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Emodin Inhibited MUC5AC Mucin Gene Expression via Affecting EGFR-MAPK-Sp1 Signaling Pathway in Human Airway Epithelial Cells

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Abstract

The aim of this study was to evaluate emodin, a natural trihydroxyanthraquinone compound found in the roots and barks of several plants including rhubarb and buckthorn, might attenuate epidermal growth factor (EGF)-induced airway MUC5AC mucin gene expression. The human pulmonary mucoepidermoid NCI-H292 cells were pretreated with for 30 min and then stimulated with EGF for the following 24 h. The effect of emodin on EGF-induced mitogen-activated protein kinase (MAPK) signaling pathway was examined. As a result, emodin blocked the expression of MUC5AC mucin mRNA and production of mucous glycoprotein via suppressing the phosphorylation of EGF receptor (EGFR), phosphorylation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1 and 2 (MEK1/2), phosphorylation of p38 MAPK, phosphorylation of ERK 1/2 (p44/42), and the nuclear expression of specificity protein-1 (Sp1). These findings imply that emodin has a potential to mitigate EGF-stimulated mucin gene expression by inhibiting the EGFR-MAPK-Sp1 signaling pathway, in NCI-H292 cells.

Key Words: Emodin, MUC5AC, EGFR, Sp1

INTRODUCTION

Mucus present in the luminal surface of respiratory tract is a thin layer of gels containing water, ions, and various molecules. It exerts anti-oxidative and anti-microbial effects. The intricate role of mucus in the pulmonary system underscores the importance of regulating its production and secretion for maintaining respiratory health. While mucus serves as a protective barrier against various harmful agents, dysregulation in its quantity or quality can contribute to a multitude of respiratory illnesses like bronchiectasis, chronic bronchitis, cystic fibrosis, and asthma. Mucous glycoproteins, called as mucins, are the major macromolecules assigning the viscoelasticity to mucus. Among the diverse subtypes of human mucins, MUC5AC mucin plays a pivotal role as the major gel-forming pulmonary mucin (Allinson *et al*., 2016; Mann *et al*., 2022; Kim *et al*., 2023a; Ryu *et al*., 2023).

Traditional approaches for controlling mucus production and secretion include the use of agents like hypertonic saline solution, S-carboxymethyl cysteine, 2-mercaptoethane sulfonate sodium, bromhexine, erdosteine, thymosin β-4, glyceryl guaiacolate, ambroxol, N-acetyl L-cysteine (NAC), mannitol dry powder, azithromycin, dornase alfa, letocysteine, myrtol, and sobrerol. However, these treatments may come with drawbacks such as rebound over-secretion of mucus or irritation of the respiratory tract lining (Li *et al*., 2020). Given these challenges, there is a prominent need for novel agents that can more effectively regulate mucin biosynthesis and/or degradation. A multitude of study suggests a potential in regulating abnormal secretion and/or production of MUC5AC mucin in pulmonary diseases, using natural products isolated from medicinal plants historically used for treating inflammation.

Over the past two decades, numerous natural products have been investigated for their effects on MUC5AC mucin

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gene expression, as reported in diverse studies (Kim *et al*., 2012; Ryu *et al*., 2013, 2014; Seo *et al*., 2014; Sikder *et al*., 2014; Lee *et al*., 2015; Kim *et al*., 2016; Li *et al*., 2020; Hossain *et al*, 2022a, 2022b; Kim *et al*., 2023a; Ryu *et al*., 2023). This research direction presents an opportunity for the development of novel therapeutic candidates aimed at controlling airway mucin secretion and production, potentially offering new opportunities for managing inflammatory pulmonary diseases. The exploration of natural products as potential treatments underscores the importance of leveraging traditional knowledge alongside modern scientific methods in drug discovery and development (Hossain *et al*, 2022a, 2022b; Huh *et al*., 2023; Hwang *et al*., 2023; Jang *et al*., 2023; Kim *et al*., 2023a, 2023c; Ko *et al*., 2023; Lee *et al*., 2023b; Ryu *et al*., 2023).

According to the literature, emodin, a trihydroxyanthraquinone derived from the roots and barks of several plants including rhubarb and buckthorn as well as molds and lichens, was reported to play a wide range of roles as a tyrosine kinase inhibitor (antineoplastic agent), laxative, diuretic, antibacterial, antimalarial, antiallergic, antiulcer, anti-inflammatory agents. With its potential anti-inflammatory effect, emodin suppresses airway hyperreactivity in mouse asthma animal model using mouse by mitigating acidic mammalian chitinase, chitinase 3-like protein 4, and MUC5AC mRNA expression *in vivo* (Chu *et al*., 2012).

However, there is no report about the activity of emodin on the gene expression of mucin in airway epithelial cells and the intracellular signaling pathway involved into the activity. Thus, in the present study, we investigate the potential activity of emodin on mRNA expression and glycoprotein production of pulmonary MUC5AC mucin stimulated by EGF in NCI-H292 cells. NCI-H292 cells, a human pulmonary mucoepidermoid cell line, is appropriate given their relevance to studying intracellular signaling pathways involved in pulmonary mucin gene expression. EGF stimulates the epidermal growth factor receptor (EGFR) signaling pathway, a major regulatory mechanism for airway mucin production, prompting investigation into whether emodin affects EGF-induced EGFR-MAPK signaling pathway, in airway epithelial NCI-H292 cells (Perrais *et al*., 2002; Shao *et al*., 2003; Evans *et al*., 2009; Le Cras *et al*., 2011; Simoes *et al*., 2019; Chen *et al*., 2021). Examining whether emodin can modulate the activation of the EGFR-MAPK signaling pathway stimulated by EGF in NCI-H292 cells could shed light on its potential therapeutic effects in pulmonary diseases. Understanding how emodin interacts with this intracellular signaling pathway could provide valuable insights into its mechanism of action and its potential as a treatment for mucin-related airway diseases.

MATERIALS AND METHODS

Materials

All the chemicals including emodin (purity: 98.0%) (Fig. 1) used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Anti-phospho-EGFR (Y1068) (#3777), anti-EGFR, anti-phospho-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 (S221) (#2338), anti-MEK1/2, anti-phospho-p44/42 MAPK (T202/Y204) (#4370), anti-p44/42 MAPK, anti-phospho-p38 MAPK (T180/Y182) (#4511), and anti-p38 MAPK antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-specificity protein-1 (Sp1) (sc-420) and anti-β-actin (sc-8432) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nuclear matrix protein p84 (ab-487) was purchased from Abcam (Cambridge, MA, USA). Either Goat Anti-rabbit IgG (#401315) or Goat Anti-mouse IgG (#401215) was used as the secondary antibody and purchased from Calbiochem (Carlsbad, CA, USA).

NCI-H292 cell culture

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 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.

Treatment of cells with emodin

After 24 h of serum deprivation, cells were pretreated with 0.1, 0.2, 0.5, and 1.0 μM of emodin for 30 min and then treated with EGF (25 ng/mL) for 24 h in serum-free RPMI 1640. Emodin 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, activity and expression of molecules involved in EGFR-MAPK 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 glycoprotein (in a 24-well culture plate). The total RNA was extracted in order to measure the expression of MUC5AC mRNA (in a 6-well culture plate) using RT-PCR. For the western blot analysis, cells were treated with emodin for 24 h, followed by EGF treatment for 15 min for upstream signal proteins (EGFR, MEKs, ERKs, and p38 MAPK) and 60 min for Sp1.

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,

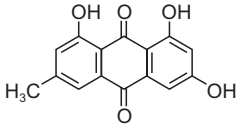

Fig. 1. Chemical structure of emodin.

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 0.1, 0.2, 0.5, and 1.0 μM of emodin, and then stimulated with EGF (25 ng/mL) for 15 min, in serum-free RPMI 1640. After the treatment of the cells with emodin, media were aspirated, and the cells were 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 emodin 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) (Kim *et al*., 2023b; Lee *et al*., 2023a; Yoon *et al*., 2023).

Computational analysis

The protein structures of EGFR, MEK1/2, ERK1/2, and p38 MAPK were determined using the Swiss model. Protein templates were selected based on BLAST analysis using the NCBI BLAST program, and protein sequences were retrieved from UniProt (https://www.uniprot.org/) (Uniprot consortium, 2023). The homology models were validated using PROCHECK. Molecular docking studies were conducted to examine the binding interactions of emodin with these specific proteins. The 3D structure of emodin (PubChem ID: 3220) was obtained in sdf file format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed 04 Jan 2024) (Kim *et al*., 2023d). All ligands' internal energies were optimized using the Chem3D Pro 12.0 computational program. Molecular docking results provided insights into the degree of ligand interaction with the active sites of the macromolecules. The active binding sites of the selected proteins were investigated within the initial protein grids (40×40×40) using PyMOL, AutoDock Vina, and Drug Discovery Studio (v.20.1.0.19295) (Morris *et al*., 2009; Trott and Olson, 2010).

Statistics

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.

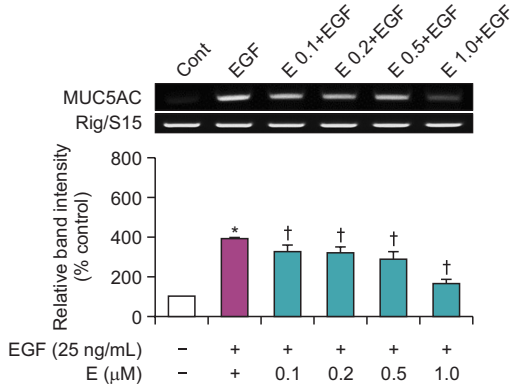

Fig. 2. Effect of emodin on EGF-induced MUC5AC mucin mRNA expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of emodin for 30 min and then stimulated with EGF (25 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin mRNA expression using RT-PCR. Three independent experiments were performed, and the representative data were shown. *Significantly different from control ($p<0.05$). †Significantly different from EGF alone ($p<0.05$). cont: control, E: emodin, concentration unit is μM.

RESULTS

Effect of emodin on EGF-induced MUC5AC mRNA expression and glycoprotein production of MUC5AC mucin

MUC5AC mRNA expression induced by EGF was inhibited by pretreatment with emodin (Fig. 2). Emodin suppressed EGF-induced MUC5AC mucin glycoprotein biosynthesis (production), in a dose-dependent fashion. The amounts of MUC5AC mucin in the cells of emodin-treated cultures were 100 ± 2% (control), 490 ± 7% (25 nM of EGF alone), 428 ± 2% (EGF plus emodin 0.1 μM), 411 ± 4% (EGF plus emodin 0.2 μM), 341 ± 7% (EGF plus emodin 0.5 μM), and 326 ± 9% (EGF plus emodin 1.0 μM), respectively (Fig. 3). Cytotoxicity was checked by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and there was no cytotoxic effect of emodin, at 0.1, 0.2, 0.5, or 1 μM concentration (Supplementary Fig. 1).

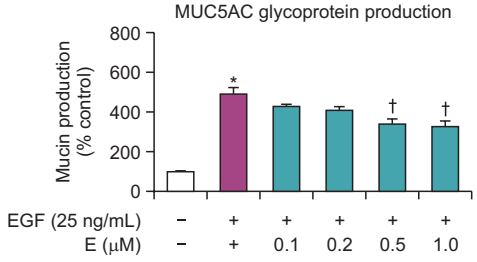

Fig. 3. Effect of emodin on EGF-induced MUC5AC mucin glycoprotein production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of emodin for 30 min and then stimulated with EGF (25 ng/mL), for 24 h. Cell lysates were collected for measurement of MUC5AC mucin glycoprotein production by ELISA. Each bar represents a mean ± SEM of 3 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 EGF alone (p<0.05). cont: control, E: emodin, concentration unit is μM.

Effect of emodin on the phosphorylation of EGFR

EGFR signaling cascade (Ras/Raf/MAPK) is recognized to be one of the major regulatory pathways for MUC5AC mucin production. EGF (25 ng/mL, 24 h) stimulated the phosphorylation of EGFR, whereas emodin inhibited EGF-stimulated phosphorylation of EGFR, as can be seen by western blot analysis in Fig. 4.

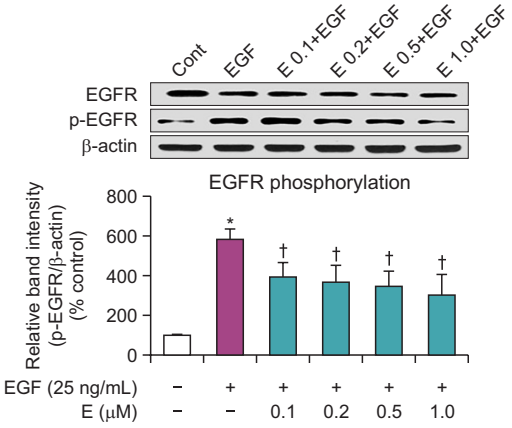

Fig. 4. Effect of emodin on the phosphorylation of EGFR in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of emodin for 24 h and then stimulated with epidermal growth factor (EGF) (25 ng/mL) for 15 min. Whole cell extract was collected and western blot analysis of the cellular proteins with anti-phospho-EGFR (Y1068) antibody was conducted. Three independent experiments were performed and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from EGF alone (p<0.05). cont: control, E: emodin, EGFR: EGF receptor, concentration unit is μM.

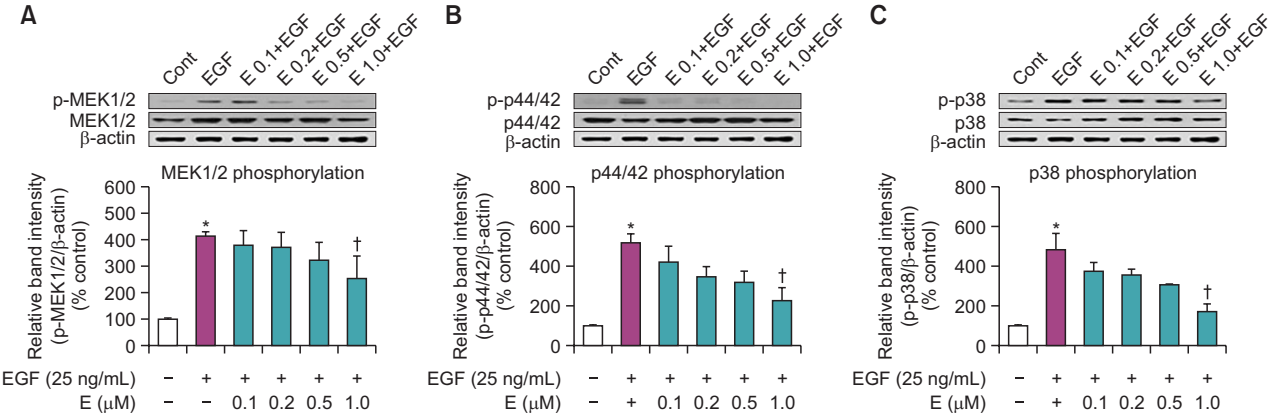

Fig. 5. Effect of emodin on the phosphorylation of MEK1/2, the phosphorylation of p44/42, and the phosphorylation of p38, in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of emodin for 24 h and then stimulated with epidermal growth factor (EGF) (25 ng/mL) for 15 min. Whole cell extract was collected and western blot analysis of the cellular proteins with anti-phospho-MEK1/2 (Ser221) (A), anti-phospho-p44/42 (Thr202/Tyr204) (B), and anti-phospho-p38 (Thr180/Tyr182) (C) antibodies was conducted. Three independent experiments were performed and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from EGF alone (p<0.05). cont: control, E: emodin, concentration unit is μM.

Effect of emodin on the phosphorylation of MAPK signaling proteins

To elucidate the effect of emodin on the downstream signaling, we investigated a potential activity of emodin on MEK1/2, ERK1/2, and p38 MAPK pathway, which are directly involved into MUC5AC mucin gene expression. EGF stimulated the phosphorylation of MEK1/2, whereas emodin suppressed the phosphorylation MEK1/2, in NCI-H292 cells (Fig. 5A). EGF stimulated the phosphorylations of p44/42 and p38, whereas emodin suppressed the phosphorylations of p44/42 (ERK1/2) and p38 MAPK (Fig. 5B, 5C), as shown by western blot analysis.

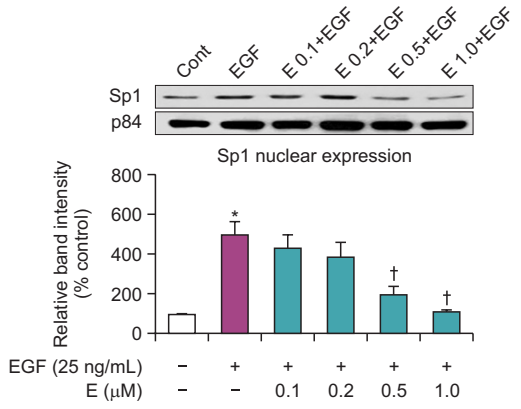

Fig. 6. Effect of emodin on the nuclear expression of Sp1 in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of emodin for 24 h and then stimulated with epidermal growth factor (EGF) (25 ng/mL) for 60 min. The nuclear protein extracts were prepared and subjected to western blot analysis using antibody against Sp1. As a loading control, p84 levels were analyzed. Three independent experiments were performed and the representative data were shown. *Significantly different from control (p<0.05). †Significantly different from EGF alone (p<0.05). cont: control, E: emodin, Sp1: specificity protein-1, concentration unit is μM.

Effect of emodin on the nuclear expression of Sp1

As a transcription factor from Sp family, Sp1 is an essential mediator in EGF-stimulated MUC5AC mucin gene expression. After activation of ERK signal, EGF upregulated nuclear expression of Sp1 and this transcriptional factor binds to DNA interacting site for MUC5AC mucin gene expression. In the present study, EGF stimulated the nuclear expression of Sp1, whereas emodin suppressed the nuclear expression of Sp1 (Fig. 6). This, in turn, led to the down-regulation of the production of MUC5AC mucin glycoprotein, in NCI-H292 cells.

Molecular interactions of emodin with EGFR and MAPK proteins

Homology models of EGFR (UniProt accession ID: P00533), MEK1 (UniProt accession ID: Q13233), MEK2 (UniProt accession ID: P36507), ERK1 (UniProt accession ID: P27361), ERK2 (UniProt accession ID: P28482), and p38 MAPK (UniProt accession ID: Q16644) were generated using the Swiss model. Molecular docking studies revealed that emodin exhibited strong binding interactions with EGFR, MEK1/MEK2, ERK1/ERK2, and p38 MAPK proteins, with binding affinities ranging from –8.3 to –9.5 kcal/mol (Table 1, Fig. 7). Among these interactions, emodin showed the highest binding affinity for MEK2 (–9.5 kcal/mol; Fig. 7C) and the lowest for ERK2 (–8.3 kcal/mol; Fig. 7E). Additionally, emodin exhibited moderate interactions with the EGFR and p38 MAPK (–8.6 kcal/mol) (Fig. 7A, 7F), forming two and four conventional hydrogen bonds, respectively (Table 1). These results suggest that emodin suppresses MUC5AC gene expression by inhibiting the activation of EGFR.

Table 1. Binding interaction of emodin (EMO) with EGFR and MAP kinase proteins

Compound- protein interaction	Docking scores (kcal/mol)	Hydrogen bond amino residues	Hydrogen bond length (Å)	Other bonding amino residues
EGFR-EMO	−8.6	GLU C:400	2.24	VAL C:336, ALA C:310
		GLU C:317	2.91	
MEK1-EMO	−7.3	ALA A:769	2.77	VAL A:776, VAL A:780, PRO A:773
		GLU A:770	3.49	
MEK2-EMO	−9.5	GLN A:157	2.71	LEU A:201, ALA A:99, LEU A:78, HIS A:149, VAL A:86
		LEU A:78	2.46	
		ASN A:199	2.01	
		ASP A:212	2.73	
		GLY A:153	3.76	
ERK1-EMO	−9.1	GLN A:121	2.63	ILE A:47, VAL A:55, LEU A:172, TYR A:52, LYS A:70
ERK2-EMO	−8.3	MET A:332	2.72	GLU A:333, VAL A:172, ARG A:171
		ARG A:69	2.17	
p38-EMO	−8.6	GLY A:109	2.56	LEU A:166, VAL A:37, LYS A:52, LEU A:166, ALA A:50
		HIS A:106	2.67	
		ALA A:110	2.02	
		ASP A:111	2.72	

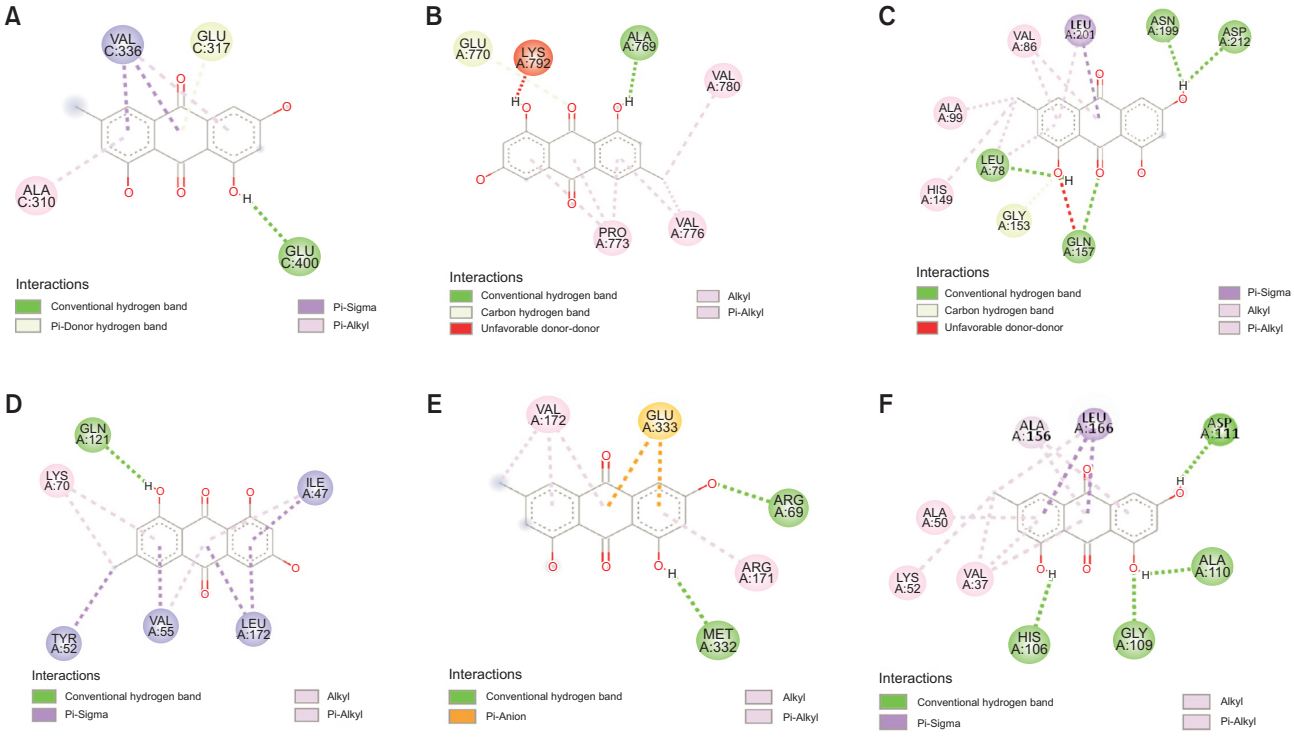

Fig. 7. Molecular interactions of emodin with EGFR and MAP kinase proteins. The 2D interaction diagram depicts emodin's interactions with (A) EGFR, (B) MEK1, (C) MEK2, (D) ERK1, (E) ERK2, and (F) p38 MAPK. Molecular docking studies revealed that emodin exhibited strong binding interactions with EGFR, MEK1/MEK2, ERK1/ERK2, and p38 MAPK proteins, with binding affinities ranging from –8.3 to –9.5 kcal/mol (Table 1, Fig. 7). Among these interactions, emodin showed the highest binding affinity for MEK2 (–9.5 kcal/mol; Fig. 7C) and the lowest for ERK2 (–8.3 kcal/mol; Fig. 7E). Additionally, emodin exhibited moderate interactions with the EGFR and p38 MAPK (–8.6 kcal/mol) (Fig. 7A, 7F), forming two and four conventional hydrogen bonds, respectively (Table 1). These results suggest that emodin suppresses MUC5AC gene expression by inhibiting the activation of EGFR-MAPK pathway through interacting with each protein.

DISCUSSION

In the current clinical settings, various agents including glucocorticoids have been used to control pulmonary diseases with airway mucus hypersecretion, although these agents often fail to provide remarkable clinical efficacy and can cause various side effects (Li *et al*., 2020). Up to date, there is no specific pharmacological agent that effectively regulates the production and/or secretion of airway MUC5AC mucin. Developing such an agent is urgent to regulate the hyperproduction and/or hypersecretion of sticky, pathologically-transformed mucus, in pulmonary diseases. Effective control of inflammatory pulmonary diseases requires regulating the inflammatory response. The current experimental results demonstrated that emodin, a natural product showing diverse biological activity including anti-inflammatory effect, suppressed the production of MUC5AC mucin glycoprotein and the expression of the MUC5AC mucin mRNA, induced by EGF (Fig. 2, 3). These results suggest that emodin can directly act on airway epithelial cells to regulate mucin gene expression, at the transcriptional and translational levels.

A comprehensive overview of the EGFR signaling pathway and its involvement into the regulation of MUC5AC mucin gene expression was published by several research groups. EGF induces MUC5AC mucin gene expression and provokes EGFR signaling pathway in NCI-H292 cells. EGFR was reported to be up-regulated in asthmatic airways and has been known to be a crucial regulator of epithelial function. It transduces extracellular signals into intracellular signaling cascades, including the MEK-MAPK pathway. Activated MAPK subsequently activates the Sp1 transcription factor and Sp1 initiates the expression of the MUC5AC mucin gene (Lemmon and Schlessinger, 1994). MAPKs are reported to be one of the pivotal signaling molecules that regulate pro-inflammatory chemokines and production of cellular macrophages (Bondeson, 1997). Among MAPKs, MEK, ERK, p38 MAPK are the most vital kinases for MUC5AC gene expression. Several studies showed that EGF induces the phosphorylation of MEK1/2, ERK1/2 (p44/42 MAPK) and p38 MAPK and MUC5AC gene (Rescan *et al*., 2005; Tashiro *et al*., 2016; Zhao *et al*., 2016). EGFR-MEK-MAPK-Sp1 signaling cascade plays a significant role in MUC5AC gene expression (Hewson *et al*., 2004). Inhibitors of EGFR tyrosine kinase can suppress EGF-stimulated MUC5AC gene expression, indicating the hyperproduction of MUC5AC mucin is due to EGFR pathway activation (Perrais *et al*., 2002; Mata *et al*., 2005; Tang *et al*., 2018). Finally, Sp1 is a well-characterized transcription factor with multiple roles in the transcription of various genes, including MUC5AC (Zhang *et al*., 2023). Our data indicated that emodin provoked a remarkable elevation of EGFR phosphorylation in airway epithelial cells (Fig. 4). Emodin also inhibited the phosphorylation of MEK, ERK, and p38 MAPK mediated by EGF (Fig. 5A-5C). Lastly, pretreatment of NCI-H292 cells with emodin blocked EGF- induced nuclear expression of Sp1, as can be

seen in Fig. 6. These findings indicate that the inhibitory activity on MAPK pathway plays a partial role in mediating the regulative effect of emodin on MUC5AC glycoprotein production and collectively, emodin showed its effect on MUC5AC gene expression through regulating the EGFR-MAPK-Sp1 signaling pathway. Additionally, computational analyses have shown that emodin exhibits strong binding interactions with EGFR, MEK2, ERK1, and p38 MAPK proteins, offering mechanistic insights downstream EGFR signaling regulation (Fig. 7).

In summary, while current treatments for airway mucus hypersecretion have limited efficacy and cause side effects, emodin shows a promise as a potential therapeutic agent due to its ability to suppress MUC5AC mucin gene expression, indicating its potential for preclinical and clinical development in controlling inflammatory pulmonary diseases. Further research and clinical trials are warranted to validate the efficacy and safety of emodin in regulating mucin production and secretion, paving the way for its potential use in clinical settings.

CONFLICT OF INTEREST

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

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