

Article

Saponins, especially Platyconic Acid A, from *Platycodon grandiflorum* reduce Airway Inflammation in Ovalbumin-induced Mice and PMA-exposed A549 Cells

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1 **Saponins, especially Platyconic Acid A, from *Platycodon grandiflorum* reduce Airway**
2 **Inflammation in Ovalbumin-induced Mice and PMA-exposed A549 Cells**

3 Short title: Saponins from *Platycodon grandiflorum* reduce airway inflammation

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ABSTRACT

We investigated the inhibitory effects of *Platycodon grandiflorum* root-derived saponins (Changkil saponins: CKS) on ovalbumin-induced airway inflammation in mice. CKS suppressed leukocytes number, IgE, Th1/Th2 cytokines, and MCP-1 chemokine secretion in bronchoalveolar lavage fluid. Also ovalbumin-increased MUC5AC, MMP-2/9, and TIMP-1/-2 mRNA expression, NF- κ B activation, leukocytes recruitment, and mucus secretion was inhibited by CKS treatment. Moreover, the active component of CKS, platyconic acid A (PA), suppressed PMA-induced MUC5AC mRNA expression (from 2.1 ± 0.2 to 1.1 ± 0.1) by inhibiting NF- κ B activation (from 2.3 ± 0.2 to 1.2 ± 0.1) via Akt (from 3.7 ± 0.3 to 2.1 ± 0.2) (respectively; $p < 0.01$) in A549 cells. Therefore, we demonstrate that CKS or PA suppressed the development of respiratory inflammation, hyperresponsiveness, and remodeling by reducing allergic responses, and they may be potential herbal drugs for allergen-induced respiratory disease prevention.

KEYWORDS: *Platycodon grandiflorum*; Saponins; Airway inflammation; Platyconic acid A; NF- κ B

43 INTRODUCTION

44 Natural agents with good efficacy and a low risk of side effects show promise as
45 preventive treatments for inflammation-related diseases. *Platycodon grandiflorum*, a food
46 and a traditional oriental medicine, is used in the treatment of chronic adult diseases and
47 inflammatory diseases. Our previous studies showed that *Platycodon grandiflorum* root-
48 derived saponins (Changkil saponins: CKS) exert anti-inflammatory,¹⁻⁴ anti-oxidant,⁵ and
49 hepatoprotective effects.⁶⁻⁸ Noh et al. reported that CKS contained triterpenoid saponins,
50 platycoside, such as deapio-platycoside E, platycoside E, deapio-platycodin D₃, platycodin D₃,
51 polygalacin D₂, platyconic acid A (PA), platycodin D₂, platycodin D, and 2'-O-
52 acetylpolygalacin D₂.⁹ Kwon et al. reported that PA improved glucose homeostasis by
53 enhancing insulin sensitivity in type 2 diabetic mice.¹⁰ However, the mechanisms underlying
54 the effects of CKS or PA as treatments for airway inflammation remain unclear.

55 Asthma is a chronic respiratory inflammatory disease caused by inappropriate responses
56 to inhaled allergens, and is clinically characterized by airway hyperresponsiveness, mucus
57 hypersecretion, and airway inflammation.¹¹⁻¹³ Allergen-induced immunoglobulin E (IgE)
58 binds to the high-affinity IgE receptor (FcεRI) on the surface of mast cells, leading to
59 activation and degranulation of the mast cells, and the release of inflammatory mediators
60 such as histamine, cytokines, resulting in immediate bronchoconstriction.¹⁴ Airway
61 inflammatory responses are mediated by Th1/2-mediated cells, together with mast cells, B
62 cells and leukocytes, as well as a number of inflammatory cytokines and chemokines.^{15,16}
63 While various stimulus related to respiratory disease can cause of mucus hypersecretion, it is
64 known that oxidative stress enhances mucus secretion.¹⁷ Respiratory tract is generated
65 oxidative stress via endogenously or exogenously exposed allergens.¹⁸ Despite the increasing
66 prevalence of respiratory disease, its pathophysiology remains unclear, and current treatments
67 are not sufficient. Plants provide a vast resource in the search for naturally effective

68 treatments with fewer side effects. For this reason, we evaluated oriental herbs suspected of
69 having inflammation-lowering effects on the airways.³

70 Airway mucus is comprised of water, ions, and cells within a glycoprotein-rich gel.¹⁹
71 Mucus plays an important role in protecting the airways from harmful inhaled
72 microorganisms, chemicals, and particles that come into contact with the respiratory tract,
73 and maintaining the normal function of the epithelium. Hypersecretion of airway mucus is an
74 important characteristic associated with severe respiratory symptoms, including airway
75 inflammation, asthma, chronic bronchitis, and cystic fibrosis.²⁰ Appropriate control of airway
76 mucin generation is important in the management of chronic respiratory symptoms. Therefore,
77 there is a need to develop new and effective therapies for mucus hypersecretion.

78 We evaluated the inhibitory effects of CKS or PA on airway inflammation in
79 ovalbumin-induced mice and PMA-exposed A549 cells. Our findings indicated that CKS or
80 PA inhibited the development of airway inflammation, hyperresponsiveness, and remodeling
81 by reducing the allergic responses. This may provide an effective alternative therapy in the
82 treatment of allergy-induced respiratory diseases.

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85 MATERIALS AND METHODS

86 **Chemicals.** Chicken egg ovalbumin (OVA) (Grade II), aluminum hydroxide gel,
87 Giemsa solution, and hematoxylin-eosin Y (H&E) staining solution were obtained from
88 Sigma Chemical Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay kits were
89 obtained from R&D Systems (Minneapolis, MN, USA) and BD Biosciences (San Diego, CA,
90 USA). Alcian blue periodic acid-Schiff (PAS) staining solution was obtained from Merck
91 KGaA (Darmstadt, Germany).³ All other chemicals and solvents were of the highest grade
92 available commercially.

Preparation of CKS from the roots extract of *Platycodon grandiflorum*. *Platycodon grandiflorum* root (CK) was supplied by Jangsaeng Doraji Co., Ltd. (Jinju, Korea). CK was extracted with MeOH. Concentration of the MeOH solution gave a brown syrupy extract which was suspended in H₂O and then divided successively with ethyl acetate and n-butanol. The n-butanol layer was suspended in H₂O and put into a Diaion HP20 column. Column was washed with H₂O and then eluted with MeOH. Also, eluted solution was concentrated in a reduced pressure to give a crude saponin mixture (CKS).²³

Preparation of platycosides by HPLC and NMR analysis. CKS extracted from the roots of *Platycodon grandiflorum* was analyzed by HPLC. The extracts were dissolved in H₂O and then put into a solid-phase-extraction cartridge (RP-C18, CREEX No. 600-3506). The cartridge was washed with H₂O and eluted with MeOH. The elution solution was injected into an OptimaPak column (4.6×250 mm, 5 μm, 100 Å) maintained at 40 °C on a Futecs NS-3000i system equipped with a light scattering detector (ELSD). Platycosides such as deapioplatycoside E, platycoside E, platyconic acid A, platycodin D, 2"-O-acetylpolygalacin D₂, and platycodin D₃ were purified from CKS and then their chemical structures were identified by direct comparison of their physical and spectral data (¹H-NMR and ¹³C-NMR).^{22,23}

Cell culture. A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified chamber with 5% CO₂.⁴ PA was dissolved in DMSO and added into the medium.

Animals and treatment. Six-week-old female ICR mice were obtained from Samtako (Osan, Korea). The animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water, and were maintained under specific pathogen-free conditions. The animals were acclimated to the temperature (22 ± 2 °C) and humidity ($55 \pm 5\%$) of the control rooms with a 12-h light/dark cycle for at least 1 week prior to experimentation.³ All experimental protocols in the use of the animals were performed according to the rules and regulations of the Animal Ethics Committee, Chungnam National University (Daejeon, Korea).

Immunization and challenge. Mice were immunized on days 1, 7, and 14 by intraperitoneal (i.p.) injection of 50- μ g chicken OVA emulsified in 1-mg aluminum hydroxide adjuvant, in a total volume of 100- μ l PBS. The CKS was dissolved in saline. The mice were intragastrically (i.g.) administered 0.5, 1 or 2 mg/kg/day (in 100 μ l) CKS each day from days 12 - 16, consecutively. The control and OVA group were administered saline (i.g.). The animals were challenged with OVA on the final day by inhalation of 1 mg/ml OVA in PBS. The control group was immunized and challenged with PBS without CKS administration (**Figure 1**).³

Bronchoalveolar lavage (BAL) fluid collection and leukocyte count. Each mouse was anaesthetized and the trachea was cannulated by gentle massaging of the throat. BAL fluid was collected by flushing 1 ml of PBS into the lungs via the trachea immediately after sacrifice; approximately 0.8 ml of BAL fluid was recovered after five lavages. The BAL fluid was centrifuged (400 $\times$ g, 4 °C, 5 min) and the supernatant was stored at -20°C until measurement of cytokines. Cells from the BAL fluid were washed three times with PBS and the pellet was resuspended in 100- μ l PBS. The total cell number was counted by cytospin, and a differential cell count was performed after staining with Giemsa solution.³

Enzyme-linked immunosorbent assay (ELISA). The levels of cytokines in the BAL fluid were measured by sandwich ELISA using the OptEIA Set mouse IL-4, IL-5, MCP-1, and IgE kits from BD Biosciences and DuoSet mouse TNF- α , IFN- γ , and IL-13 kits from R&D Systems, according to the manufacturer's instructions. Each concentration was calculated using a linear-regression equation obtained from the standard absorbance values.³

Histopathological studies. Right lung tissues were sliced and fixed in 10% buffered-neutral formalin for 24 h. The fixed lung tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Sections 4 μ m thick were subjected to H&E staining for general morphological analysis. Airway mucus hypersecretion was evaluated using alcian blue-PAS to stain infiltrated goblet cells. Pulmonary histopathological changes were assessed on a five-point score by three independent examiners. The scores were: 0, no inflammatory cells; 1, minimal accumulation of inflammatory cells; 2, moderate accumulation of inflammatory cells; 3, severe accumulation of inflammatory cells; 4, extreme accumulation of inflammatory cells. Infiltrated goblet cells were also assessed on a five-point score by three independent examiners. The scores were: 0, no goblet cells; 1, minimal infiltration of goblet cells; 2, moderate infiltration of goblet cells; 3, severe infiltration of goblet cells; 4, extreme infiltration of goblet cells.^{3,24} All lung tissue was processed according to the histopathological protocols of the pathological laboratory, College of Veterinary Science, Chungnam National University (Daejeon, Korea).

Western blot analysis. Western blot analysis was performed as described previously.³ The intensity of the western blot bands was measured using the ImageJ software (NIH Image J program).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

analysis. Total RNA was extracted from the left lung tissues or A549 cells using RNAiso reagent (Takara, Kyoto, Japan), according to the manufacturer's instructions. RT-PCR analysis was performed as described previously.^{3,25} The intensity of the RT-PCR bands was measured using the ImageJ software (NIH Image J program). The PCR primer sequences are listed in **Table 1**.

Quantitative real-time RT-PCR (qRT-PCR).

Cells were pretreated with PA (0.5, 1, or 2 μ M) and then treated with PMA (20 nM). PCR product formation was monitored continuously during amplification using the Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). The accumulated products were detected directly by monitoring the signal from the SYBR® Green reporter dye. MUC5AC mRNA expression was compared between the treated and control cells at each time point using the comparative cycle threshold (Ct) method. The quantity of each transcript was calculated as described in the instrument manual, and normalized to the GAPDH mRNA expression.³⁴ The PCR primer sequences are listed in **Table 1**.

Transient transfection and luciferase assays.

To determine the promoter activity, a dual-luciferase reporter assay system (Promega, WI, USA) was used. The cells were seeded into 48-well plates and incubated at 37°C. At 70-80% confluence, the cells were incubated with RPMI1640 without serum or antibiotics for 6 h. The cells were transiently co-transfected with a wild-type MUC5AC promoter luciferase construct, NF- κ B reporter vector, and pRL-SV plasmid (Promega, WI, USA) using LipofectAMINE™ 2000 reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. After 5 h, the medium was replaced with

basal medium. The cells were pretreated with PA for 1 h and then treated with PMA for 24 h. After incubation, the cells were lysed and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron, Waltham, MA, USA). For each sample, the luciferase signal was normalized to Renilla luciferase activity and expressed relative to the control value.⁴

Statistical analyses. All experiments were repeated at least three times. Results reported are means $\pm$ the standard error of the mean (S.E.M.). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test, with $p < 0.05$ or $p < 0.01$ indicating significance.

RESULTS

CKS inhibited OVA-induced leukocyte recruitment and histopathological changes in mice. Leukocytes play an important role in airway inflammation. To evaluate the effectiveness of CKS in airway inflammation, OVA-induced leukocytes were counted in bronchoalveolar lavage (BAL) fluid. As shown in **Table 2**, repeated ovalbumin (OVA) application increased the numbers of total and differential leukocytes (i.e., eosinophils, basophils, lymphocytes, and macrophages) in the BAL fluid. The numbers of OVA-induced total and differential leukocytes were reduced by CKS administration in a dose-dependent manner. Continuously, we determined the inhibitory effect of CKS on OVA-induced histopathological changes in the lung tissue. Repeated OVA application increased bronchoconstriction in sensitized mice, with the trachea characterized by goblet cell hyperplasia and mucus hypersecretion from the goblet cells.^{26,27} Histopathological analysis by H&E and alcian blue-PAS staining of the lung tissue showed inflammatory cell infiltration

and goblet cell hyperplasia in the OVA-treated mice. CKS inhibited these OVA-induced pathological changes in the lung tissue (**Figure 2A,B**).

CKS inhibited OVA-induced IgE, Th1/Th2 cytokines, and MCP-1 secretion in mice.

Mast cells are key effector cells in IgE-mediated allergic disorders. IgE-mediated allergic disorders are regulated by mast cells. During mast cells activation, mast cells undergo degranulation and secrete a variety of biologically active substances, which play an important role in the development of airway inflammation.^{28,46} We evaluated the effectiveness of CKS against total IgE secretion in BAL fluid. CKS reduced OVA-induced IgE level in a dose-dependent manner (**Table 3**). Furthermore, airway inflammation is associated with the secretion of Th1/Th2 cytokines and monocyte chemoattractant protein-1 (MCP-1) chemokine.²⁹ We examined the effectiveness of CKS against OVA-induced TNF- α , IFN- γ , IL-4, -5, -13, and MCP-1 secretion in BAL fluid. Repeated OVA application increased TNF- α , IFN- γ , IL-4, -5, -13, and MCP-1 secretion levels. CKS reduced OVA-elevated Th1/Th2 cytokines and MCP-1 secretion in a dose-dependent manner (**Table 3**). These results demonstrated that CKS decreased the OVA-induced inflammatory response, affecting leukocyte recruitment and secretion of IgE, Th1 and Th2 cytokines, and MCP-1 chemokine in mice.

CKS inhibited OVA-increased MUC5AC and MMP-2/-9 mRNA expression in mice.

Airway mucus hypersecretion is associated with goblet cell hyperplasia in mice.³⁰ We determined the inhibitory effects of CKS on OVA-increased MUC5AC mRNA expression in lung tissues. Repeated OVA application increased MUC5AC mRNA expression, similar to the histopathological changes observed. CKS inhibited OVA-increased MUC5AC mRNA expression in a dose-dependent manner (**Figure 3A,B**). Airway remodeling is associated with

MMPs and TIMPs modulation in mice.²⁵ We also determined the inhibitory effects of CKS on OVA-mediated MMP-2/9 and TIMP-1/2 mRNA expression in lung tissues. MMP-2/9 mRNA expression increased with OVA application, but TIMP-1/2 mRNA expression decreased with OVA application. CKS significantly inhibited OVA-increased MMP-2/9 mRNA expression and OVA-decreased TIMP-1/2 mRNA expression (**Figure 3A,B**). These results demonstrated that CKS inhibited OVA-induced mucus hypersecretion- and airway remodeling-related MUC5AC and MMPs mRNA expression in mice.

CKS inhibited OVA-increased NF- κ B activation in mice. Nuclear factor-kappa B (NF- κ B) activation plays an important role in airway inflammation. Various stimuli lead to the phosphorylation, ubiquitination, and proteolytic degradation of I κ B α , resulting in the release of NF- κ B from its inhibitory protein and its translocation to the nucleus.³¹ NF- κ B activation has been observed in allergic airway inflammation in human and animal.³³ We determined the inhibitory effect of CKS on NF- κ B p65 level during OVA-increased allergic airway inflammation in lung tissues. OVA-sensitized and -challenged mice showed a notably increased NF- κ B p65 nuclear protein and a decreased cytoplasmic NF- κ B p65 cytosolic protein compared to control mice. CKS treatment inhibited OVA-increased these alterations in a dose dependent manner (**Figure 4A,B**).

Platyconic acid A inhibited PMA-induced MUC5AC expression by inhibiting the activation of NF- κ B in A549 cells. We determined the inhibitory effects of CKS triterpenoid saponins, platycosides, on PMA-induced MUC5AC expression in A549 cells. First, the cytotoxicity of CKS triterpenoid saponins, platycosides, such as deapioplatycoside E, platycoside E, platyconic acid A (PA), platycodin D, 2"-O-acetylpolygalacin D₂, and platycodin D₃ were determined in A549 cells (**Supporting Information Figure 2**).

Treatment with each platycoside at concentrations of 0-8 μ M for 24 h showed that platycoside concentrations < 4 μ M did not affect cell viability, and cytotoxicity was determined by MTT reduction and LDH release assays. We further evaluated the inhibitory effects of platycosides on PMA-increased MUC5AC expression in cells. Among the platycosides tested, PA significantly decreased PMA-increased MUC5AC mRNA expression in the cells (**Table 4**). The chemical structure of platyconic acid A (PA) is shown in **Figure 5A**. PA is the most potent CKS platycoside, which improves glucose homeostasis by enhancing insulin sensitivity in type 2 diabetic mice.¹⁰ To assess the inhibitory effects of PA on MUC5AC expression, A549 cells were treated with PMA in the presence of PA for 24 h, and MUC5AC mRNA expression was then evaluated by qRT-PCR. PA treatment inhibited PMA-increased MUC5AC mRNA expression in the cells in a concentration-dependent manner (**Figure 5B**). PA also inhibited PMA-induced MUC5AC luciferase activity by inhibiting the NF- κ B promoter-driven luciferase reporter activity (**Figure 5C,D**). PA inhibited PMA-induced NF- κ B p65 phosphorylation, I κ B α phosphorylation and degradation in a concentration-dependent manner (**Figure 5E,F**). These results demonstrated that PA inhibited PMA-increased MUC5AC expression by suppressing NF- κ B activation in cells.

Platyconic acid A inhibited PMA-induced MUC5AC expression by inhibiting the activation of Akt in A549 cells. We demonstrated that PA inhibited NF- κ B activity of PMA-treated cells at the transcriptional level (**Figure 5**). Akt pathway is well-known upstream regulator of NF- κ B signaling in MUC5AC transcription factor.⁴⁰ We evaluated the inhibitory effect of PA on PMA-induced Akt phosphorylation in cells. PA inhibited PMA-induced Akt phosphorylation in a concentration-dependent manner (**Figure 6A,B**).

DISCUSSION

There is widespread interest in developing new, and less toxic, anti-inflammatory agents from natural sources. Natural immune modulators from herbal extracts or herbal derivatives have been reported to have inhibitory effects on inflammation.^{21,34,41-45} We investigated the inhibitory effects of *Platycodon grandiflorum* root-derived saponins (CKS), especially platyconic acid A (PA), on airway inflammation in ovalbumin-induced mice and PMA-exposed A549 cells.

Asthma is one of the most common respiratory inflammatory diseases worldwide, and is characterized by airway hyperresponsiveness. Asthma is caused by inappropriate responses to inhaled allergens, and the strength of the hyperresponsiveness correlates with the level of airway inflammation, which is predominated by inflammatory cell infiltration, mucus hypersecretion, allergen-specific IgE, and overproduction of Th1/Th2-mediated cytokines and chemokines.³²

Allergen exposure in the airway induces a Th2-dominant response by activating inflammatory cells and increasing Th2 cytokines. IL-4, IL-5, and IL-13 play important roles in causing the symptoms, such as airway hyperresponsiveness, airway inflammation, and goblet cell hyperplasia via eosinophil activation and IgE secretion.³³ Th2 cytokines are important therapeutic targets for the inhibition of airway inflammation. We evaluated the inhibitory effects of CKS on the secretion of Th1/Th2 cytokines in OVA-induced mice. OVA-induced secretion of Th2 cytokines (IL-4, IL-5, and IL-13) as well as secretion of Th1 cytokines (TNF- α and IFN- γ) was reduced by CKS treatment. The Th2 immune response is associated with IgE hypersecretion. In IgE-mediated airway inflammation, IgE released from B cells binds to mast cells, leading to degranulation of the mast cells and the release of various biological mediators.¹⁴ We confirmed that OVA-induced IgE production was reduced by CKS treatment, and we previously reported on the inhibitory effects of CKS on IgE-

mediated allergic responses in mast cells.³⁴ Chemokines play an important role in the development of airway inflammatory through their participation in leukocyte recruitment. MCP-1 plays a role in the recruitment and activation of leukocytes in response to various stimuli.³⁵ We demonstrated that CKS treatment reduced OVA-induced MCP-1 secretion. These results suggest that CKS reduced OVA-induced development of airway inflammation by suppressing IgE-mediated mast cell activation, Th1/Th2 inflammatory cytokines, and MCP-1 chemokine release.

Airway inflammation occurs due to an infiltration of leukocytes into the bronchial and lung tissues. It is associated with the production of Th2 cytokines, which play a critical role in the differentiation, infiltration, and activation of eosinophils.²⁵ We evaluated the inhibitory effects of CKS on the number of leukocytes in OVA-inhaled mice. We demonstrated that the number of total and differential inflammatory cells was reduced by CKS treatment. Asthma is characterized by the infiltration of inflammatory cells, as well as hyperplasia of goblet cells into the lung tissues. Goblet cell hyperplasia is associated with bronchial inflammation and narrowing of the airways.³⁶ Pathological changes showed that CKS treatment inhibited OVA-induced inflammatory cell infiltration and goblet cell hyperplasia in the lung tissue.

Airway remodeling often occurs during the initial stages of asthma and may facilitate the continuation of damage to tissues and the inflammatory response. Airway remodeling is characterized by inflammatory cell infiltration, goblet cell hyperplasia, and MMP-2/-9 production.³⁷ MMPs are proteases that cleave components of the extracellular matrix. MMPs play an important role in tissue remodeling associated with pathological processes such as morphogenesis, angiogenesis, tissue repair, and migration.³⁷ A recent study reported that MMP-2/-9 overexpression was related to an accumulation of airway inflammatory cells in an animal model.³⁷ We evaluated OVA-induced MMP-2/9 and MUC5AC mRNA expression associated with airway remodeling in lung tissues. We demonstrated that OVA-induced

MMP-2/9 and MUC5AC mRNA expression was reduced by CKS treatment. However, OVA-reduced TIMP-1/-2 mRNA recovered with CKS treatment. Expression of MUC5AC and MMP-2/-9 is controlled by a NF- κ B transcription factor. We examined the OVA-induced NF- κ B nuclear translocation in lung tissues. OVA-induced NF- κ B nuclear translocation was reduced by CKS treatment. We previously reported the inhibitory effects of CKS on cellular MMP-2/-9 and MUC5AC expression via inhibition of NF- κ B activity.^{4,38} CK inhibited OVA-induced MMP-2/-9 production via inhibition of NF- κ B nuclear translocation.³ Finally, we evaluated the effects of PA, a component of the CKS triterpenoid saponins, on MUC5AC expression via NF- κ B activation in the cells. The inhibition of iNOS and COX-2 mRNA expression by PA, through blocking of NF- κ B activation has been reported previously.³⁹ We showed that PA treatment inhibited PMA-induced MUC5AC expression by suppressing NF- κ B activity. These results suggested that CKS reduced OVA-induced airway remodeling by suppression of MMP-2/-9 and MUC5AC expression via inhibition of NF- κ B activity. PA potentially inhibited MUC5AC expression through the inactivation of NF- κ B.

In conclusion, we demonstrated that CKS and its active component, PA, reduced the severity of airway inflammation in an experimental animal model and in cells. CKS inhibited OVA-induced airway inflammation symptoms, including infiltration of inflammatory cells, hypersecretion of goblet cells, levels of IgE, Th1/Th2, and MCP-1, mRNA expression of MMP-2/-9 and MUC5AC, and NF- κ B activation in mice. PA inhibited MUC5AC expression via suppression of NF- κ B activation in cells. These results demonstrated that CKS and PA have a potent preventive effect against the progression of airway inflammation related to mucin production.

Abbreviations: IgE, immunoglobulin E; MMP, matrix metalloproteinase; MCP-1, monocyte

chemoattractant protein-1; MUC5AC, mucin 5 subtype A and C; NF- κ B, nuclear factor
kappaB; PMA, phorbol 12-myristate 13-acetate; Th, T helper; TIMP, tissue inhibitor of
metalloprotease.

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Notes

The authors declare no competing financial interest.

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Figure captions

Figure 1. Schematic diagram of the experimental protocol in mice. Mice were divided into five groups. Mice were immunized on days 1, 7, and 14 by intraperitoneal (i.p.) injection of 50 μ g chicken OVA emulsified in 1 mg aluminum hydroxide adjuvant in a total volume of 100 μ l PBS. CKS was dissolved in saline. Mice were intragastrically (i.g.) administered 0.5, 1 or 2 mg/kg/day (in 100 μ l) CKS each day from days 12 - 16, consecutively. The control and OVA group were administered saline (i.g.) without CKS. The animals were challenged with OVA on the final day by inhalation of 1 mg/ml OVA in PBS. The control group was immunized and challenged with PBS without drug administration.

Figure 2. Inhibitory effects of CKS on OVA-induced leukocytes and goblet cells infiltration in mice. (A) Histopathological changes in the lung tissues. Lung tissue was cut and stained with H&E or alcian blue-PAS. Lung tissue was obtained from PBS-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CKS2 (OVA + CKS2). (B) Inflammatory cell infiltration (closed bar) and mucus production (open bar) in the lung tissue were scored as described in the materials and methods section. Results shown are representative of five observations. Results are presented as means $\pm$ S.E.M. of three independent experiments. Magnification $\times 100$. [#] $p < 0.05$, significantly different from the control group; ^{*} $p < 0.05$, significantly different from the OVA-treated group.

Figure 3. Inhibitory effects of CKS on OVA-induced airway remodeling in mice. (A) Total RNA was extracted from the lung tissue and mRNA expression of MUC5AC, MMP-2, MMP-9, TIMP-1, TIMP-2, and GAPDH were determined by semi-quantitative RT-PCR. (B)

The intensities of the PCR bands were calculated using the ImageJ software. Results shown are representative of five observations. Results are presented as means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.05$, significantly different from the control group; * $p < 0.05$, significantly different from the OVA-treated group.

Figure 4. Inhibitory effects of CKS on OVA-induced NF- κ B activation in mice. (A) Mice were pretreated with CKS (0.5, 1, or 2 mg/kg) for 1 h and then treated with OVA for 2 h. Cytosolic and nuclear protein was extracted from lung tissue treated with OVA, and NF- κ B activation was determined by western blotting. (B) The intensities of the western blot bands were calculated using the ImageJ software. Results shown are representative of five observations. Results are presented as means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.05$, significantly different from the control group; * $p < 0.05$, significantly different from the OVA-treated group.

Figure 5. Inhibitory effects of PA on PMA-induced MUC5AC mRNA expression in cells. (A) Chemical structure of platyconic acid A. (B) Total RNA was extracted from the cells and MUC5AC mRNA expression was measured by qRT-PCR. (C and D) MUC5AC or NF- κ B luciferase activity was measured by a promoter construct. (E) Cells were pretreated with PA (0.5, 1, and 2 μ M) and then treated with PMA (20 nM). After 30 min, total protein was extracted from the cells and phospho-NF- κ B p65, phospho-I κ B α , and I κ B α level was measured by western blotting. (F) The intensities of the western blot bands were calculated using the ImageJ software. Results are presented as means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.01$, significantly different from the control group; * $p < 0.01$, significantly different from the PMA-treated group.

Figure 6. Inhibitory effect of PA on PMA-induced Akt activation in cells. (A) Cells were pretreated with PA (0.5, 1, and 2 μ M) and then treated with PMA (20 nM). After 30 min, total protein was extracted from the cells and phospho-Akt and Akt level was measured by western blotting. (B) The intensities of the western blot bands were calculated using the ImageJ software. Results are presented as means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.01$, significantly different from the control group; * $p < 0.01$, significantly different from the PMA-treated group.

Table 1. Primer sequences for semi-quantitative RT-PCR and qRT-PCR

Gene	Primer sequence (5'→3')	Gene bank no.
mMUC5AC	(F) AAA GAC ACC AGT AGT CAC TCA GCA A	NM_010844.1
	(R) CTG GGA AGT CAG TGT CAA ACC A	
mMMP-2	(F) AGA TCT TCT TCT TCA AGG ACC GGT T	NM_008610.2
	(R) GGC TGG TCA GTG GCT TGG GGT A	
mMMP-9	(F) GTA TGG TCG TGG CTC TAA GC	NM_013599.3
	(R) AAA ACC CTC TTG GTC TGC GG	
mTIMP-1	(F) CAC CAC CTT ATA CCA GCG TT	NM_001044384.1
	(R) GTC ACT CTC CAG TTT GCA AG	
mTIMP-2	(F) CCA GGT CCT TTT CAT CCT GA	NM_011594.3
	(R) TCC ATT CGC TGA AGT CTG TG	
mGAPDH	(F) CAT CTT CCA GGA GCG AGA CC	NM_008084.2
	(R) TCC ACC ACC CTG TTG CTG TA	
hMUC5AC	(F) CTG AGG GTC TCA GGA ATG ACG C	XM_006726526.1
	(R) TTT ATG CAA CAG ATT GGC CGT G	
hGAPDH	(F) CCA CAT CGC TCA GAC ACC AT	NM_002046.5
	(R) CCA GGC GCC CAA TAC G	

Table 2. Effects of CKS on OVA-induced total and differential leukocytes in BAL fluid [§]

	Control	OVA	OVA+CKS 0.5	OVA+CKS 1.0	OVA+CKS 2.0
Total cells (1 × 10 ⁴ cells/ml)	14.0 ± 1.00	59.3 ± 6.43 [#]	26.0 ± 2.65 [*]	21.3 ± 2.31 [*]	15.0 ± 2.00 [*]
Eosinophils (1 × 10 ⁴ cells/ml)	5.00 ± 1.00	57.0 ± 5.57 [#]	27.0 ± 3.61 [*]	18.3 ± 2.52 [*]	11.7 ± 1.53 [*]
Basophils (1 × 10 ⁴ cells/ml)	5.30 ± 1.53	24.0 ± 2.00 [#]	16.0 ± 2.00 [*]	12.3 ± 2.08 [*]	9.70 ± 1.53 [*]
Lymphocytes (1 × 10 ⁴ cells/ml)	14.3 ± 1.53	46.0 ± 2.00 [#]	35.7 ± 2.52 [*]	26.3 ± 3.21 [*]	18.7 ± 1.53 [*]
Macrophages (1 × 10 ⁴ cells/ml)	11.0 ± 1.00	26.0 ± 2.65 [#]	17.7 ± 2.08 [*]	14.7 ± 2.08 [*]	13.0 ± 1.00 [*]

[§] Recovered total cells or differential leukocytes were counted using standard morphological criteria for cytopsin (cells number × 10⁴). At least 200 cells were examined in each cytopsin. PBS-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered CKS 0.5 mg/kg/day (OVA + CKS 0.5), OVA-inhaled mice administered CKS 1.0 mg/kg/day (OVA + CKS 1.0), and OVA-inhaled mice administered CKS 2.0 mg/kg/day (OVA + CKS 2.0). Results represent the means ± S.E.M. of five mice per group.

[#] *p* < 0.05, significantly different from the control group.

^{*} *p* < 0.05, significantly different from the OVA-treated group.

Table 3. Effect of CKS on OVA-induced levels of IgE, Th1 and Th2 cytokines, and MCP-1 chemokine in BAL fluid [§]

	Control	OVA	OVA+CKS 0.5	OVA+CKS 1.0	OVA+CKS 2.0
IgE (ng/ml)	2.80 ± 0.50	16.3 ± 0.52 [#]	7.90 ± 0.70 [*]	5.80 ± 0.31 [*]	4.60 ± 0.54 [*]
TNF- α (pg/ml)	141 ± 3.40	538 ± 56.2 [#]	254 ± 15.4 [*]	203 ± 17.8 [*]	150 ± 14.0 [*]
IFN- γ (pg/ml)	49.7 ± 1.35	66.4 ± 4.04 [#]	59.4 ± 2.37 [*]	53.6 ± 1.81 [*]	49.9 ± 3.04 [*]
IL-4 (pg/ml)	6.50 ± 0.75	35.4 ± 2.38 [#]	21.3 ± 2.50 [*]	17.3 ± 1.12 [*]	12.1 ± 1.75 [*]
IL-5 (pg/ml)	83.9 ± 5.11	562 ± 17.7 [#]	287 ± 21.6 [*]	224 ± 11.7 [*]	163 ± 8.86 [*]
IL-13 (pg/ml)	231 ± 17.8	789 ± 81.3 [#]	493 ± 54.4 [*]	316 ± 27.0 [*]	264 ± 8.62 [*]
MCP-1 (pg/ml)	72.9 ± 1.63	237 ± 28.3 [#]	107 ± 10.2 [*]	88.8 ± 6.37 [*]	74.8 ± 5.98 [*]

[§] Cytokine level in the BAL fluid were measured using ELISA kits. PBS-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), OVA-inhaled mice administered CKS 0.5 mg/kg/day (OVA + CKS 0.5), OVA-inhaled mice administered CKS 1.0 mg/kg/day (OVA + CKS 1.0), and OVA-inhaled mice administered CKS 2.0 mg/kg/day (OVA + CKS 2.0). Results represent the means ± S.E.M. of five mice per group.

[#] $p < 0.05$, significantly different from the control group.

^{*} $p < 0.05$, significantly different from the OVA-treated group.

Table 4. Effect of platycosides and CKS on PMA-induced MUC5AC mRNA expression in A549 cells [§]

Treatments	Relative MUC5AC mRNA to GAPDH (Fold of control)
Control	1.0 ± 0.11
PMA	2.1 ± 0.22 [#]
PMA + Deapioplatycoside E	2.0 ± 0.19
PMA + Platycoside E	2.0 ± 0.20
PMA + Platyconic acid A	1.2 ± 0.11 [*]
PMA + Platycodin D	1.6 ± 0.17 [*]
PMA + 2"-O-Acetylpolygalacin D ₂	1.5 ± 0.16 [*]
PMA + Platycodin D ₃	1.4 ± 0.15 [*]
PMA + CKS	1.3 ± 0.14 [*]

[§] A549 cells were pretreated with platycosides (1 μM) or CKS (1 μg/ml) and then treated with PMA (each 20 nM). After 24 h, total RNA was extracted from the cells and MUC5AC mRNA levels were determined by qRT-PCR. Results represent the means ± S.E.M. of three independent experiments.

[#] *p* < 0.01, significantly different from the control group.

^{*} *p* < 0.01, significantly different from the PMA-treated group.

Fig. 1

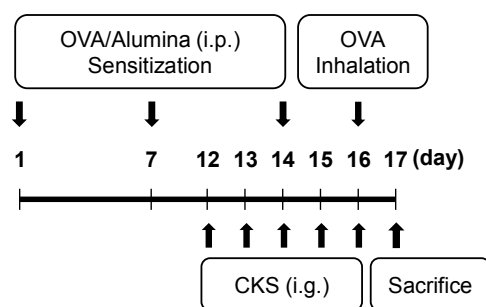


Fig. 2

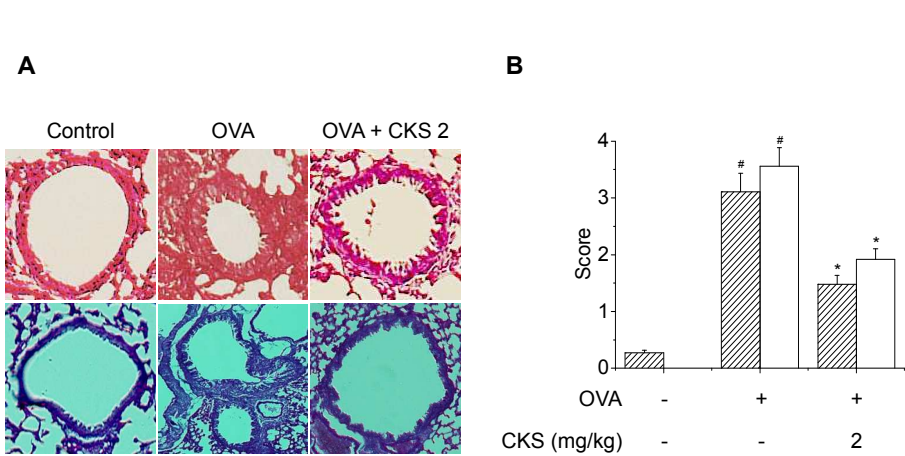


Fig. 3

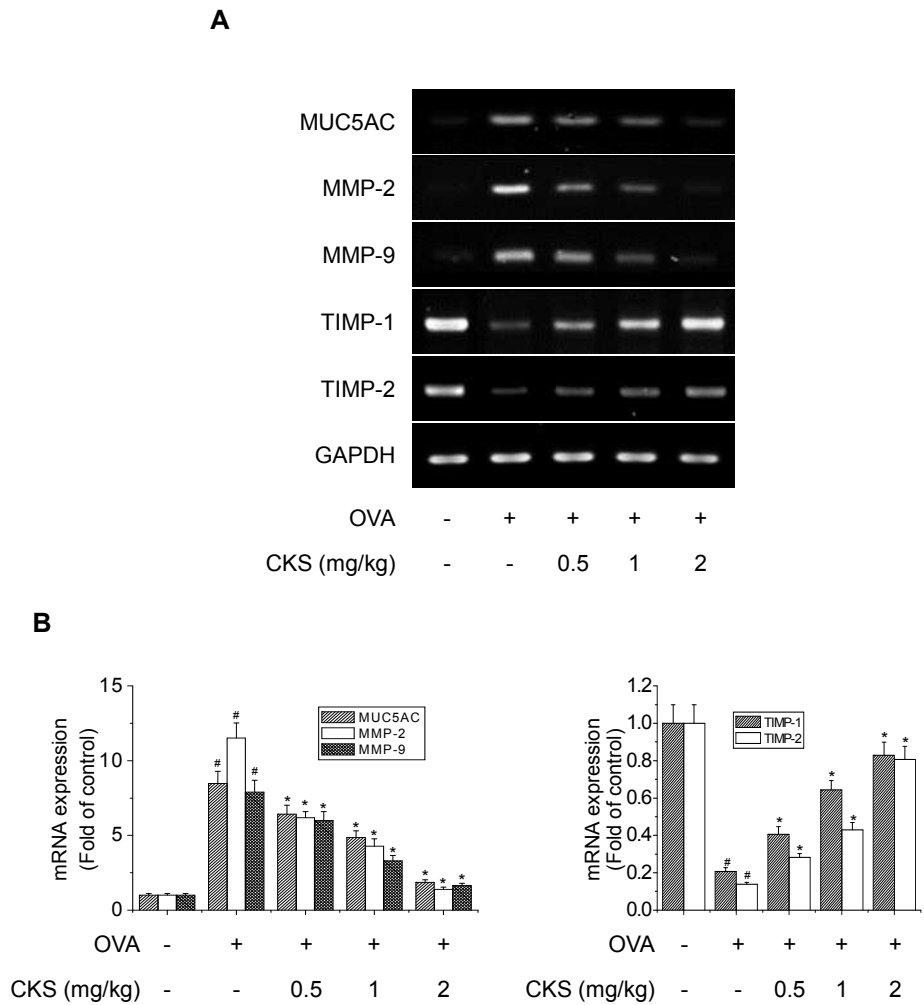


Fig. 4

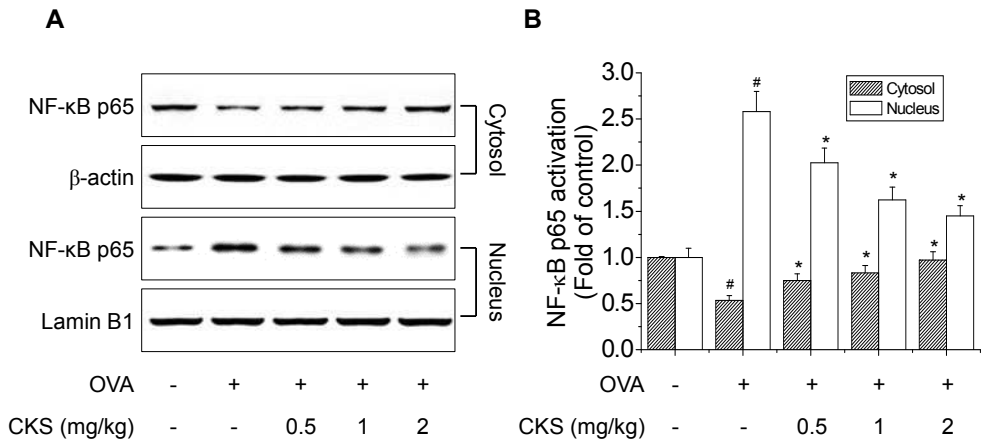


Fig. 5

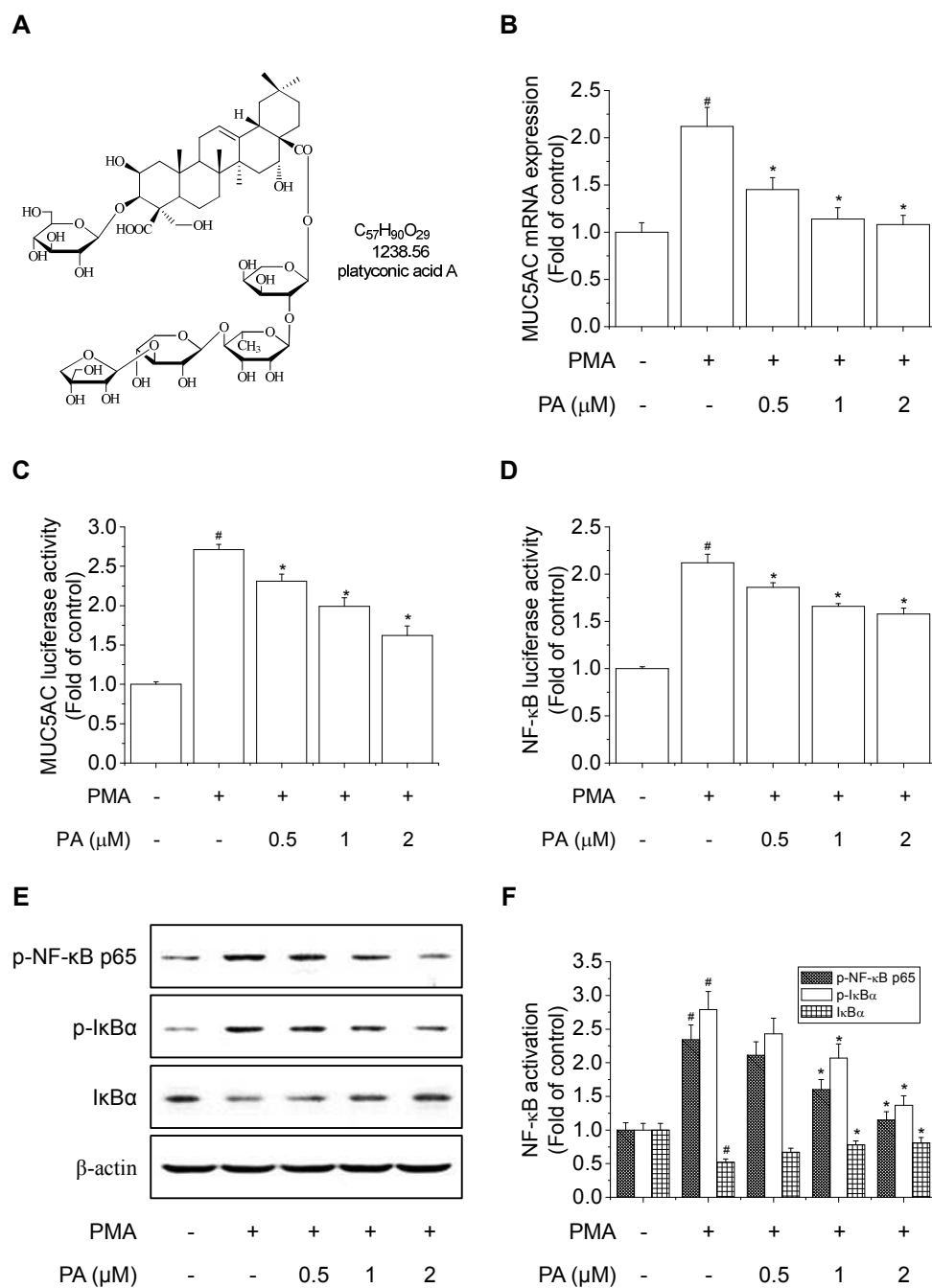


Fig. 6

