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Punicalagin alleviates protease-activated receptor 2-mediated allergic respiratory diseases

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Punicalagin alleviates protease-activated receptor 2-mediated allergic respiratory diseases

Directed by Professor Hyung-Ju Cho

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

Miran Kang

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This certifies that the Master's Thesis
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ABSTRACT

Punicalagin alleviates protease-activated receptor 2-mediated allergic respiratory diseases

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Allergic respiratory diseases such as allergic rhinitis (AR) and asthma are chronic diseases caused by exposure to allergens such as house dust mites (HDM).[1] Protease-activated receptor 2 (PAR2), a member of 7 transmembrane receptors that bind to G-protein coupled receptor, is activated by cleavage by serine proteases.[2,3] When PAR2 is activated, inflammatory cytokines are secreted, causing inflammation and allergic respiratory diseases. We investigated the effect of punicalagin (PCG), a specific PAR2 antagonist in primary human nasal epithelial (HNE) cells and allergic respiratory mouse models to determine its therapeutic ability. First, as a result of treating PCG in

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cells by concentration, it was confirmed that the treatment was effectively inhibited when PCG was treated from 3 μM. In addition, an allergic respiratory model was created in 8-week-old male mice of Par2-wild type (WT) and Par2-knockout (KO) and pretreated with PCG 10 mg/kg. As a result, by treating HDM extract, AR and asthma could be modeled simultaneously, but modeling was not possible when PCG was pretreated. We found that punicalagin specifically inhibits PAR2 in HNE cells. Therefore, we suggest that PCG can be used as a therapeutic agent for allergic respiratory diseases.

Keywords: allergic rhinitis, asthma, protease-activated receptor 2, house dust mite, punicalagin

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Punicalagin alleviates protease-activated receptor 2-mediated allergic respiratory diseases

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I. INTRODUCTION

Allergic rhinitis (AR) is a disease caused by hypersensitivity immune response to exposure to allergens such as house dust mites (HDM).[4] Symptoms such as sneezing, runny nose, stuffy nose, and itching nose appear due to hypersensitivity of the nasal mucosa to antigens.[5] Allergic asthma is a chronic pulmonary disease in which the airways become swollen and narrow, and excessive mucus is produced, making it difficult to breathe. Asthma causes symptoms such as coughing, shortness of breath, tightness, and chest pain.[6] Allergic respiratory diseases such as AR or asthma are chronic diseases characterized by airway hyper-responsiveness and Th2-cytokine-mediated inflammation.

Allergic respiratory diseases are caused environmental factors such as HDM. HDM acts as a major allergen in the pathogenic mechanisms of allergic diseases by inducing immunoglobulin E (IgE) production, cytokine secretion, and induce innate immune activation such as protease-activated receptor 2 (PAR2) that initiates inflammatory signaling.[7,8] PAR2, also called coagulation factor II (thrombin) receptor-like 1 (F2RL1), composed 7 transmembrane receptor binding to G-protein coupled receptor. PAR2 is cleaved at the N-terminal by

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proteases such as trypsin and HDM.[2] The newly exposed tethered ligand binds to the extracellular loop 2 (ECL2) site and is activated.[9] This causes specific changes in morphology and alters the affinity for G-protein in the cell.[10] PAR2 is expressed in airway epithelial cells, fibroblasts, myocytes, sensory neurons, and endothelial cells. It is also present in immune cells such as mast cells, macrophages, dendritic cells, and T cells.[11] When PAR2 is activated, nuclear factor kappa B (NF-κB) and extracellular signal-regulated kinases 1 and 2 (ERK 1/2) are activated, cytokines are expressed and secretions are released, causing inflammation and proliferation.[2,12] In addition, the Ca^{2+} release-activated Ca^{2+} (CRAC) channel is activated by store-operated calcium entry (SOCE) and calcium flows in. For this reason, it regulates the production of major inflammatory cytokines such as interleukin-6 (IL-6), IL-8, and thymic stromal lymphopoietin (TSLP) through Ca^{2+} signaling. The CRAC channel acts as a major regulator for the activation of allergic inflammation, and Ca^{2+} influx regulates various functions including gene expression, cell proliferation, and differentiation.[13,14] PAR2 is activated by inhaled allergens and stimulates the production of various allergen-induced inflammatory mediators involved in pathogenesis. In addition, PAR2 agonists induce contraction of bronchioles, a major site of resistance to the bronchi and airflow.[15-17] Therefore, PAR2 acts as one of major factors in allergic respiratory diseases.

PAR2 inhibitors were developed and have been evaluated for the treatment of PAR2-mediated diseases and their pathophysiology. However, an effective drug capable of selectively inhibiting PAR2 at a low concentration as an actual drug has not yet been developed. Recently, a candidate qualified for selectively inhibiting PAR2 has been discovered, suggesting the application of a therapeutic agent for diseases related to PAR2.[12] Therefore, in this study, we aimed to verify its beneficial effect on allergic respiratory inflammation and investigate the PAR2-mediated mechanism of allergic respiratory inflammation.

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II. MATERIALS AND METHODS

1. Animal

Experiments were performed with the approval of the Institutional Animal Care and Use Committee of Yonsei University (2018-0168). Par2-transgenic mice were provided by the Korea Research Institute of Bioscience and Biotechnology. Animals were reared in accordance with the environmental stipulations outlined by the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine. Experiments were conducted with wild type (WT) and knockout (KO) mice produced by mating the parental generation. The genotype of the offspring was confirmed through polymerase chain reaction. DNA electrophoresis was performed with Mupid-exU (Takara Bio, Seoul, Korea) to confirm the genotype of mice. It was confirmed that the band appeared at 345 bp in Par2-wt and 198 bp in Par2-ko mice (Table 1).

2. Mouse modeling of allergic rhinitis and asthma

The mouse model was developed in accordance with a previously published method.[18] In brief, 8-week-old male C57BL/6 mice were used as pups of Par2-wt and Par2-ko mice. AR and asthma were simultaneously time induced by performing a total of 3 sensitizations and 4 challenges under sedation. For sensitization, a mix HDM extract (100 μg/ml) (Greer Laboratories Inc, NC, USA) and Alum (2 mg/ml) (Thermo Scientific, MA, USA) in phosphate-buffered saline (PBS) on 0 day (d), 7 d, and 14 d and injected 200 μl by intraperitoneally once a week for a total of 3 times. After seven days, the challenging shot were injected for 4 consecutive days from 21 d to 24 d. Because we had to model AR and asthma together, anesthesia was performed with isoflurane (Ifrane, Hana Pharm, Gyeonggi, Korea). After anesthesia the, mice injection of 50 μl of PBS or HDM extract 1 mg/ml into the nasal were

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performed. Punicalagin (PCG) was administered to determine the therapeutic effect of PAR2 inhibition. The PCG, which was discovered through high-throughput screening[12], was provided by Prof. Namkung. The treatment group was injected intraperitoneally with PCG (10 mg/kg, 50 μl) from day 20 to 24 (Figure 1). Finally, the mice were euthanized by inhalation of CO_2 gas and then nasal and lung tissues were harvested.

3. Measurement of airway hyper-responsiveness

Airway hyper-responsiveness (AHR) to inhaled metacholine was measured in mouse model. Before measurement, adaptation for 2 weeks to prevent stress during measurement. On day 25 after modeling protocol, AHR was measured. After putting each mouse in the chamber, AHR to inhaled PBS (Lonza, Basel, Switzerland) or methacholine (Sigma, Kanagawa, Japan) was measured. Methacholine (0, 6.25, 12.5 25, and 50 mg/ml) was administered sequentially from low to high concentrations. At the end of one cycle of measurement, the inside of the machine was cleaned and used before starting the next cycle.

4. Immunoglobulin E level measurement by ELISA

On day 26, blood serum was collected from mice, serum was separated and the serum immunoglobulin (Ig) E levels were ascertained using total IgE and HDM-specific IgE enzyme-linked immunosorbent assay (ELISA). Total IgE ELISA was measured according to the product's protocol of the mouse IgE uncoated ELISA kit (Invitrogen, MA, USA). HDM-specific IgE ELISA was measured according to the product's protocol of the mouse serum anti-HDM IgE antibody assay kit (Chondrex, WA, USA).

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5. Histopathology

After the blood is collected, perfusion is performed. It serves to drain the blood with PBS and fixes it with 4% paraformaldehyde (PFA) (Biosesang, Gyeonggi, Korea). The head and lung were harvested and stored in 4% PFA for fixation for 4 days. Decalcification was performed by replacing with 10% Ethylene-diamine-tetraacetic acid (EDTA) (Biosesang, Gyeonggi, Korea) for 20 days. Finally, tissue samples were fixed again with 4% PFA. The head including the nasal cavity, and the lung tissues were sectioned and embedded in paraffin blocks. The paraffin blocks were trimmed and sectioned in 4 μm slices and mounted on slides. Thereafter, hematoxylin and eosin (H&E) staining, Periodic-acid-Schiff (PAS) staining, and Sirius red (SR) staining were performed for each group, and the differences between the groups were observed and compared with an Olympus camera.

6. Immunofluorescence

The sectioned slides were placed in xylene (Duksan, Gyeongju, Korea) and 100% to 70% ethanol (Duksan) for deparaffinization. Steaming antigen retrieval was performed for 40 minutes with epitope retrieval solution (IW-1100-1L, IHC-TeK™). We marked a circle around the tissue using a PAP pen (IHC WORLD, Gyeonggi, Korea) to preserve the solution on the tissue during cooling. We inactivated endogenous peroxidase with 3%, peroxidase-blocking solution (DAKO, CA, USA)**.** The slides were blocked with 5% bovine serum albumin (BSA, Sigma) for 1 hour at room temperature and incubated with the primary antibody, Muc5ac (Invitrogen, MA, USA) at 1:500 at 4 ˚C overnight. The secondary antibody was rinsed three times with 1X tris-buffered saline (TBS), diluted 1:500 with goat anti-mouse Alex 568, and incubated for 30 minutes. Afterward, mount with Fluoromount™ with DAPI (F6057-20ML, Sigma). Confocal images were taken with a 40X lens using a Carl Zeiss LSM 700 microscope and processed using ZEN software.

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7. Bulk RNA sequencing

The nasal mucosa and lung lobe of the mice were isolated for bulk RiboNucleic Acid-sequencing (RNA-seq). It is necessary to measure as accurately as possible so that there is no blood on the tissue. Tissue samples were rinsed gently in the saline to remove blood as much as possible and were placed in the RNA later solution (Invitrogen, Carlsbad, CA) and then stored at -20˚C. Analyses were conducted one by one for each specimen in each group. The samples were sent to Macrogen© (Seoul, Korea) for the analysis. We classified and targeted cytokines, goblet cell markers, chemokines, signaling pathways, ion channels, and epithelial-mesenchymal transition (EMT) markers. Among the cytokines, Interleukin 4 (Il-4), Il-5, Il-6, Il-10, Il-13, Il-17, Il-33, and interferon gamma (Ifn-ɣ) were mainly analyzed. The following goblet cell markers were analyzed: Muc5ac, Cst1, Spdef, Fetub, Tspan8, Agr2, Ifi35, Serpinb2, and Timp1. The following chemokines were analyzed: Ccr5, Ccl2, Ccl11, Ccl20, Ccl24, Cxcr4, and Cxcl12 were analyzed. In the signaling pathway, Gata3, Tlr2, Tlr4, Mapk, Rela, phospho65, NF-κB, Tnf, Pi3k-Akt, Egfr, and Jak-Stat were analyzed. Cftr, Soce, and ENaC-β were analyzed in the ion channel. Finally, PD-L1, E-cadherin, Vimentin, and Tgf-β were interpreted as EMT markers.

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8. Primary human nasal epithelial cell culture

Primary human nasal epithelial (HNE) cells were obtained from nasal polyp. The harveste nasal polyp was moved in the transfer media from operation room the red blood celland incubated in the 5 ml of 0.1% protease overnight at 4˚C. Epithelial cells were isolated by scraping the surface of polyp and cultured in a 100 ø dish (Corning, NY, USA). In the process of passage-0 and passage-1, 100 ø dishes were used for culture, and they were moved to Costar 3460 transwell 12mm plate (Corning, ME, USA) for passage-2. Subculture medium included a mixture of bronchial epithelial cell growth medium (BMGM) Bulletkit (Lonza, Basel, Switzerland), 150 mg/ml of bovine serum albumin (BSA) (Sigma, MO, USA), and epidermal growth factor and it was used for passage-0 and -1 culture. Cell culture for passage-2 was performed using a filtered culture medium by mixing 150 mg/ml of BSA in the BEGM Bulletkit, called +RA+BPE medium. In this case, BEGM is mixed with Dulbecco modified eagle medium in a 1:1 ratio. After each passage, cells were removed from the plate with 0.25% trypsin-EDTA (TE) (Gibco, Seoul, Korea) and neutralized with trypsin neutralizing solution (TNS) (Lonza, Basel, Switzerland) after 1-2 min. When the cells were full at passage-2, airway liquid interface (ALI) was started by removal of culture medium in the apical part of cells. During the ALI, only the basolateral side was filled with a culture medium for 2 weeks to be fully differentiated.

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9. Western blot and immunoblotting

Phosphor-ERK1/2 (p-ERK1/2), total ERK1/2 (t-ERK1/2), phosphor-P65 (p-P65), and total P65 (t-p65) were analyzed by western blot. Indicated concentrations of PCG were applied 30 min before PAR2 activation by PAR2-activating peptide (PAR2-AP). The indicated concentrations of PCG were applied 30 min before PAR2 activation by PAR2-AP. (mean ± SEM, n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

HNE cells were washed twice PBS and lysed using a cell lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, and protease inhibitor mixture) on ice. Whole-cell lysates were centrifuged at 13,000x g for 20 min at 4˚C, and protein extracts were separated on 4-12% Tris-glycine precast gels (Komabiotech, Seoul, Korea) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 3% BSA in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h and then, incubated with anti-ERK1/2 (9101, 1:1000; Cell signaling, Danvers, MA, USA), anti-p-ERK1/2 (9102, 1:1000; Cell Signaling), anti-P65 (sc-8008, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-p-P65 (sc-136548, 1:1000; Santa Cruz Biotechnology) antibodies. After washing the membranes three times with TBST, the membranes were incubated at room temperature for 1 h with the appropriate HRP-conjugated secondary antibody. Protein levels were detected with an ECL reagent using the FUSION SOLO imaging system (Vilber Lourmat, Marne-la-Vallée, France). Image J was used to quantify band intensity.

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10. Multiplex assay

After differentiating the HNE cells, the apical and basolateral medium were ++RA+BPE medium to hold the zero point for 12 hours. In apical and basolateral baths, PCG was treated by concentration for 30 minutes, and HDM 100 μl is treated for 6 hours, and each soup is collected. All collected samples were entrusted to KomaBiotech Inc. for cytokine analysis according to the Human XL Cytokine Premixed Kit (R&D, catalog number FCSTM18).

11. Statistical analysis

All data were conducted in two or more replicate experiments. Statistical comparisons were performed based on Student's t-test or one-way analysis of variance (ANOVA) using Graphpad Prism 8.0 software (San Diego, CA, USA). A p-value less than 0.05 was considered statistically significant.

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Table 1. Primer sequence information used to identify the genotype of protease-activated receptor 2 transgenic mice

No.	Primer sequence (5' to 3')
olMR7415	GCC AGA GGC CAC TTG TGT AG
olMR7419	TCA AAG ACT GCT GGT GGT TG
olMR7420	GGT CCA ACA GTA AGG CTG CT

The table shows sequences (5' to 3') required to represent the Par2 transgenic mouse genotype. Information was provided by the Korea Research Institute of Bioscience and Biotechnology (KIRBB).

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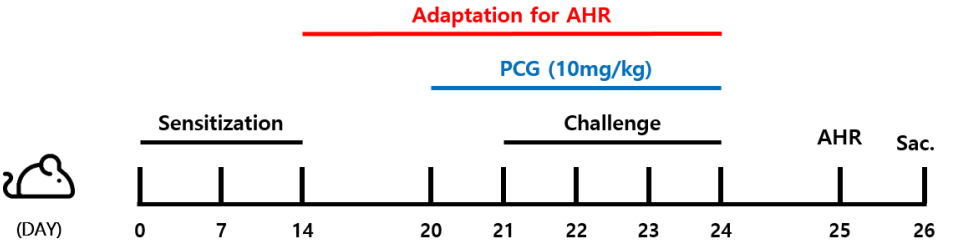

Figure 1. Experimental protocol for allergic rhinitis and asthma mouse model

Mice were sensitized three times and challenged for four consecutive days after a week following sensitization. For sensitization, 200 μl of a mixture HDM extract (100 μg/ml) and Alum (2 mg/ml) in PBS was injected intraperitoneally. For the challenge, 50 μl of HDM extract 1 mg/ml or PBS were administered intranasally under anesthesia. Punicalagin (PCG) 50 μl were pretreated by intraperitoneal administration from one day to the end of the challenge. AHR was conducted on the day after last challenge. Mice were sacrificed at the end of the whole schedule for obtaining tissue and blood samples were harvested.

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III. RESULTS

1. Measurement of airway hyper-responsiveness in allergic mouse model

Using methacholine, five consecutive measurements were performed sequentially from low concentration to high concentration (0, 6.25, 12.5, 25, 50 mg/ml). All results were expressed in enhanced pause (Penh) values. As a result of comparing the differences between the groups as a whole, it was confirmed that the Penh level was increased when allergic asthma developed **(Fig. 2A)**. However, pretreatment with PCG lowered Penh levels in response to 25 mg/ml **(Fig. 2B)** or 50 mg/ml **(Fig. 2C)** of methacholine inhalation in WT allergy group. Also, no significant difference in Penh level was noted in Par2-ko mouse regardless of allergy status.

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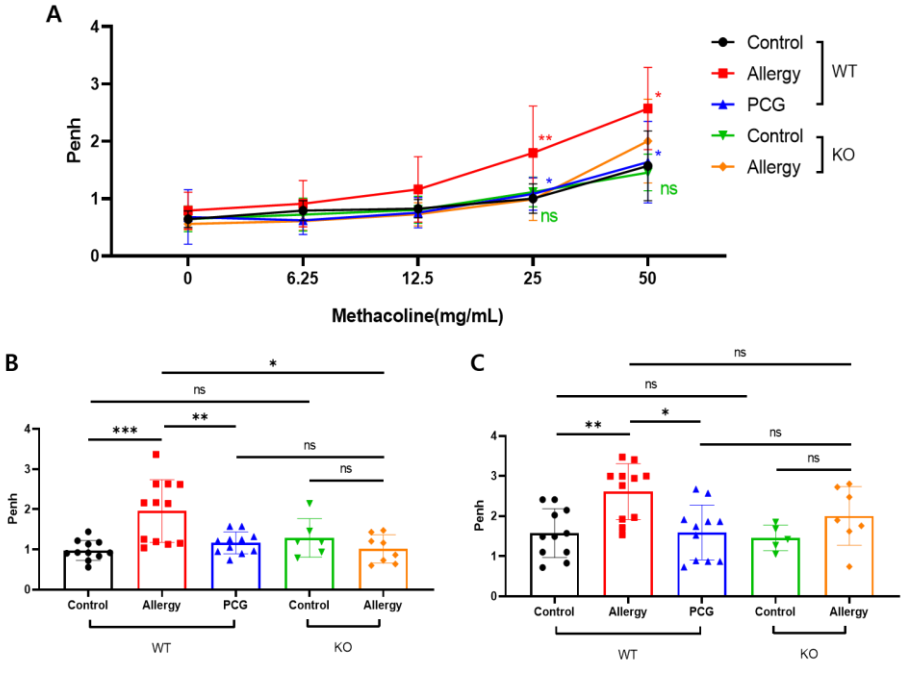

Figure 2. Analysis of airway hyper-responsiveness in asthma model

Penh was measured in response to inhaled methacholine from 0 mg/ml to 50 mg/ml (group n=5). **(A)** Plots showing Penh levels to the dose-dependent stimulation of methacholine in all groups. From 25 mg/ml, asthma models developed higher airway resistance compared to control group. The Penh was suppressed when treated with PCG or Par2-knouked out. **(B)** Penh measured at 25 mg/ml of methacholine. The airway resistance level was significantly higher in the asthma group than in control and PCG group in WT mouse. In Par2-ko mouse, asthma group showed no significant difference in Penh level compared to control group. **(C)** Penh measured at 50 mg/ml of methacholine. The airway resistance level was significantly higher in the asthma group than in control and PCG group in WT mouse. In Par2-ko mouse, asthma group showed no significant difference in Penh level compared to control group.

**P<0.05; **P<0.01; ***P<0.001*

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2. Levels of IgE in serum

The IgE levels in mouse serum were determined through total and HDM-specific IgE ELISA to confirm the allergy model had been well prepared **(Fig. 3)**. As a result of establishing the level of total IgE by ELISA, the value significantly increased in the allergy model of Par2-wt. Whereas, the PCG group showed a significant decrease compared to the allergy group in Par2-wt and it was similar to control group in Par2-wt and Par2-ko AR/asthma group were similar. Similar results of HDM-specific IgE were also noted as shown in **Fig.3B**. In Par2-ko mice, the AR/asthma group showed similar level of HDM-specific IgE as the control group **(Fig. 3B)**.

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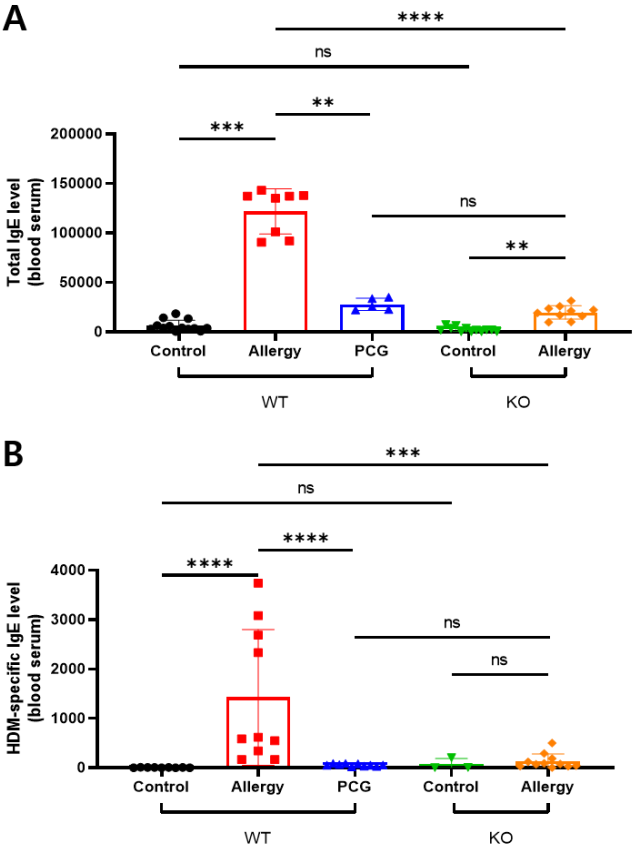

Figure 3. Levels of total and HDM-specific IgE in serum in Par2-wt and –ko mouse models

(A) Comparison of serum total IgE levels. In Par2-wt mice, the AR/asthma group showed increased total IgE levels but this was suppressed in the PCG group. In Par2-ko mice, AR/asthma group showed similar total IgE levels to the PCG group in Par2-wt mice, although levels were higher than those of the control group. **(B)** Comparison of serum HDM-specific IgE levels. In Par2-wt mice, AR/asthma group showed increased HDM-specific IgE level and it was suppressed in the PCG group. In Par2-ko mice, HDM-specific IgE levels were not significantly lower than those of the AR/asthma group in Par2-wt mice.

**P<0.05; **P<0.01; ***P<0.001*

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3. Histopathologic results of nasal and bronchial epithelium in mouse model

In Par2-wt mice, the allergy group showed thickened nasal and bronchial epithelium by H&E staining and increased numbers of PAS-positive goblet cells compared to the control and PCG groups **(Fig. 4A, 5A)**. To confirm that allergic inflammation developed in nasal and bronchial epithelium, eosinophils were stained with SR. The allergy group in Par2-wt mice also showed increased number of eosinophils compared to control or PCG groups. In Par2-ko mice, the allergy group showed increased epithelium thickness, and higher number of goblet cells, and eosinophils than the control group, but they were still lower than the allergy group in Par2-wt mice **(Fig. 4B, 5B)**. We quantified these by counting their numbers in the histologic slides to compare the change of goblet cells and eosinophils number and statistically confirmed that Par2 mediates goblet cell hyperplasia and tissue eosinophilia in the allergic respiratory diseases mouse model **(Fig. 4C, 4D)**.

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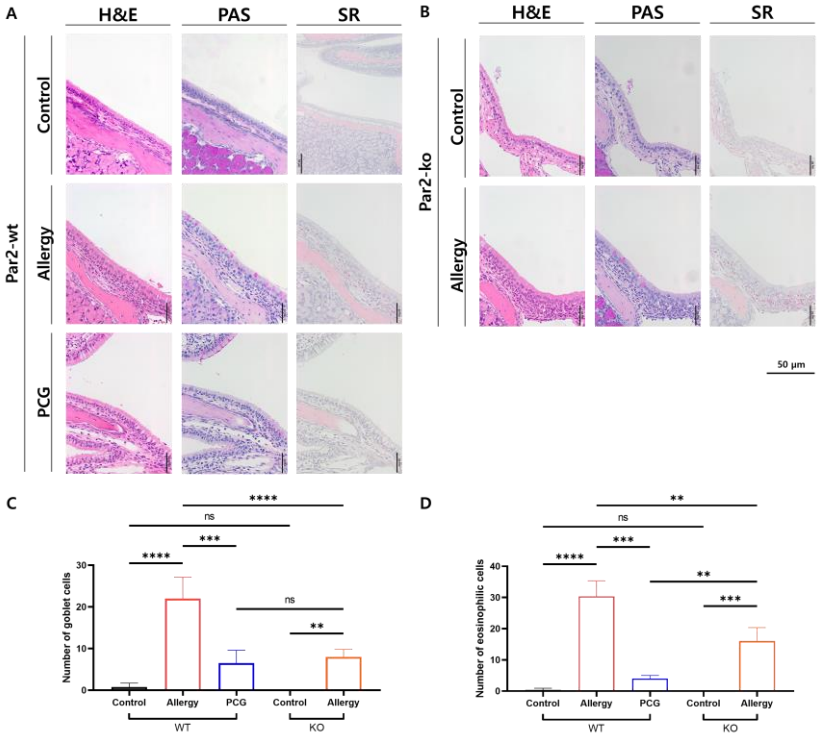

Figure 4. Histopathologic results of nasal epithelium in mouse model

(A) In Par2-wt mice, the allergy group showed thickened nasal epithelium by H&E staining, increased goblet cells by PAS staining, and increased eosinophils by Sirius red (SR) staining compared to the control or PCG group. **(B)** In Par2-ko mice, the allergy group showed a similar number of goblet cells to the PCG group in Par2-wt mice, although it was higher than the control group in Par2-ko mice. **(C)** Comparison of goblet cell number among groups in Par2-wt and –ko mice. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower goblet cell number than the allergy group in Par2-wt mice. **(D)** Comparison of eosinophil number among groups in Par2-wt and –ko mice. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower eosinophil number than the allergy group in Par2-wt mice.

*$P \leq 0.05$; **$P \leq 0.01$; ***≤ 0.001; ns> 0.05

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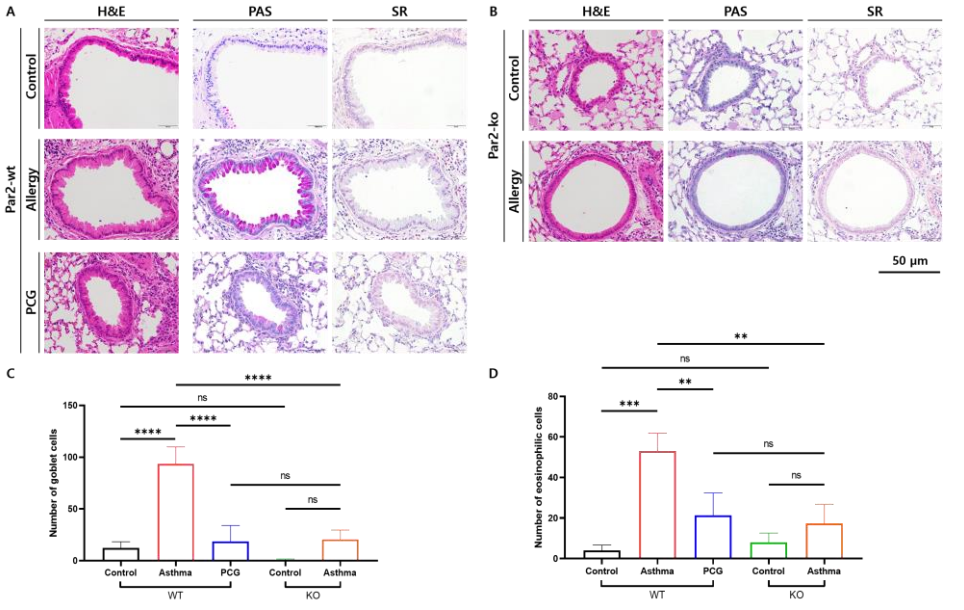

Figure 5. Histopathologic results of bronchial epithelium in mouse model

(A) In Par2-wt mice, the allergy group showed thickened bronchial epithelium by H&E staining, increased goblet cells by PAS staining, and increased eosinophils by Sirius red (SR) staining compared to the control or PCG group. **(B)** In Par2-ko mice, both control and allergy group showed a low number of goblet cells and eosinophil. **(C)** Comparison of goblet cell number among groups in Par2-wt and –ko mice. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower goblet cell number than the allergy group in Par2-wt mice. **(D)** Comparison of eosinophil number among groups in Par2-wt and –ko mice. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower eosinophil number than the allergy group in Par2-wt mice.

*Scale bar, 50 μm; *P<0.05; **P<0.01; ***P<0.001*

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4. Muc5ac expression in nasal and bronchial epithelium in mouse model

Expression of Muc5ac, one of major secretor mucin, was determined in nasal and bronchial epithelium through confocal microscopy imaging. The allergy group in Par2-wt mice showed overexpression of Muc5ac and it was suppressed in PCG group **(Fig. 6A, 6B)**. In Par2-ko mice, Muc5ac expression in the allergy group was few and similar to the control group **(Fig. 6C, 6D)**. These results were quantified using ImageJ through image analysis. In Par2-wt mice, the PCG group showed significantly suppressed of Muc5ac expression than the allergy group in both nasal and bronchial epithelial cells **(Fig. 6E, 6F)**. The allergy group in Par2-ko mice also showed suppressed Muc5ac expression, similar to the control group or PCG group in Par2-wt mice **(Fig. 6E, 6F)**. These results suggest that Muc5ac overexpression can be regulated through PAR2-midiated mechanism in allergic respiratory diseases.

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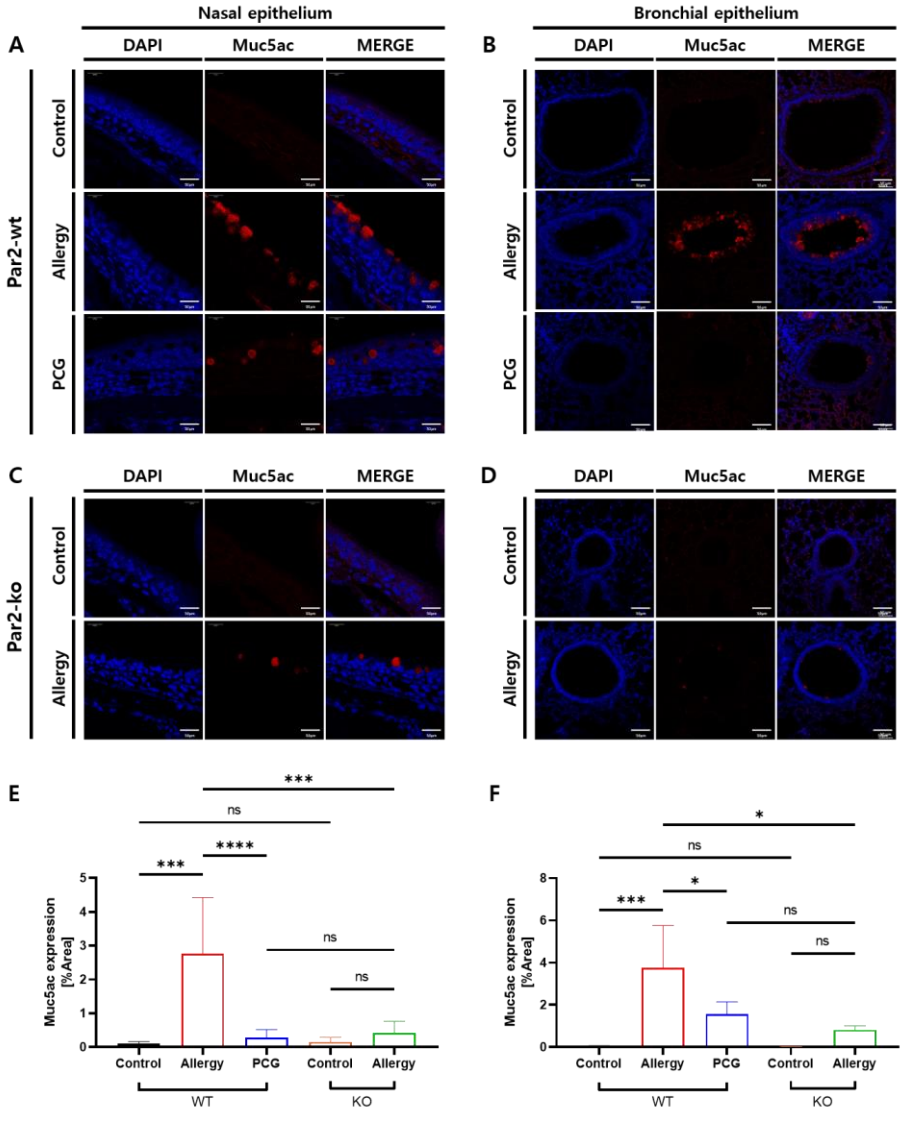

Figure 6. Muc5ac expression in nasal and bronchial epithelium in mouse model

(A) In Par2-wt mice, the PCG group showed more suppressed Muc5ac expression in nasal epithelium than the allergy group. **(B)** In Par2-wt mice, the PCG group showed suppressed Muc5ac expression in the bronchial epithelium than the allergy group. **(C)** In Par2-ko mice, the allergy group showed similar Muc5ac expression in nasal epithelium to the control group. **(D)** In Par2-ko

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mice, the allergy group showed similar Muc5ac expression in the bronchial epithelium to the control group. **(E)** Comparison of Muc5ac expression in the nasal epithelium among groups. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower Muc5ac expression than the allergy group in Par2-wt mice. **(F)** Comparison of Muc5ac expression in the bronchial epithelium among groups. The PCG group in Par2-wt mice and the allergy group in Par2-ko mice showed a significantly lower Muc5ac expression than the allergy group in Par2-wt mice.

Scale bar, 50 μm; Blue, DAPI; Red, Muc5ac;

**P<0.05; **P<0.01; ***P<0.001*

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5. Bulk RNA-seq analysis in the nasal and lung tissues

Bulk RNA sequencing was performed to compare the alteration of genes which are associated with PAR2. The nasal turbinate mucosa and lung tissues from mouse models were harvested and analyzed. Major genes which are related to allergic inflammation were selected as following categories such as Th2 cytokines, goblet cell markers, chemokines, signaling pathways, ion channels and EMT for the comparison of gene transcriptomes. The nasal and lung tissues of Par2-wt mice, the PCG group had lower expression of most genes than the allergy group. The allergy group in Par2-ko mice showed higher gene expression than control group, but they were still lower than allergy group in Par2-wt mice. These results imply that Par2 is associated with various inflammatory process including Th2 cytokines, airway remodeling, cell proliferative inflammation, and mucus production.

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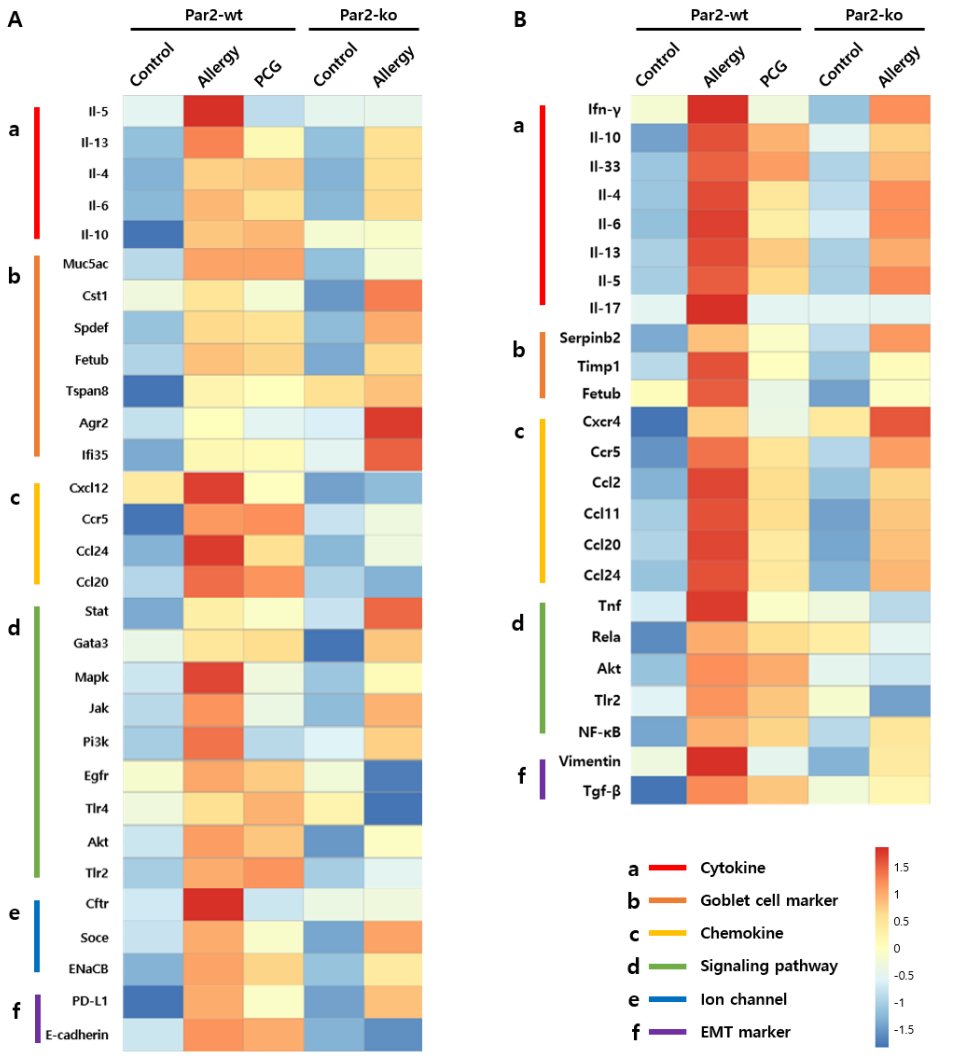

Figure 7. Bulk RNA-seq analysis in nasal and lung tissues of mouse model

(A) Heatmap comparing gene expression of nasal tissue among groups. **(B)** Heatmap comparing gene expression of lung tissue among groups.

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6. PAR2-mediated ERK1/2 and NF-κB signaling pathway

To determine the important signaling pathway which is associated with cell proliferation and inflammation, p-ERK and p-P65 were identified whether they are related PAR2 under HDM extract stimulation in HNE cells.

Western blot was performed to determine whether pretreatment with PCG inhibited the protein levels of p-ERK and p-P65. It was also shown whether the protein of total ERK 1/2 and total P65 was also affected. Stimulation with HDM extract activated p-ERK 1/2 and it was suppressed by PCG in dose dependent manner. The inhibitory effect of PCG was noted significantly from 3 μm concentration **(Fig. 8A, 8B)**. The protein level of P65, a type of NF-κB, was also confirmed. Stimulation with HDM extract activated p-P65 and it was suppressed by PCG in dose dependent manner. The inhibitory effect of PCG was also noted significantly from 3 μM concentration **(Fig.8C, 8D)**. These results indicate that PAR2 mediates the signaling pathways of ERK 1/2 and NF-κB, which are involved in proliferation and inflammation.

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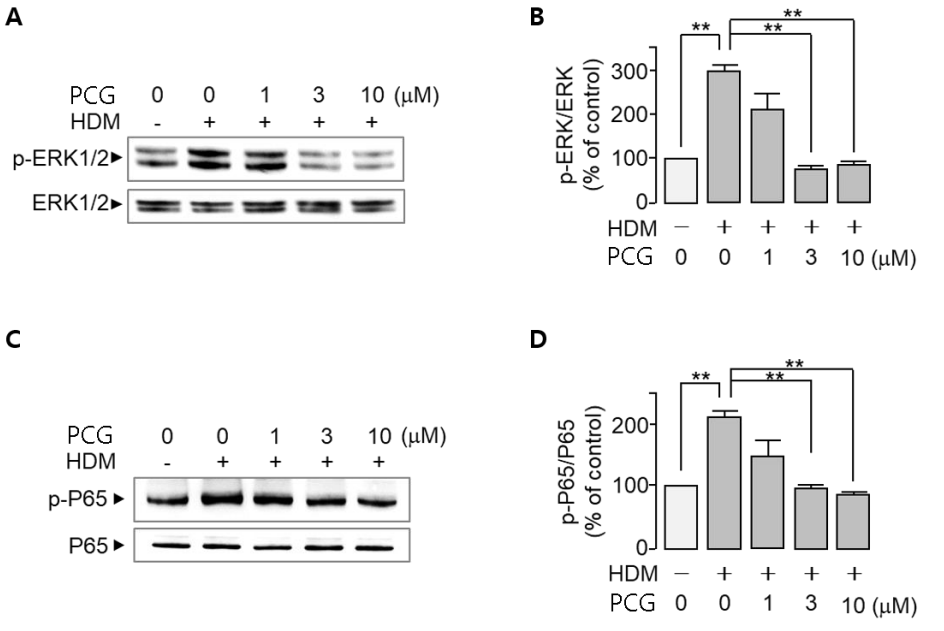

Figure 8. Inhibition of p-ERK and p-P65 by PCG in HNE cells

(A) The p-ERK was overexpressed by HDM stimulation and it was suppressed by PCG in dose-dependent manner. **(B)** The inhibitory effect of PCG was noted significantly from 3 μM. **(C)** The p-P65 was overexpressed by HDM stimulation and it was suppressed by PCG in dose-dependent manner. **(D)** The inhibitory effect of PCG was noted significantly from 3 μM.

***P<0.01*

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7. PAR2-mediated cytokines released from human nasal epithelial cells

The respiratory epithelial cell has an essential role in secreting various cytokines which are associated with allergic inflammation. To find essential cytokines secreted from epithelial cells by HDM stimulation, we performed Multiplexing cytokine assays from the apical and basolateral secretion of HNE cells. In addition, PCG was treated to reveal a PAR2-mediated mechanism. When stimulated by HDM extract, the most significant cytokine from the HNE cell was IL-33, followed by TNF-alpha, IL-7 and IL-6 in apical and basolateral sides **(Fig. 9A, 9B)**. The HDM-induced cytokines were suppressed by PCG in a dose-dependent manner from 1 μM. The protein level of TSLP was measured separately by ELISA assay because TSLP was not involved in the Multiplexing cytokine panel. The TSLP was measured in apical and basolateral secretion and cell lysate as well. The stimulation with HDM extracts induced TSLP expression significantly from 1hr in the cell lysate and basolateral side. In the apical secretion, TSLP was increased considerably by HDM stimulation from 6hr **(Fig. 9C, 9D, 9E)**. Subsequently, PCG was co-treated with HDM extract and TSLP level was measured in apical and basolateral side. The PCG suppressed TSLP secretion significantly from 3 μM on the apical side and 1 μM on the basolateral side **(Fig. 9F, 9G)**. These results also indicate that TSLP secretion in the respiratory epithelial cells is mediated through the PAR2 signaling pathway.

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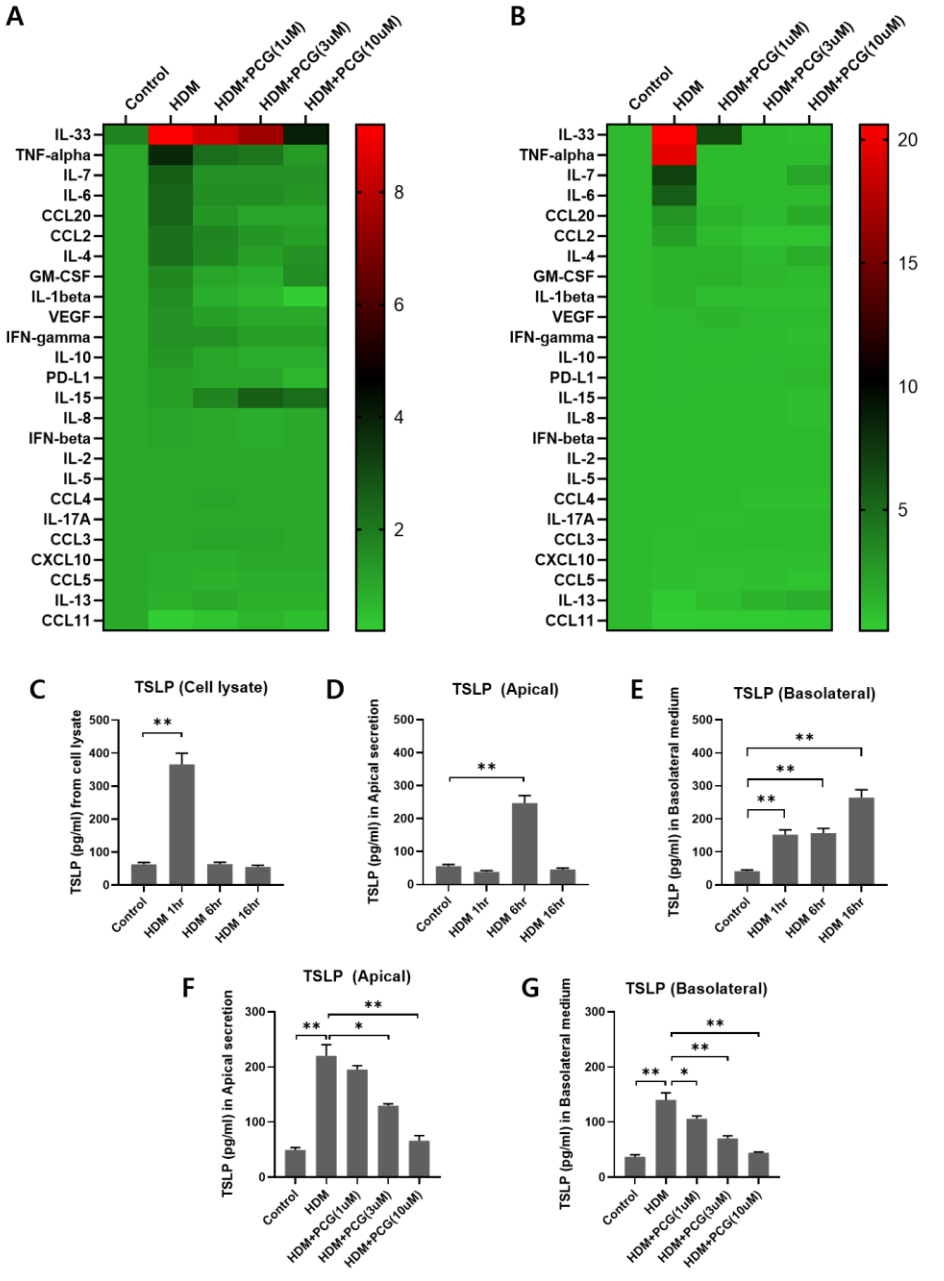

Figure 9. PAR2-mediated cytokine secretion from human nasal epithelial cells

(A) Cytokine profiles in the apical secretion measured by Multiplexing cytokine

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assay. **(B)** Cytokine profiles in the basolateral secretion measured by Multiplexing cytokine assay. **(C)** TSLP protein level in cell lysate measured by ELISA when stimulated with HDM extracts. **(D)** TSLP protein level in apical secretion measured by ELISA when stimulated with HDM extracts. **(E)** TSLP protein level in basolateral secretion measured by ELISA when stimulated with HDM extracts. **(F)** TSLP protein level in apical secretion measured by ELISA when HDM was co-treated with PCG in dose-dependent manner. **(G)** TSLP protein level in basolateral secretion measured by ELISA when HDM was co-treated with PCG in dose-dependent manner.

*$P<0.05$, **$P<0.01$

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IV. DISCUSSION

PAR2 is known to induce allergic respiratory diseases such as AR and asthma when the N-terminal is cleaved and activated by allergens.[3] Cytokines that cause inflammation are expressed through various signaling pathways. There have been some studies proving PAR2-mediated pathophysiology in allergic asthma mouse models. A study which used cockroach extract-induce allergic asthma mouse model.[17] They showed that pretreatment with ENMD-1068, a small molecule antagonist of PAR2, effectively suppresses asthma in an allergic asthma phenotype.[17] ENMD-1068 was demonstrated to inhibit PAR2-mediated Ca2+ signaling and inhibited p38, a type of MAPK.[17] In other studies, inflammation was strongly inhibited by selective PAR2 antagonists AZ3451 and AZ8838, and PAR2-mediated signaling pathways and related functions were also inhibited by compound I-191.[9,19] However, these antagonists have a limitation in that they consists of a compound that non-selectively inhibits PAR2.

We used PCG, a novel PAR2-specific antagonist with high efficacy, extracted from natural products discovered through high-throughput drug screening. As proved by a previous report, PCG could specifically block PAR2 activation with an IC_{50} less than 3 μM.[12] In addition, PCG had a therapeutic effect on kidney disease by inhibiting PAR2.[12] In the current study, we tested primary HNE cells to determine the effective treatment concentration of PCG. Activation of PAR2 promotes cell proliferation and migration through intracellular signaling pathways by activating MAPK signaling pathways including ERK, p-38, and JNK.[20] P38 promotes the expression of NF-κB.[21] Accordingly, inhibition of PAR2 has a therapeutic effect by suppressing those signaling pathways.

We also confirmed PAR2-mediated secretion of alarming cytokines such as IL-33, and TSLP in the human HNE cells. Those cytokines were released from

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the cell onto the apical or basolateral sides. These cytokines are key factors for the initiation of allergic inflammation when epithelial cells exposed to exogenous allergens. Therefore, inhibition of alarming cytokine release can be an important therapeutic method for allergic respiratory inflammation.

A prior study which investigated whether asthma was generated in Par2-ko mice using HDM extract. They confirmed that the Par2-ko mice showed decreased mucus overproduction compared to Par2-wt mice.[16]

In our study, we established the HDM-induced AR/asthma model and applied the protocol in Par2-wt and Par2-ko mice. In addition, we also investigated PAR2-specific inhibitor effect using PCG in AR/asthma mouse model and compared its phenotype. Treatment with PCG, a PAR2-specific antagonist, showed similar results to PAR2-KO mice in airway hyper-responsiveness, histology, and various allergy-related genes. Another interesting finding in our study was the suppression of total and HDM-specific IgE in serum by PAR2 inhibition. This means that PAR2 inhibition can block HDM-induced sensitization, which is a first step of allergic inflammation. Therefore, PAR2 inhibition can have a preventive and therapeutic effect on allergic respiratory inflammation.

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V. CONCLUSION

We conducted experiments on primary human nasal epithelial cells and allergic airway mouse models using PAR2-ko to determine the therapeutic effect of punicalagin, a novel specific PAR2 inhibitor, in allergic respiratory diseases and screened its associated mechanism. This PCG inhibits the activity of PAR2 by preventing allergens such as HDM from cleaving the N-term. First, the optimal efficacy was demonstrated by treating various concentrations of PCG in HNE cells. Since most asthmatics also suffer from rhinitis, we prepared allergic rhinitis and asthma model together in mice. We successfully established a mouse model of allergic rhinitis and asthma simultaneously by installing HDM into the nostril under anesthesia using isoflurane. In PAR2-WT, the PCG group showed suppressed phenotypes of allergic inflammation in both nose and lungs. In addition, PCG showed similar results to PA2-ko mice suggesting PCG as a potential drug candidate for reducing allergic airway inflammation.

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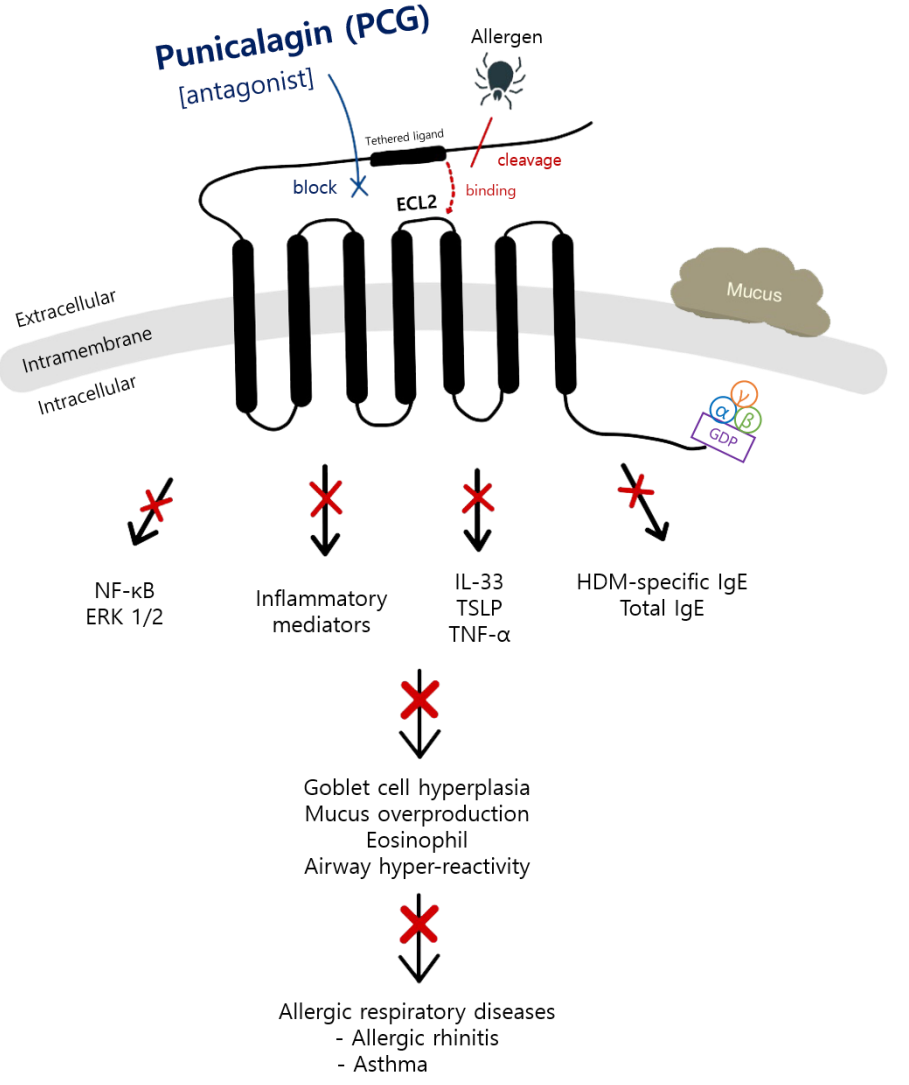

Figure 10. Summary of the effect of punicalagin in PAR2-mediated allergic respiratory inflammation

When the antigen cleaves the N-term, the tethered ligand binds to ECL2, and PAR2 is activated, resulting in several signaling pathways. Downstream by activation, an immune response is induced by several genes involved in the Th2 cytokine. However, it is inactivated by treatment with PCG, which inhibits PAR2.

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ABSTRACT (IN KOREAN)

Punicalagin이 PAR2 매개 알레르기 호흡기 질환을 완화함

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강 미 란

알레르기성 비염 및 천식과 같은 알레르기성 호흡기 질환은 공기중에 있는 집먼지진드기와 같은 알레르겐에 노출되어 발생하는 만성 질환이다. Protease-activated receptor 2 (PAR2)는 G 단백질 연결 수용체 (G protein coupled receptor, GPCR) 중의 하나로 세린 프로테아제에 의해 수용체의 N 말단이 절단되면 활성화되며, 집먼지진드기 등에 들어있는 프로테아제에 의해서도 활성화된다. 활성화된 PAR2에 의해 염증성 사이토카인이 분비되어 염증을 일으켜 알레르기성 호흡기 질환을 유발한다. 고처리량 약물 스크리닝을 통해 천연물에서 추출한 PAR2에 특이적으로 억제하는 길항제인 푸니칼라진 (punicalagin, PCG)을 제공받아 실험을 진행했다. 우리는 PCG로

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전처리하고 집먼지진드기를 처리하여 1차 세포인 사람비강상피세포에서 길항제의 효과를 시험했다. 이러한 결과를 바탕으로 *in vivo*에서 그 효과를 시험하기로 결정했다. 먼저, *in vitro*로 PCG를 농도 별로 처리한 결과, PCG 3 μM부터 처리하면 효과적으로 염증이 억제됨을 확인했다. 또한 8주령 수컷의 PAR2-WT 와 PAR2-KO 마우스에서 알레르기성 호흡기 질환 모델을 만들고 PCG 10 mg/kg을 전처리하여 효과를 시험했다. 그 결과 HDM으로 알레르기 비염과 천식 질환을 동시에 만들 수 있었지만, PCG를 전처리하면 알레르기 호흡기 질환이 생성되지 않은 것을 확인했다. 우리는 여러 분석을 통해 PCG가 PAR2를 특이적으로 억제할 수 있고 수용체를 비활성화한다는 것을 알아냈다. 따라서 우리는 PAR2에 특이적으로 억제하는 길항제인 PCG를 알레르기성 호흡기 질환의 치료제로 사용될 수 있음을 제안한다.

핵심되는 말 : 알레르기 비염, 천식, 집먼지진드기, PAR2, 푸니칼라진