designed at least two exons in order to avoid binding to genomic DNA. Real-time PCR was performed using the qSYBR Green PCR Kit (Dongsheng, Guangzhou, China) with the cycling conditions as follows: 1 min at 94 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 45 s. The identity and purity of PCR products were assessed by melting curve analysis. The relative quantity of MUC5AC mRNA was obtained using a comparative cycle threshold method and was normalized using β-actin as an endogenous control.

2.6. Western-blotting analysis

Protein (20 μg) in cell lysate or nucleus was separated by SDS-PAGE electrophoresis, and then transferred onto a nitrocellulose membrane. After blocking into 1% bovine serum albumin for 1 h, the membrane was incubated with antibodies (diluted 1:1000) against P-EGF receptor, EGF receptor, P-p38 MAPK, p38 MAPK, P-ERK1/2, ERK1/2, P-JNK, JNK, NF-κB p65, AP-1 and β-actin, respectively, on an oscillator at 37 °C for 2 h. After washing 3 times with PBS, the membrane was dealt with HRP-anti-mouse or HRP-anti-rabbit monoclonal antibody (diluted 1:1000) on an oscillator at 37 °C for 1 h. After removing the secondary antibody, the membrane was washed at least 5 times with PBS, infiltrated by BeyoECL Plus (Beyotime, Shanghai, China), and then developed in the ECL western detection reagents at last.

2.7. Statistical analysis

Data of MTT, ELISA, and real-time rt-PCR were expressed as mean $\pm$ S.E.M, while data of western-blotting were expressed as mean $\pm$ S.D. A significant difference between normal control and

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