Heliyon 9 (2023) e20154

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Methyl lucidone inhibits airway inflammatory response by reducing TAK1 activity in human bronchial epithelial NCI–H292 cells

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ARTICLE INFO

Keywords:
Methyl lucidone
Inflammatory lung disease
TAK1
NF-κB
p38 MAP kinase
Human bronchial epithelial cell

ABSTRACT

Background: Methyl lucidone (ML), a methyl derivative of lucidone, has anti-inflammatory properties. However, the molecular mechanisms that reduce the inflammatory effect of ML in human lung epithelial cells remain unkown. This study aimed to elucidate the molecular mechanisms underlying the anti-inflammatory effect of ML.

Methods: Four compounds (ML, methyl linderone, kanakugiol, and linderone) from *Lindera erythrocarpa* Makino were evaluated for their ability to reduce MUC5AC secretion levels in phorbol-12-myristate-13-acetate (PMA)-stimulated NCI–H292 cells using ELISA. The expression and secretion levels of inflammatory response-related proteins were analyzed using quantitative reverse transcription-PCR, ELISA, and western blotting. To determine whether ML directly regulates TGF-β-activated kinase 1 (TAK1), we performed an *in vitro* kinase assay.

Results: ML treatment effectively reduced the levels of inflammatory cytokines, including interleukin-1β and TNF-α, increased by stimulation. Furthermore, ML downregulated the pathway cascade of both IκB kinase (IKK)/NF-κB and p38 mitogen-activated protein (MAP) kinase/CREB by inhibiting the upstream kinase TAK1. An *in vitro* kinase analysis confirmed that ML treatment significantly reduced the kinase activity of TAK1.

Conclusion: ML pretreatment repressed the PMA-stimulated inflammation reaction by reducing the TAK1-mediated IKK/NF-κB and p38 MAP kinase/CREB signaling. These findings suggest that ML may improve respiratory health and can be used as a dietary supplement or functional food to prevent inflammatory lung diseases.

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https://doi.org/10.1016/j.heliyon.2023.e20154
Received 19 April 2023; Received in revised form 14 July 2023; Accepted 13 September 2023
Available online 16 September 2023

1. Introduction

Patients with worsening pulmonary inflammatory diseases, such as asthma, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (COPD), have a common symptom of cytokine and mucus hypersecretion [1,2]. Inhaled corticosteroids relieve these symptoms, but side effects such as osteoporosis, obesity, and hyperglycemia can occur when they are used in the long term [3]. Thus, studies aimed at finding safer therapeutic alternatives for asthma and COPD are attracting attention [4,5].

Mucus secretion from bronchial epithelial cells is a primary defense mechanism against exposure to inhaled pathogens, irritants, and toxins at the surface layer of the mucosa [6]. However, mucus hyperproduction and oversecretion are common pathophysiological hallmarks of inflammatory lung diseases [7]. MUC5AC is a known and prominent mucin protein expressed in airway epithelium. Its production is notably increased in the pulmonary tissues of asthma mice models exposed to ovalbumin [8] and in the bronchial epithelia of patients with COPD [9]. Moreover, MUC5AC secretion is upregulated by diverse inflammatory cytokines such as IL-1β and TNF-α [10].

Inflammatory processes are controlled by multiple signaling pathways, including the mitogen-activated protein (MAP) kinases and nuclear factor (NF)-κB pathways. NF-κB plays a key regulatory role in the expression of pro-inflammatory mediators, including MUC5AC and TNF-α [11,12]. To activate NF-κB, the IKK complex (comprising IKKα, IKKβ, and IKKγ) phosphorylates IκBα, which is subsequently broken down by the proteasome. The released NF-κB translocates to the nucleus and expresses inflammatory genes [13]. MAP kinases with conserved serine and threonine residues are composed of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase. MAP kinases are involved in controlling crucial cellular processes and inflammatory diseases [14]**.** In particular, there are reports that p38 MAP plays a central role in regulating COPD and asthma pathology by turning on cAMP response element-binding protein (CREB) [15]. Thus, investigating compounds that can inhibit both IKK/NF-κB and p38 could help identify therapeutics for inflammatory lung diseases.

TGF-β-activated kinase 1 (TAK1) is a serine/threonine kinase that functions upstream of MAP kinases [16]. It is also an important regulator for the activation of IKK/NF-κB and p38 MAP kinase pathway cascades. TAK1 is emerging as an important target in various diseases, including pulmonary diseases, cancers, and fibrosis [17,18]. Indeed, our previous research demonstrated that a TAK1 inhibitor (5Z-7-oxozeaenol, 5OZ) reduced MUC5AC expression and secretion [19]. Thus, approaches to modulate TAK1 activity can be applied to reduce the expression of genes associated with respiratory inflammatory diseases.

The *Lindera (L.)* plants have great medicinal and therapeutic value because they are a source of many secondary metabolites, such as sesquiterpenoids, alkaloids, butanolides, lucidones, flavonoids, and phenylpropanoids [20]. *L. erythrocarpa* Makino is cultivated in China, Japan, and Korea and is used as a traditional remedy owing to its analgesic, digestion-promoting, and antibacterial properties [21]. It also exerts protective effects against oxidants [22] and inflammation [23]. Among the active constituents of *Lindera* plants, lucidone and its derivatives have diverse functions, including antioxidant, dermatoprotective, hepatoprotective, hypolipidemic reaction, and anti-inflammatory effects [24]. The present study aimed to elucidate the molecular mechanisms underlying the anti-inflammatory effect of methyl lucidone (ML) isolated from *L. erythrocarpa* in human airway epithelial cells.

2. Materials and methods

2.1. Chemicals and reagents

Growth medium for NCI–H292 cell maintenance was obtained from WELGENE company (Daegu, South Korea). The materials added to the growth medium were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Specific antibodies against β-actin, phosphorylated (p)-JNK, *p*-ERK, p-p38, and p38 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). p–NF-κB, NF-κB, p-IKKα/β, IKKα, p-IκBα, p-CREB, CREB, p-TAK1, and TAK1 antibodies were purchased from Cell Signaling Technology **(**Beverly, MA, USA). SB203580 was acquired from Selleckchem (Houston, TX, USA). Phorbol 12-myristate 13-acetate (PMA), 5Z-7-oxozeaenol (5OZ), and Bay 11–7082 were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell lines and culture conditions

NCI–H292 cells were sourced from the American Type Culture Collection (CRL-1848*;* ATCC, Manassas, VA, USA). The cells were grown in complete media, containing RPMI 1640 medium, 10% FBS and 100 mg/L streptomycin plus 100 U/mL penicillin, at 37 °C and 5% CO_2 under humidified conditions.

2.3. Plant material and preparation of Lindera erythrocarpa fruit

For this study, the same *L. erythrocarpa* Makino fruit as the sample KRIB 0000372, collected on Jeju Island, South Korea, in October 2013, was collected in October 2021 by Dr. Jin-Hyub Paik. The target compounds were derived from dried fruits of *L. erythrocarpa* as described previously [25]. Briefly, the extracts (770.0 g, yield 15.4%) were fractionated on a silica gel column (10 × 90 cm, JEO prep 60, 40–63 μm, 2.3 kg) and eluted using hexane−EtOAc mixtures (20:1 → 15:1 → 10:1 → 8:1 → 6:1 → 4:1 → 2:1 → 1:1, *v/v*) to obtain 10 pooled fractions (LE No. 1–10). LE No. 4 (105.0 g) was chromatographically separated using reversed-phase open column chromatography (M.S.GEL®, 75 μm, H_2O/CH_3OH, 40%→50%→60%→70%→75%→80%→90%→100%, *v/v*) to yield LE04_I–K (kanakugiol 3, 4.1 g). LE No. 6 (47.1 g) was subjected to preparative HPLC (YMC-Pack ODS-AQ-HG, 10 mm, 70% MeOH isocratic system, 100 mL/min, 55.0 min) to obtain LE06_B (methyl linderone 2, 2.5 g). LE No. 8 (35.4 g) was chromatographically separated using

reversed-phase open column chromatography (M.S.GEL®, 75 μm, H_2O/CH_3OH, 30–100%, *v/v*) to yield six fractions (LE08_A-F). LE08_B was separated by MPLC (Spot Prep II 250, Armen, Paris, France, flow rate: 100 mL/min) using a YMC ODS AQ HG (10 × 250 mm, 10 μm, Kyoto, Japan) and a gradient solvent system (0–50.0 min, 60% MeOH; 50.0–70.0 min, 60–100% MeOH) to provide LE08B_P2B (ML 1, 2.4 g). LE No. 9 (80.6 g) was chromatographically separated by reversed-phase open column chromatography (M.S. GEL®, 75 μm, H_2O/CH_3OH, 40–100%, v/v) to yield 10 fractions (LE09_A-J), and LE09_F-G was separated by preparative HPLC (YMC ODS AQ HG, 50–100% CH_3OH, 12 mL/min) to give LE09P2 (linderone 4, 0.45 g). The compound was distinguished by spectroscopic data, such as HRMS, ^1H, and ^{13}C NMR, and compared with the earlier reported data [25].

An ACQUITY UPLC™ system (Waters Corporation, Milford, MA) was used for UPLC (ultra-performance liquid chromatography) analysis. HRMS analysis was carried out using an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometer (UPLC-QTOF-MS) equipped with an electrospray ionization (ESI) interface (Waters Q-TOF PremierTM, Waters Corporation). NMR analysis was performed using a Fourier transform (FT)-NMR spectrometer (JEOL ECZ500R, JEOL Ltd., Akishima, Tokyo, Japan) to obtain 1D spectra (^1H NMR and ^{13}C NMR).

2.4. Cytotoxicity analysis

To measure cell viability, NCI–H292 cells were seeded, at a density of 1×10^4 cells/well, in complete media in 96-well microplates for 1 d. The complete media was replaced with a reduced-serum (0.1% FBS) medium and incubated for 16 h. Cells pretreated with various concentrations of four compounds (ML, methyl linderone, kanakugiol, and linderone) were cultured for 2 h prior to PMA stimulation treatment. After 1 d, cell viability was analyzed using the CCK-8 assay (CK04, Dojindo Laboratories, Kumamoto, Japan) in triplicate, referring to the technical manual. The optical density at 450 nm was measured using a microplate spectrophotometer (Epoch, Bio tek Instruments, Winooski, Vermont, USA) and expressed as a relative percentage compared with the control value.

2.5. ELISA

NCI–H292 cells were grown, at a density of 1×10^4 cells/well, in 96-well microplates for 1 d. The cells were starved by changing reduced-serum (0.1% FBS) medium after 16 h and then treated with ML for 2 h prior to activation by PMA for 16 h. The IL-1β and TNF-α proteins secreted in the supernatant were estimated according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). MUC5AC protein levels were measured as described previously [26].

2.6. Evaluation of mRNA expression levels

The seeded NCI–H292 cells were incubated in 24-well plates 1 d prior and then placed in a low-serum medium. After starvation, the cells were exposed to several doses of ML for 2 h and treated with PMA for 3 h. Total RNA from cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the instruction manual. cDNA synthesis and SYBR green-based quantitative reverse transcription-PCR (qRT-PCR) were performed as reported previously [26]. The primers used are listed in Table 1. Each experiment was conducted in triplicate, and results were calculated using the $2^{-\Delta\Delta CT}$ method.

2.7. Western blot analysis

The cells were homogenized in the CETi lysis buffer (TransLab, Daejeon, South Korea) under cold conditions. Protein samples were processed via electrophoresis and electrotransferred onto a PVDF membrane, which was blocked with 5% skim milk and incubated with the target protein antibodies overnight. The membrane was rinsed with TBST and incubated with secondary antibodies. The blots were visualized using Amersham Imager 600 (Amersham, Little Chalfont, UK), and quantifiable data were obtained using the Multi Gauge software version 3.0 (Fujifilm, Tokyo, Japan).

2.8. In vitro TAK1 kinase assay

The inhibitory effect of the ML on TAK1 kinase activity was determined using a commercial TAK1-TAB1 kinase enzyme system (V4088) and an ADP-Glo kinase assay kit (V9101) following the manufacturer's instructions (Promega Corporation, Wisconsin, USA). Briefly, the human recombinant TAK1-TAB1 fusion protein was diluted to 100 ng/μL using reaction buffer A containing 50 μM DTT. Diluted TAK1-TAB1 was mixed with the substrate at 1 μg/μL MBP protein. The mixture was then incubated for 20 min. Next, the reaction was started by introducing ATP. After 40 min of incubation, the ADP-Glo reagent and the detection buffer were added to each

Table 1
Primer sequences using for evaluation of mRNA expression levels.

Target gene	Accession no.	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)
IL-1β	NM_000576.3	AGCCAGGACAGTCAGCTCTC	ACTTCTTGCCCCCTTTGAAT	241	55
TNF-α	NM_000594.4	CCTACCAGACCAAGGTCAAC	AGGGGGTAATAAAGGGATTG	279	52
MUC5AC	NM_001304359.2	TGATCATCCAGCAG	CCGAGCTCAGAGGACATATGGG	409	58
β-actin	NM_001101.5	CATGTACGTTGCTATCCAGG	CTCCTTAATGTCACGCACGA	250	55

well and incubated for 30 min. Luminescence was measured with a luminometer (SpectraMax M4, Molecular Devices, Sunnyvale, CA, USA).

2.9. Statistical analysis

Data are presented as average ± standard deviation. Statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) using Student's *t*-test.

3. Results

3.1. Inhibitory effect of four compounds isolated from L. erythrocarpa on MUC5AC secretion levels in PMA-treated NCI–H292 cells

Since MUC5AC is a pathological marker of airway inflammatory disease, we performed MUC5AC ELISA to determine whether four compounds (ML, methyl linderone, kanakugiol, and linderone) isolated from the fruit of *L. erythrocarpa* inhibit inflammation in human epithelial cells (NCI–H292). Cell viability was assessed to determine the non-cytotoxic concentrations of compounds before the experiments. None of the compounds showed cytotoxicity at concentrations <2 μM in the presence of 100 nM PMA (Fig. 1A). Next, we investigated whether the four compounds reduced the PMA-stimulated MUC5AC secretion. Of these compounds, ML, methyl linderone, and linderone significantly inhibited MUC5AC secretion, but kanakugiol had no effect (Fig. 1B). Notably, ML, methyl linderone, and linderone have a common chemical structure, which contains lucidone derivatives. Since ML most strongly suppressed MUC5AC secretion in a concentration-dependent manner (range of 0.5–2 μM), we focused on the anti-inflammatory effect of ML in further experiments.

3.2. ML treatment inhibits the PMA-induced pro-inflammatory responses in NCI–H292 cells

As ML treatment effectively inhibited MUC5AC secretion , we examined whether this compound could inhibit the expression and secretion of other inflammatory mediators. We found that ML pretreatment decreased the levels of TNF-α and IL-1β in a dose-dependent manner in NCI–H292 cells (Fig. 2B and C). Consistent with this, ML treatment significantly inhibited the mRNA expression of PMA-induced *TNF-α*, *IL-1β*, and *MUC5AC* (Fig. 2D–F). These data suggest that ML exerts potent anti-inflammatory effects by reducing the transcriptional levels of various inflammatory mediators, including MUC5AC, IL-1β, and TNF-α.

3.3. ML inhibits NF-κB signaling and p38 MAP kinase activation in PMA-stimulated NCI–H292 cells

NF-κB is a key transcription factor that regulates the expression of inflammatory mediators, including cytokines and *MUC5AC*. We performed western blotting to determine whether ML inhibits the PMA-stimulated activation of NF-κB. First, we confirmed that PMA significantly increased the phosphorylation of IKKα/β at Ser 176/180, IκBα at Ser 32, and p65 at Ser 536 (Fig. 3A, lanes 1 and 2). ML treatment markedly reduced IκBα, IKKα/β, and NF-κB phosphorylation in a concentration-dependent manner (Fig. 3A, lanes 3–5). As the total levels of IKK and p65 proteins were stable, the decrease in phosphorylation upon ML pretreatment was not due to a change in their protein levels. Moreover, when we performed Western blot analysis to verify whether ML regulated an activity of MAPKs, our results showed that ML treatment specifically inhibited the PMA-induced p38 phosphorylation but not ERK or JNK phosphorylation.

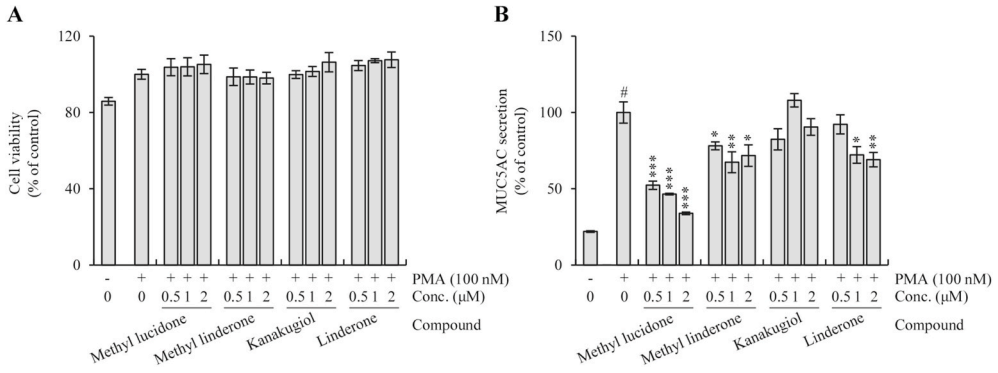

Fig. 1. Effects of four compounds derived from the *L. erythrocarpa* Makino fruit on PMA-stimulated MUC5AC secretion in NCI–H292 cells. (A) The toxicity of four compounds isolated from *L. erythrocarpa* Makino fruit, namely methyl lucidone (ML), methyl linderone, kanakugiol, and linderone, in NCI–H292 cells was measured following treatment with different concentrations of these compounds (0.5, 1, and 2 μM) in the presence of PMA for 24 h using the CCK-8 assay. (B) The inhibitory effect of the four compounds on MUC5AC secretion in NCI–H292 cells was determined using ELISA. The cells were pretreated with the compounds for 2 h and then with PMA for 16 h. Error bars indicate the average ± standard deviation of values from three independent experiments. #, $p < 0.01$ (compared with the group without PMA treatment); *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ (compared with the group with PMA only).

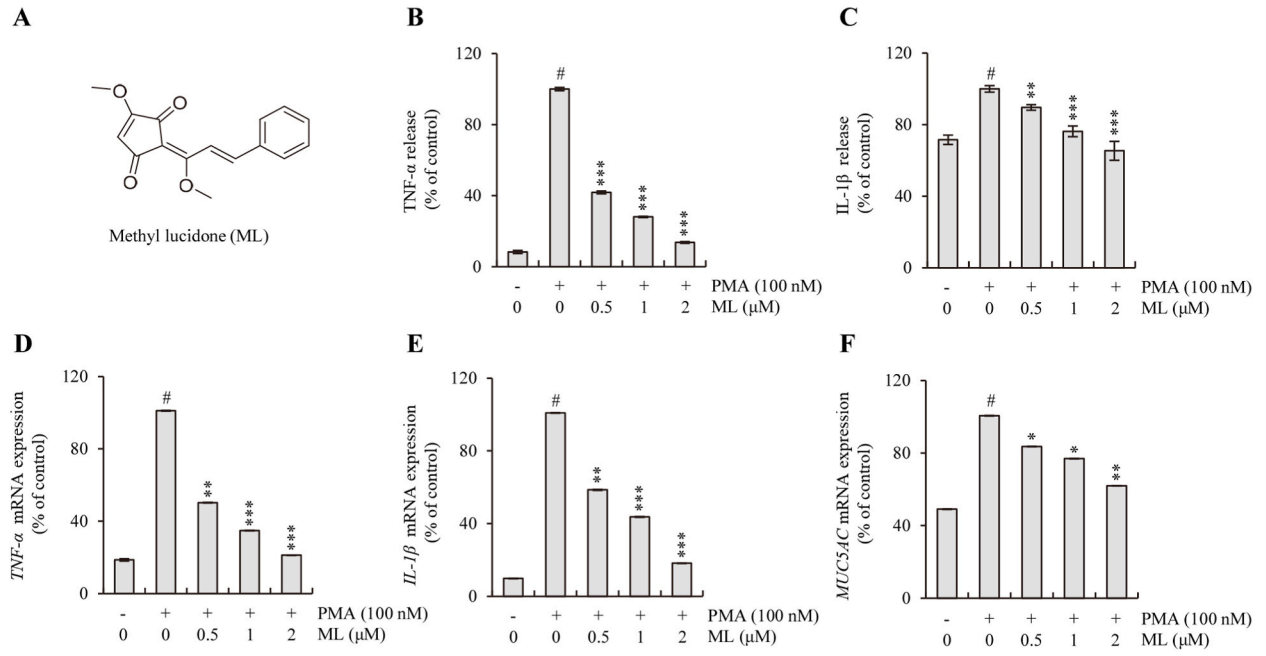

Fig. 2. ML strongly inhibits the expression of inflammatory mediators in PMA-treated NCI–H292 cells. (A) Chemical structure of ML. (B–C) The inhibitory effects of ML on PMA-induced cytokine (IL-1β and TNF-α) secretion levels were measured using ELISA. (D–F) The inhibitory effect of ML on the mRNA expression of pro-inflammatory cytokines (*IL-1β* and *TNF-α*) and *MUC5AC* was evaluated using qRT-PCR. Error bars indicate the average ± standard deviation of values from three independent tests. #, $p < 0.01$ (compared with the group without PMA treatment); *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ (compared with the group with PMA only).

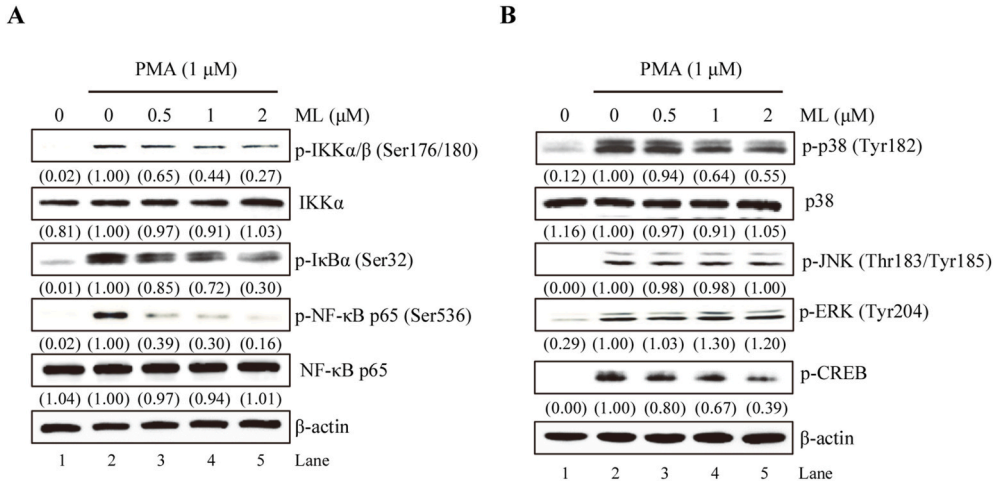

Fig. 3. ML inhibits activation of the NF-κB and p38 pathways in NCI–H292 cells. The cells were treated with different ML doses for 2 h and then with PMA (1 μM) for 30 min. (A) The reduction effects of ML on NF-κB signaling phosphorylation were determined using specific antibodies against p-IKKα/β, IKKα, p-IκBα, p–NF–κB, and NF-κB. (B) The reduction effect of ML on PMA-stimulated MAPKs signaling and CREB was detected using antibodies against p-CREB, p38, p-ERK, p-JNK, and p-p38. The signal of protein expression was normalized to that of actin. The numbers above each lane indicate the relative band intensities estimated using the Multi Gauge software version 3.0 (Fujifilm, Tokyo, Japan).

ML treatment also reduced CREB phosphorylation (Fig. 3B). These results show that ML may exert anti-inflammatory effects by negatively modulating both NF-κB and p38 MAP kinase signaling.

3.4. ML suppresses TAK1-mediated NF-κB and p38 activation

Since TAK1 is an important upstream kinase that regulates NF-κB and p38, we examined whether ML affects TAK1 phosphorylation

using Western blot analysis. Our results showed that ML treatment reduced the PMA-induced TAK1 phosphorylation in a concentration-dependent manner, without affecting the total TAK1 levels (Fig. 4A). Next, we used the TAK1 inhibitor 5OZ to determine whether PMA-induced NF-κB and p38 activation is mediated by TAK1 in NCI–H292 cells. Treatment with 5OZ reduced NF-κB, p38, CREB, and TAK1 phosphorylation. Combination treatment with two compounds targeting TAK1 may have a synergistic inhibitory effect compared to the individual treatment with each compound. As expected, the combined treatment with ML and 5OZ significantly reduced PMA-stimulated TAK1, NF-κB, p38, and CREB phosphorylation compared to treatment with each agent alone (Fig. 4B). Consistently, PMA-induced MUC5AC secretion was strongly reduced by the combination of the two compounds compared to that by ML or 5OZ alone without affecting cell viability (Fig. 4C and 4D). Similar reduction in MUC5AC secretion was observed when using the combination of two compounds compared to that achieved with either ML or SB203580 (p38 inhibitor) alone and in the combination of two compounds compared to that with either ML or Bay11-7082 (NF-κB inhibitor) alone (Fig. 4D). These results suggest that ML treatment reduces TAK1 phosphorylation, thereby reducing MUC5AC secretion by negatively regulating NF-κB and p38.

3.5. ML treatment significantly inhibits TAK1 activity under cell-free conditions

To assess whether ML directly binds to TAK1, we performed a TAK1 kinase activity assay in a cell-free system using a recombinant human TAK1-TAB1 fusion protein. The activity of TAK1 kinase, evaluated by the rate of ATP conversion to ADP, was reduced by ML treatment and was strongly reduced by 5OZ. It is well known that 5OZ binds to the ATP-binding pocket of the catalytic domain of TAK1 and inhibits the activity of this enzyme. In our study, 5OZ inhibited enzyme activity by approximately 60% at 0.25 μM. In contrast, ML showed 20% inhibition at 2.5 μM, which was approximately 10 times higher than that obtained with 5OZ.

Catalytic properties of the TAK1 enzyme were markedly reduced upon ML treatment in a concentration-dependent manner and were strongly inhibited by 5OZ, which is already known to attach to the ATP-binding site of TAK1 (Fig. 5A). These data show that ML

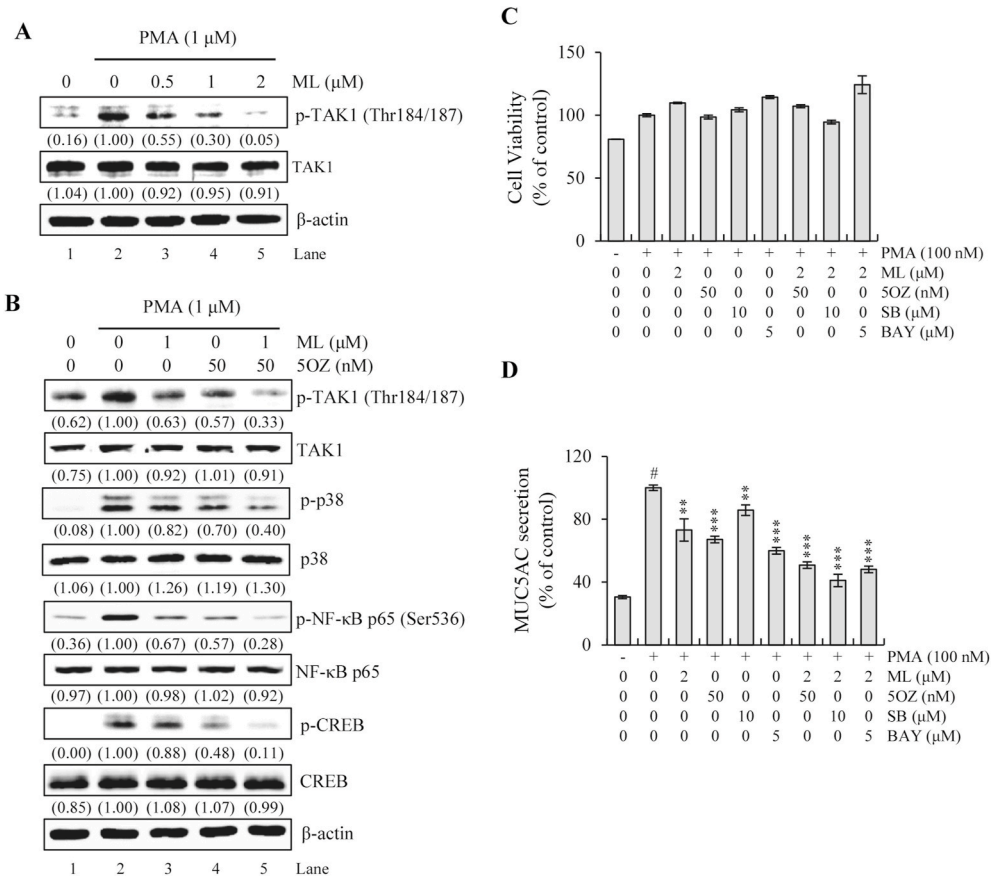

Fig. 4. ML suppresses activated TAK1 and its downstream molecules in NCI–H292 cells. (A) The inhibitory effect of ML on PMA-induced TAK1 activation was detected using antibodies against p-TAK1 and total TAK1. (B) The inhibitory effect of ML and a TAK1-specific inhibitor (5Z-7-oxozeaenol, 5OZ) was evaluated via Western blot analysis using antibodies against p-TAK1, TAK1, p-p38, p38, p–NF–κB, NF-κB, p-CREB, and CREB. The signal of protein expression was normalized to that of actin. The numbers above each lane indicate the relative band intensities estimated using the Multi Gauge software version 3.0. (C) The cellular toxicity of each compound was determined by CCK-8 assay. (D) Inhibitory effect of the combination of ML and 5OZ, p38 inhibitor (SB203580), and NF-κB specific inhibitor (Bay11-7082) on MUC5AC secretion in NCI–H292 cells was determined using ELISA.

can inhibit kinase activity by blocking the ATP-binding site or other regions of TAK1. Collectively, our results suggest that the repression effects of ML on NF-κB signaling and p38 MAP are mediated by the suppression of the activity of TAK1, which is an upstream IKK/NF-κB and p38/CREB regulator in human bronchial epithelial cells as well as in cell-free conditions (Fig. 5B).

4. Discussion

The long-term administration of corticosteroids to reduce chronic lung inflammation has serious limitations with unfavorable effects, including pneumonia and osteoporosis [27,28]. Thus, the development of adjuvant therapies or alternative medicines using natural products could be a strategy to overcome the side effects of long-term administration. Tiotropium (trade name Spiriva®), derived from Datura plants, is widely used as an inhaled anticholinergic bronchodilator to treat COPD [29]. Coenzyme Q10 [28], ginseng [30], and resveratrol [31] are effective in relieving systemic and respiratory inflammation and have been suggested as adjuvant therapies.

NCI–H292 cells are derived from bronchial mucoepidermoid carcinomas. As such, their characteristics and responses may not fully represent those of normal cells or tissues, limiting the generalizability of the findings to non-cancerous conditions. Meanwhile, NCI–H292 is a well-established and widely used cell line, which is readily available from cell banks. It has been extensively characterized and has established protocols for culturing, which makes it a convenient and standardized model for experimentation. The ease of reproducibility of results in these cells by other researchers serves as an advantage. Additionally, the cells are widely employed in studies related to bronchial-associated disorders and inflammatory responses. This cell line serves as a valuable model for pulmonary diseases, particularly those associated with mucin production activity [32,33]. In our previously published paper, we have already demonstrated that the levels of MUC5AC, a major component of mucin, are significantly increased when NCI–H292 cells are treated with PMA, EGF, TNF, cigarette smoke extract, or acrolein [34].

PMA is a potent inflammation/cytokine inducer that has been widely used in various cell types, including NCI–H292 cells. PMA activates the protein kinase C (PKC) signaling pathway, leading to the production of inflammatory cytokines and the initiation of inflammatory responses. Studies have reported that PMA treatment of NCI–H292 cells leads to the upregulation of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and TNF-α [35,36]. PMA-induced inflammation in NCI–H292 cells is accompanied by activation of the NF-κB signaling pathway, a key regulator of inflammatory responses. Consistent with previous findings, PMA treatment in THP-1 cells has been shown to increase the expression of TLR4 pathway-related molecules (such as MyD88, MAPKs, and NF-κB), resulting in the upregulation of inflammatory proteins (including IL-1β, IL-6, and TNF-α) [37,38].

Plants of the genus *L. erythrocarpa* (family Lauraceae) have potent antioxidant activities [39]. However, the therapeutic potential of *L. erythrocarpa* Makino extract for relieving inflammatory lung disease and its molecular mechanisms of action have not been studied in detail. In this study, we isolated four compounds (ML, methyl linderone, kanakugiol, and linderone) from *L. erythrocarpa* Makino extract and found that only three lucidone derivative compounds (ML, methyl linderone, and linderone) significantly reduced

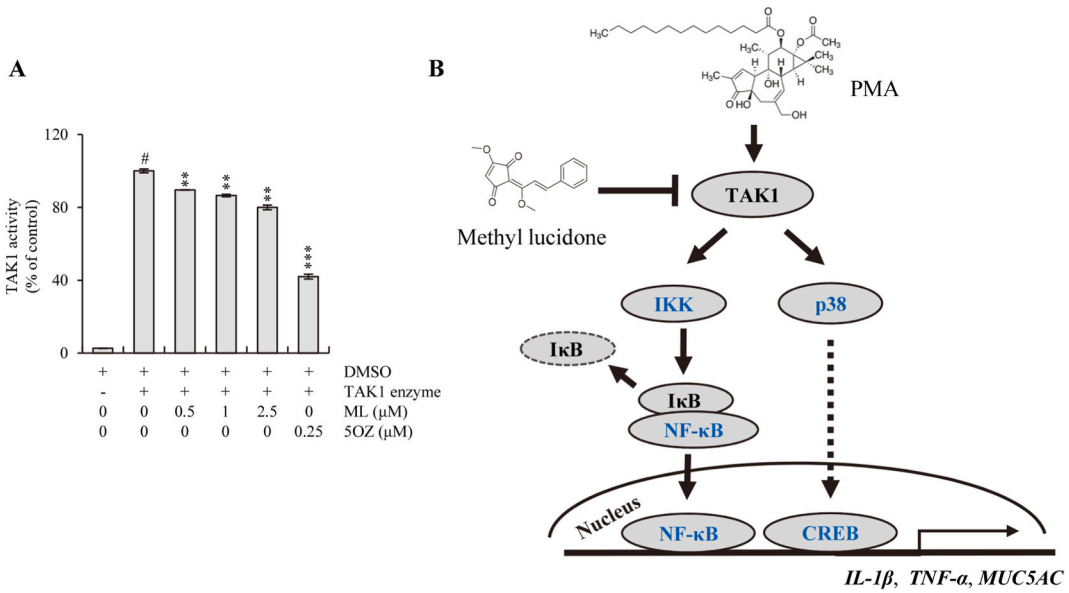

Fig. 5. ML significantly reduces the activity of TAK1 kinase in cell-free conditions. (A) The reduction effect of ML on TAK1 activity was evaluated using an *in vitro* TAK1 kinase assay. Error bars represent the mean ± S.D. of values from three separate experiments (#, $p < 0.01$ compared with the group with DMSO alone; **, $p < 0.01$, and ***, $p < 0.001$, compared with the group with TAK1 enzyme without drug treatment). (B) Schematic of the molecular mechanism by which ML suppresses PMA-increased MUC5AC, IL-1β, and TNF-α expression in airway epithelial cells. ML inhibits the activation of TAK1 and its downstream IKK/NF-κB and P38/CREB signaling pathways, thereby suppressing the expression of inflammation-related genes, including *MUC5AC*, *IL-1β*, and *TNF-α*. TM, transmembrane.

MUC5AC secretion levels, while kanakugiol had no effect. Notably, ML effectively inhibited the expression and secretion of inflammatory mucin or cytokines, such as TNF-α, MUC5AC, and IL-1β, in PMA-induced NCI–H292 cells. These results suggest that ML may inhibit the molecular mechanism(s) controlling the transcriptional expression of various inflammatory mediators.

Furthermore, we showed that ML exerted anti-inflammatory effects by blocking the phosphorylation of IKK/NF-κB and p38/CREB signaling in PMA-treated NCI–H292 cells. Activation of NF-κB or p38 is associated with the worsening of asthma or COPD [40]. Our results showed that ML treatment significantly inhibited the phospho-activation of NF-κB components, such as IKKα/β, IκBα, and NF-κB p65 (Fig. 3A). Interestingly, ML treatment selectively reduced only the activation of p38 and not that of JNK and ERK (Fig. 3B). p38 is a pharmacologically important target involved in restoring corticosteroid responsiveness and ameliorating the advanced stages of COPD and asthma [41]. Our data fully support that ML is a promising drug candidate for downregulating lung inflammatory responses by negatively modulating the upstream factors of the IKK/NF-κB and p38/CREB pathways, thereby decreasing the expression of lung inflammatory mediators.

Since TAK1 is a critical upstream molecule that initiates the IKK/NF-κB pathway [42], we investigated whether ML inhibits TAK1 phosphorylation in PMA-stimulated NCI–H292 cells. Our data indicate for the first time that ML pharmacologically inhibits TAK1 kinase activity. In support of this conclusion, phosphorylation of Thr187, an important residue for the TAK1 activation loop [43], showed a synergistic inhibitory effect when combined with ML to 5OZ, a TAK1-selective inhibitor (Fig. 4B). Furthermore, we demonstrated that ML can directly regulate TAK1 kinase using an *in vitro* TAK1 enzymatic assay (Fig. 5A). ML weakly inhibits TAK1 activity compared to 5OZ, which targets reactive cysteines at the Asp-Phe-Gly (DFG) motif of the ATP-binding site [44]. Therefore, it is highly likely that ML acts allosterically rather than directly on the ATP-binding pocket of TAK1. Referring to other similar studies, as one of the anti-inflammatory mechanisms of resveratrol, TAK1 amino acid residues, including Asp 161 and Ala 107 surrounding Thr187 of TAK1, are essential for the resveratrol-TAK1 interaction [17]. This suggests the need for additional molecular docking prediction experiments to identify the TAK1 docking pocket in ML.

5. Conclusion

In conclusion, our study is the first to demonstrate that ML isolated from the fruit of *L. erythrocarpa* Makino inhibits TAK1-mediated p38 MAP kinase and NF-κB activation to exert anti-inflammatory effects. ML inhibits MCU5AC secretion and inflammatory mediators, which are biomarkers of respiratory inflammatory disease. Taken together, we propose that ML could serve as a promising agent for treating pulmonary inflammatory diseases such as asthma and COPD.

Author contribution statement

Eun Sol Oh: Performed the experiments; Wrote the paper. </p>

Hyunju Ro: Su Ui Lee:Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper. </p>

Hyung Won Ryu: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. </p>

Ji-Yoon Park: Yu Na Song: Namho Kim: Hae-Young Kim: Seon Min Oh: Su-Yeon Lee: Doo-Young Kim: Performed the experiments. </p>;

Sooil Kim: Analyzed and interpreted the data. </p>

Sung-Tae Hong: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. </p>;

Mun-Ock Kim: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the KRIBB Research Initiative Program funded by the Ministry of Science and ICT of Republic of Korea (MSIT, KGM5522322), the National Research Foundation of Korea (NRF) grants funded by the MSIT (2020R1A2C2006664, 2020R1C1C1011146, RS-2023-00252236), and research grants funded by the Ministry of Education (MOE) of Republic of Korea and Chungnam National University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20154.

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