



The therapeutic efficacy of α -pinene in an experimental mouse model of allergic rhinitis



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ABSTRACT

In the present study, the therapeutic effect and underlying mechanism of α -pinene (α -PN) in the ovalbumin (OVA)-sensitized allergic rhinitis (AR) model were investigated. Our results showed that pretreatment with α -PN caused a decrease in clinical symptoms, including a decrease in the number of nasal, eye, and ear rubs, and spleen weight in the OVA-sensitized mice. The level of interleukin (IL)-4 was decreased on the spleen tissue of α -PN treated mice. Pretreatment with α -PN significantly decreased levels of nasal immunoglobulin E. Protein levels of tumor necrosis factor- α , intercellular adhesion molecule-1, and macrophage inflammatory protein-2 were decreased by the administration of α -PN in the nasal mucosa of the OVA-sensitized mice. The increased numbers of eosinophils and mast cells infiltrating the nasal mucosal tissue of mice with AR were decreased following oral administration of α -PN. Post-treatment with α -PN 1 h after OVA challenge also resulted in a significant reduction of clinical symptoms and IgE levels. In addition, the expression and phosphorylation of receptor-interacting protein 2 (RIP2) and I κ B kinase (IKK)- β and activation of nuclear factor- κ B (NF- κ B), and caspase-1 were all increased in the activated human mast cell line, HMC-1 cells, however, increased activations of RIP2, IKK- β , NF- κ B, and caspase-1 were inhibited by treatment with α -PN. Taken together, we suggest that α -PN is a promising anti-allergic agent and may be useful in the clinical management of AR.

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1. Introduction

Allergic rhinitis (AR) is a very common and debilitating disease of the nasal membranes, triggered by the interaction of ubiquitous environmental proteins and specific immunoglobulin E (IgE) in sensitized patients [1,2]. The incidence of AR is increasing worldwide. Epidemiologic data shows that AR affects up to 50% of the world's population [3]. This rapid increase in prevalence has been associated with multiple environmental changes, including improvements in hygiene, exposure to environmental toxicants, and a Western lifestyle [4,5]. Clinical symptoms such as nasal congestion, sneezing, itching, and rhinorrhea have a detrimental effect on the quality of life and work performance of affected individuals [6]. In some cases, AR can lead to morbidity or even mortality, although AR is generally not life-threatening.

A series of cellular interactions plays an important role in orchestrating AR reactions [7]. Antigen presenting cells activate type-2 T helper (Th2) cells to produce Th2 cytokines, which promote IgE synthesis as well as infiltration of other immune cells [8]. Mast cells

degranulate and release a spectrum of inflammatory mediators upon the cross-linking of a multivalent allergen to an IgE-Fc ϵ R1 complex on their surface [7]. Eosinophils, basophils, and T lymphocytes regulate the late allergic reaction. Therefore, the current drug treatment for AR, including antihistamines, topical corticosteroids, and leukotriene receptor antagonists has been developed to prevent their cellular and molecular processes [9]. However, all current drug treatments have adverse side effects with long-term use. There is significant interest in the development of a safe and affordable alternative therapy against allergic disorders, and recent attention has focused on bioactive compounds based on natural products, which have provided a source of medicines for treating a wide spectrum of diseases.

α -Pinene (α -PN, Fig. 1A) is a monoterpene compound, found mainly in *Eucalyptus* oils, *Camphor*, *Bupleurum frutescens*, and *Psidium* [11]. It is also present in oils of aromatic plants such as coniferous trees and rosemary [12,13]. Various studies have described α -PN as an antimicrobial, apoptotic, antimetastatic, and antibiotic agent [14,15]. Its anti-inflammatory activities have also been demonstrated on rat hindpaw edema induced by carrageenan or prostaglandin E, on cerulean-induced acute pancreatitis, and on IL-1 β induced inflammation in chondrocytic cells [16–18]. In addition, Zhou et al. reported that the

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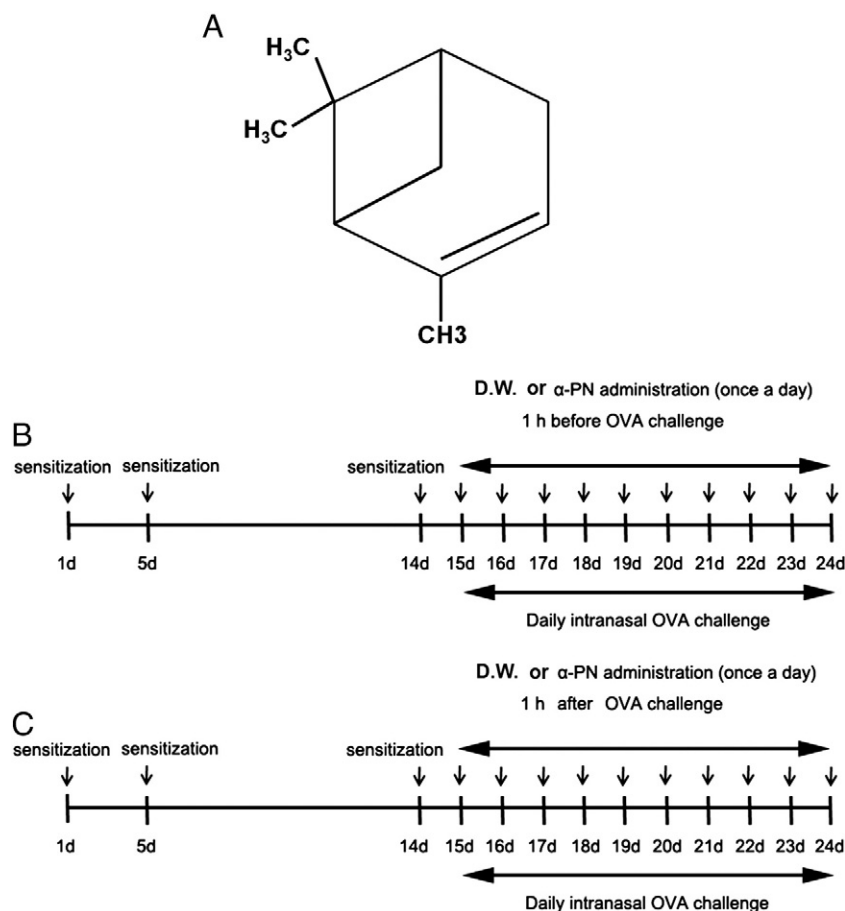


Fig. 1. (A) Chemical structure of α -PN. (B–C) Schematic protocols of an AR animal model. D.W., distilled water.

anti-inflammatory activity of α -PN is dependent on nuclear factor- κ B (NF- κ B) pathways in THP-1 cells [10]. However, thus far, no study has addressed the question of whether α -PN has an in vivo protective effect against AR or the possible mechanisms involved. Therefore, we studied the effects of α -PN on an AR murine model induced by an OVA challenge and further clarified the mechanisms involved. Mast cells play an important role in an allergic reaction. Human mast cell line, HMC-1 cells have been widely used to investigate the beneficial effects of anti-allergic drugs [19]. To elucidate the mechanisms underlying the effects of α -pinene on AR, we also performed an in vitro assay using HMC-1 cells.

2. Material & methods

2.1. Reagents

We purchased α -PN (>98.0% purity), OVA, phorbol 12-myristate 13-acetate (PMA), calcium ionophore (A23187), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), bovine serum albumin (BSA), bicinchoninic acid (BCA), and avidin–peroxidase from Sigma Chemical Co. (St Louis, MO, USA); Isocove's Modified Dulbecco's Medium (IMDM), penicillin, streptomycin, and fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). Anti-mouse immunoglobulin E (IgE)/interleukin (IL)-1 β /tumor necrosis factor (TNF)- α /interferon (IFN)- γ /macrophage inflammatory protein-2 (MIP-2)/intercellular adhesion molecule-1 (ICAM-1) antibodies (Ab), biotinylated anti-mouse IgE/IL-1 β /TNF- α /IFN- γ /MIP-2/ICAM-1 Ab, recombinant mouse IgE/IL-1 β /TNF- α /IFN- γ /MIP-2/ICAM-1, anti-human IL-1 β /TNF- α /IL-6 Ab, biotinylated anti-human IL-1 β /TNF- α /IL-6 Ab, and recombinant human IL-1 β /TNF- α /IL-6 Ab were purchased from BD Biosciences

Pharmingen (San Diego, CA, USA). Ab for NF- κ B (p65), I κ B- α , receptor-interacting protein-2 (RIP2), phosphorylated (p)-RIP2, I κ B kinase β (IKK- β), p-IKK- β , histone, procaspase-1, caspase-1, actin, and α -tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. OVA-induced AR animal model

Six-week-old female BALB/c (Charles River Laboratories, Inc., Wilmington, MA, USA) mice were maintained under a pathogen-free condition. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University [approval no. KHUASP (SE)-10-016]. Mice were immunized consistently with previous studies [20]. The experimental procedure for the animal model of allergic rhinitis is summarized in Fig. 1B–C. Mice were sensitized i.p. with 100 μ g of OVA emulsified and 20 mg of aluminum hydroxide (Sigma-Aldrich) in 100 μ l of PBS on days 1, 5, and 14. Then, local immunization was performed once a day from day 15 to day 24 by instilling the OVA. Mice were treated with α -PN (0.1, 1, or 10 mg/kg) once a day for 10 days 1 h before or 1 h after intranasal OVA challenge. Normal and OVA-induced AR mice were given distilled water alone on the same schedule. Nasal symptoms were only evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA intranasal provocation at the 10-day mark after the challenge. We measured OVA-specific IgE as the relevant endpoint for AR. Each group included five mice.

2.3. Cell culture and stimulation

HMC-1 cells were generously provided by Eichi Morri (Osaka University, Osaka, Japan). HMC-1 cells were grown in an IMDM medium

supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µM monothioglycerol, and 10% heat-inactivated FBS at 37 °C, in 5% CO₂ and 95% humidity. Cells were treated with α-PN for 1 h prior to stimulation with 20 nM of PMA and 1 µM of A23187 (PMACI), which plays an important role in activation of protein kinase C and increasing cytosolic calcium concentration in mast cells and incubated at 37 °C for 8 h.

2.4. MTT assay

Cell viability was assessed using an MTT assay. HMC-1 cell suspension was cultured in 24-well plates for 24 h after treatment with each concentration of α-PN. 20 µl of MTT solution (5 mg/ml) was added and the cells were incubated at 37 °C for 4 h. The insoluble formazan product was dissolved in DMSO after washing the supernatant. Then, the optical densities of 96-well culture plates were measured using an ELISA reader at 540 nm.

2.5. ELISA assay

Cytokines of mucosa, spleen tissue, and cell supernatant were measured using an enzyme-linked immunosorbent assay (ELISA). Nasal mucosa and spleen tissues were homogenized with protease and phosphatase inhibitor in RIPA lysis buffer (Sigma, St. Louis, MO, USA). The homogenates were transferred to 1.5 ml tubes, centrifuged at 13,000 ×g for 10 min at 4 °C, and the supernatant was stored at –80 °C until analyzed. ELISA was performed by coating 96-well plates with 1 µg/well capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with 1 × PBS containing 0.05% Tween 20. All reagents and coated wells used in this assay were incubated for 2 h at room temperature. A standard curve was generated from the known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the biotin-conjugated secondary antibodies and an avidin peroxidase and 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablet substrate solution containing 30% H₂O₂ were used. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine levels in the spleen and nasal mucosa were divided according to the total protein.

$$\% \text{Inhibition} = (a - b) \times 100/a.$$

where 'a' is a cytokine secretion without α-PN and 'b' is a cytokine secretion with α-PN.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

HMC-1 cells were treated with α-PN (0.1, 1, and 10 µg/ml) for 1 h before stimulation with PMACI and incubated for 6 h. The total RNA was isolated from the cells and nasal mucosa according to the manufacturer's specification using an easy-BLUE RNA extraction kit (iNtRON Biotechnology, Kyunggi-do, Korea). The concentrations of total RNA in the final elutes were determined by spectrophotometry. Total RNA (2.5 µg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 42 °C using a cDNA synthesis kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Polymerase chain reaction (PCR) was performed using the following primers for the human TNF-α (5'-CAC CAG CTG GTT ATC TCT CAG CTC-3'; 5'-CGG GAC GTG GAG CTG GCC GAG GAG-3'); IL-1β (5'-CCG GAT CCA TGG CAC CTG TAC GAT CA-3'; 5'-GGG GTA CCT TAG GAA GAC ACA AAT TG-3'); IL-6 (5'-GAT GGATGC TTC CAAT CT GGAT-3'; 5'-AGT TCT CCATAG AGA ACA ACA TA-3'); and GAPDH (5'-CAA AAG GGT CAT CAT CTC TG-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3'). GAPDH was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. Saturation curves for PCR were obtained

from various experimental conditions (RNA concentrations, annealing temperatures, and PCR cycle numbers). We determined the optimal amplification conditions (annealing temperature and PCR cycle number) of primers for PCR. The annealing temperature was 60 °C for TNF-α and GAPDH, 50 °C for human IL-1β, and 56 °C for IL-6, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.7. Preparation of nuclear extracts and cytoplasmic extracts

Briefly, after cell activation, the cells were washed with ice-cold PBS and resuspended in 60 µl of buffer A (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The cells were left on ice for 15 min, lysed gently with 2.5 µl of 10% Nonidet P-40, and centrifuged at 2000 ×g for 10 min at 4 °C. The supernatant was collected and used as the cytoplasmic extract. The nuclei pellets were resuspended in 40 µl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9), left on ice for 20 min and inverted. The nuclear debris was then spun down at 15,000 ×g for 15 min. The supernatant (nuclear extract) was collected and stored at –70 °C until the analysis was performed.

2.8. Western blot analysis

Cell extracts were prepared using a detergent lysis procedure. Samples were heated at 95 °C for 5 min and briefly cooled on ice. After centrifugation, 50 µg aliquots were resolved by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred to nitrocellulose membranes and then the membranes were blocked for 2 h with 5% skim milk. The primary antibodies were diluted with PBS containing Tween-20 (PBST) and incubated overnight at 4 °C. Afterwards, the nitrocellulose membrane was washed five times for 15 min with PBST. For protein detection, the blot was incubated with secondary antibodies (1:3,000 in PBST) conjugated with peroxidase for 40 min. Finally, the protein bands were visualized using an enhanced chemiluminescence assay purchased from Amersham Co. (Newark, NJ, USA) following the manufacturer's instructions.

2.9. Histological examination

The nasal mucosa tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. The sections of the sample were 4 µm thick. Each section was stained with hematoxylin and eosin (for eosinophils) and alcian blue and sarganine O (A&S, for mast cells) before dewaxing and dehydration. The numbers of eosinophils on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

2.10. Statistical analysis

All results are expressed as the mean ± S.E.M. Statistical analyses were performed using SPSS statistical software (SPSS 11.5, Armonk, NY, USA). Treatment effects were analyzed by an independent *t*-test and one-way ANOVA with a Tukey post hoc test. Significance was determined for *P* < 0.05.

3. Results

3.1. Inhibitory effect of α-PN on the clinical symptoms and inflammation-related protein of AR mice

To examine the regulatory effect of α-PN in the AR mouse model, we sensitized mice on days 1, 5, and 14 by intraperitoneal injections

of OVA. Various doses of α -PN (0.1, 1, and 10 mg/kg) were administered orally 1 h before the intranasal OVA challenge once a day from days 15 to 24. We measured the rub score of the nose, eye, and ear for 10 min in the OVA-induced AR mice. The OVA-induced AR mice showed significantly increased rub scores of the nose, eye, and ear (Fig. 2A, $P < 0.05$). However, increased rub scores were significantly inhibited in the α -PN administered mice (Fig. 2A, $P < 0.05$). The symptom scores revealed significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals. Significantly elevated spleen weights were observed in the AR mice compared with the normal mice (Fig. 2B, $P < 0.05$). These increased spleen weights were subsequently reduced by administration of α -PN (Fig. 2B). Next, we determined IL-4 and IFN- γ levels in the spleen owing to identification of a Th1/Th2 immune reaction in the α -PN administered mice. As shown in Fig. 2C, IFN- γ levels were significantly decreased by OVA, but α -PN had no effect. The AR mice showed significantly increased levels of IL-4 compared to the normal mice (Fig. 2C). Increased IL-4 levels were significantly decreased in the α -PN administered mice (Fig. 2C). The IL-4 and IFN- γ levels in the spleen revealed no significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals. Significantly higher levels of IgE were observed in the AR mice than in the nasal mucosa tissues of the OVA-unsensitized mice (Fig. 2D). The IgE levels

increased by OVA in nasal mucosa were reduced by administration of α -PN (Fig. 2D, $P < 0.05$). The IgE levels revealed significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals.

3.2. Inhibitory effect of α -PN on the levels of TNF- α , IL-1 β , IL-6, ICAM-1, and MIP-2 in nasal mucosa tissue of AR mice

To evaluate the regulatory effect of α -PN on the levels of proinflammatory cytokines, TNF- α , IL-1 β , and IL-6, we measured the protein levels of TNF- α , IL-1 β , and IL-6 in the nasal mucosa of the AR model. The protein levels of TNF- α , IL-1 β , and IL-6 in nasal mucosa tissue were increased in the AR mice compared with those levels in the control mice (Fig. 3A–C, $P < 0.05$). We observed that the protein levels of TNF- α and IL-6 were reduced by the administration of α -PN. However, levels of IL-1 β were not reduced by the administration of α -PN. Treatment with α -PN (10 mg/kg) alone had no effect on levels of TNF- α , IL-1 β , or IL-6 (Fig. 3A–C). TNF- α (at 10 mg/kg) and IL-6 (at 0.1 mg/kg) levels revealed significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals. We also analyzed the ICAM-1 and MIP-2 levels in the OVA-induced AR mice. Significantly higher levels of ICAM-1 and MIP-2 were observed in the OVA-induced AR mice than in the nasal mucosa tissues of OVA-unsensitized mice

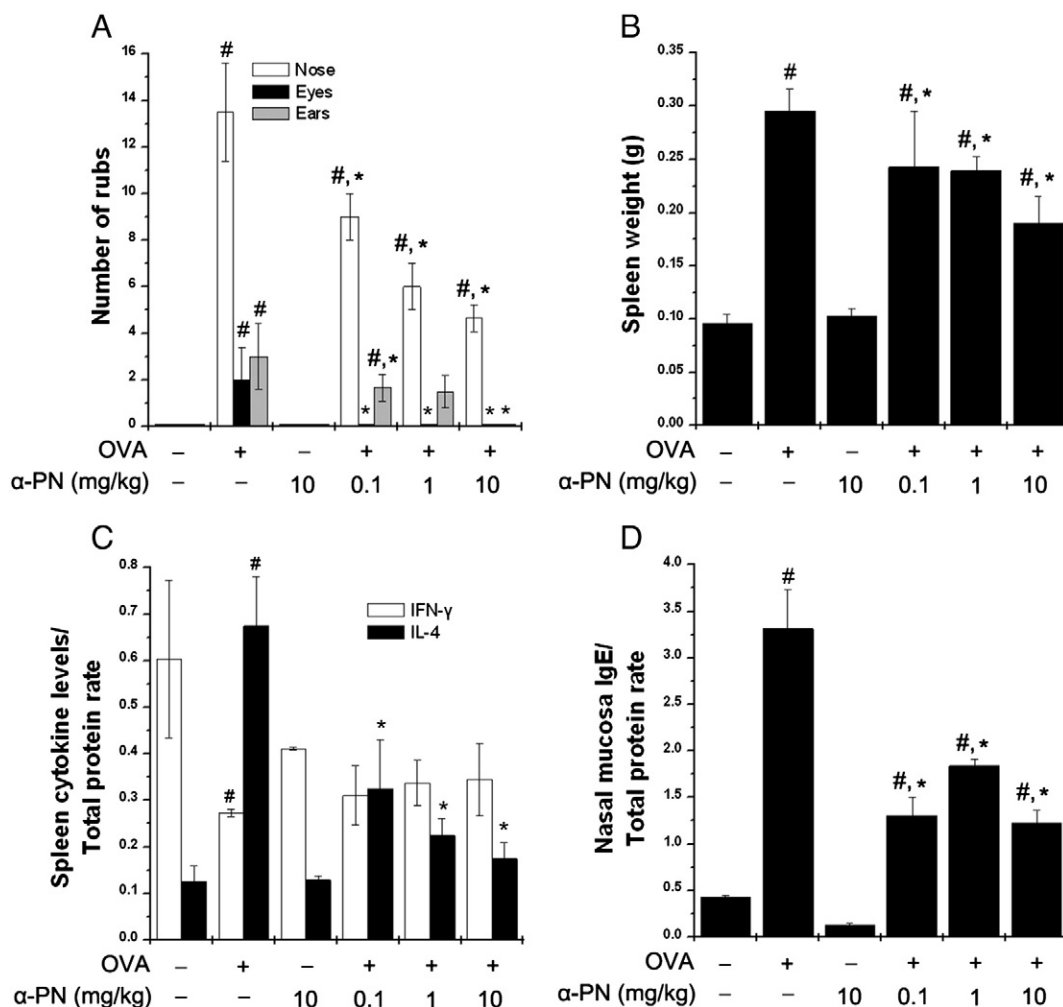


Fig. 2. Effects of α -PN on clinical symptoms, IFN- γ , IL-4, and IgE in the AR animal model. Mice were sensitized on days 1, 5, and 14 by intraperitoneal injections of 100 μ g of OVA emulsified in 20 mg of aluminum hydroxide and then challenged mice with 1.5 mg of OVA. Mice were administered α -PN 1 h before the OVA challenge daily for 10 days. (A) The rub number of the nose, eyes, and ears that occurred 10 min after OVA intranasal provocation. (B) Spleen weight. (C) The levels of IFN- γ and IL-4 in the spleen were measured using the ELISA method. (D) OVA-specific IgE levels in nasal mucosa tissues were measured using the ELISA method. Data are represented as the mean $\pm$ S.E.M. with $n = 5$ mice per group. # $P < 0.05$; significantly different from OVA-unsensitized mice. * $P < 0.05$; significantly different from OVA-sensitized mice.

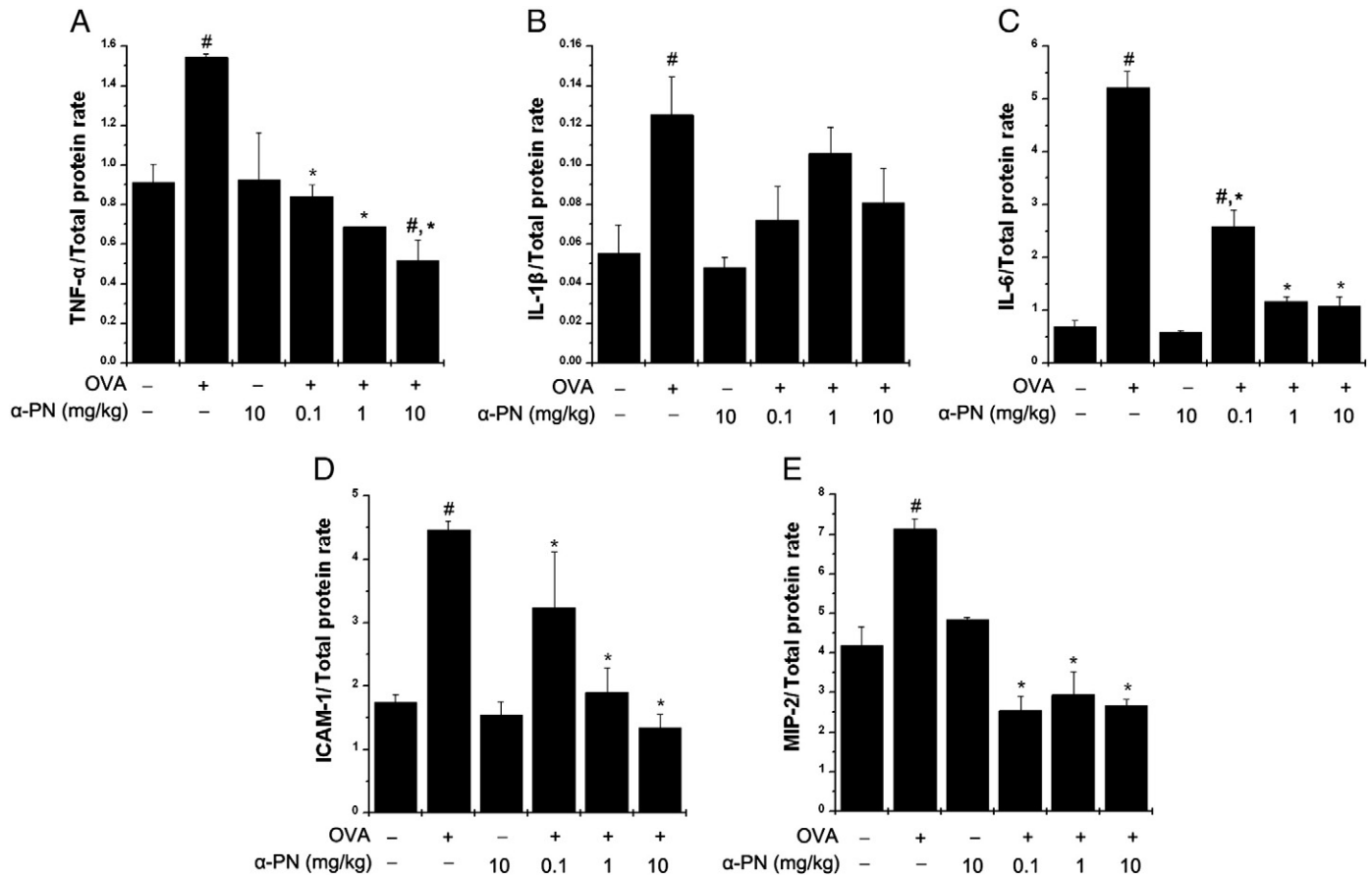


Fig. 3. α -PN reduced inflammation-related protein in the AR animal model. Mice were sensitized on days 1, 5, and 14 by intraperitoneal injections of 100 μ g of OVA emulsified in 20 mg of aluminum hydroxide and then challenged mice with 1.5 mg of OVA. Mice received α -PN 1 h before the OVA challenge for 10 days. (A–C) The levels of cytokines in nasal mucosa were measured using the ELISA method. (D–E) The levels of MIP-2 and ICAM-1 in nasal mucosa tissue were measured using the ELISA method. Data are represented as the mean $\pm$ S.E.M. with $n = 5$ mice per group. # $P < 0.05$; significantly different from OVA-unsensitized mice. * $P < 0.05$; significantly different from OVA-sensitized mice.

(Fig. 3D and E, $P < 0.05$). Increased levels of ICAM-1 and MIP-2 were significantly inhibited by α -PN (Fig. 3D and E, $P < 0.05$). Treatment with α -PN (10 mg/kg) alone had no effect on the levels of ICAM-1 and MIP-2 (Fig. 3D and E). ICAM-1 and MIP-2 levels revealed no significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals.

3.3. Inhibitory effect of α -PN on infiltration of eosinophils and mast cells in AR nasal mucosa tissue

The numbers of inflammatory cells (eosinophils and mast cells) in the nasal mucosa of the AR mice were significantly higher than those in the OVA-unsensitized mice. In the α -pinene administered mice, infiltration of eosinophils and mast cells increased by OVA sensitization was significantly decreased (Fig. 4A–C). Treatment with α -PN (10 mg/kg) alone had no effect on the numbers of infiltrating eosinophils and mast cells (Fig. 4A–C). The numbers of eosinophils and mast cells revealed no significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals.

3.4. α -PN inhibited the production and mRNA expression of pro-inflammatory cytokines in HMC-1 cells

We examined the regulatory effect of α -PN on the production of TNF- α , IL-1 β , and IL-6. PMACI induced a significant increase in the pro-inflammatory cytokine production compared with the media control (Fig. 5A–C). Production of TNF- α and IL-6 increased by PMACI was significantly inhibited by α -PN (Fig. 5A–C, $P < 0.05$).

The inhibition rates for TNF- α , IL-1 β , and IL-6 at 10 μ g/ml were approximately 90.2%, 103.8%, and 87.8%, respectively. We examined cell viability using a MTT assay. α -PN had no effect on the cell viability (Fig. 5D). Next, we examined the effect of α -PN on the mRNA expression of TNF- α , IL-1 β , and IL-6 in the PMACI-stimulated mast cells. The mRNA expressions of TNF- α , IL-1 β , and IL-6 were decreased by α -PN (Fig. 6A and B). Treatment with α -PN (10 μ g/ml) alone had no effect on the mRNA expression and production of TNF- α , IL-1 β , and IL-6 (Fig. 6).

3.5. Inhibitory effect of α -PN on the activation of NF- κ B in HMC-1 cells

To investigate the regulatory mechanism of α -PN on allergic inflammation, we analyzed NF- κ B activation. α -PN inhibited the PMACI-induced activation of NF- κ B (Fig. 7A and B). As a marker of NF- κ B activation, degradation of I κ B- α in the cytoplasm lysates was detected. α -PN also inhibited the PMACI-induced degradation of I κ B- α (Fig. 7A and B). Treatment with α -PN (10 μ g/ml) alone had no effect on the PMACI-induced activation of NF- κ B (Fig. 7A and B).

3.6. Inhibitory effects of α -PN on the PMACI-induced activation of RIP2/IKK- β /Caspase-1 in HMC-1 cells

RIP2, a caspase recruitment domain (CARD)-containing kinase, interacts with caspase-1 through CARD–CARD interaction [21]. Thereafter, RIP2 recruits the IKK β complex for activation of NF- κ B [22]. Expression and phosphorylation of RIP2 and IKK- β were inhibited by α -PN in the PMACI-stimulated HMC-1 cells (Fig. 7C and D). We performed a

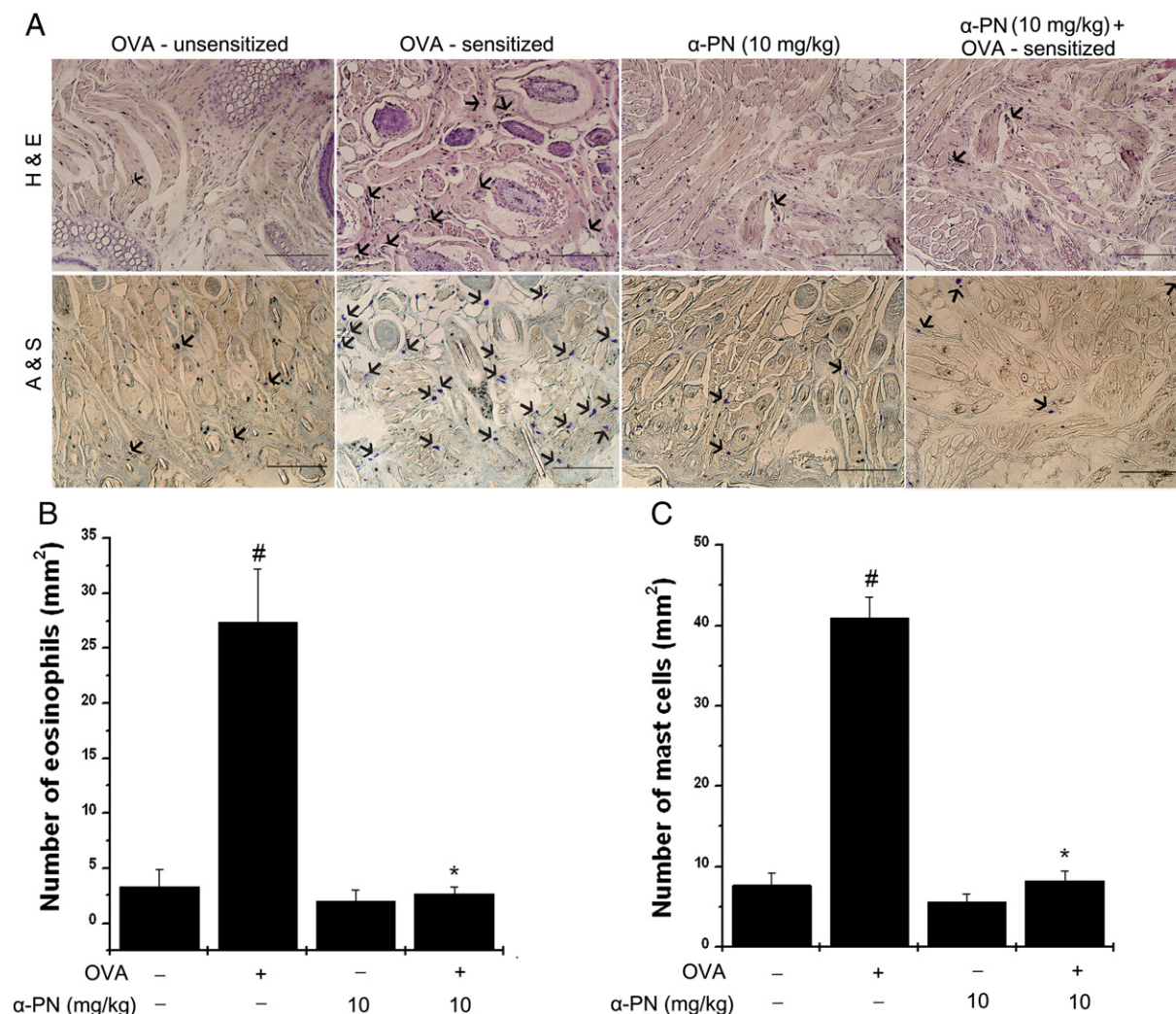


Fig. 4. α -PN reduced infiltration of eosinophils and mast cells in AR nasal mucosa tissue. Mice were sensitized on days 1, 5, and 14 by intraperitoneal injections of 100 μ g of OVA emulsified in 20 mg of aluminum hydroxide and then challenged with 1.5 mg of OVA. Mice were administrated α -PN 1 h before the OVA challenge daily for 10 days. (A) Nasal mucosa tissue was stained with hematoxylin and eosin (H&E) for eosinophils (black arrow, upper panel) and alcian blue and safranin O (A&S) for mast cells (black arrow, lower panel). (B–C) Eosinophils and mast cells were counted by three individuals. Five randomly selected tissue sections per mouse were counted. Data are represented as the mean $\pm$ S.E.M. with $n = 5$ mice per group. $^{\#}P < 0.05$, significantly different from OVA-unsensitized mice. $^*P < 0.05$, significantly different from OVA-sensitized mice. Original magnification $\times 400$, scale bar = 100 μ m.

Western blot analysis to determine for α -PN regulated caspase-1 activation. As shown in Fig. 7E and F, the caspase-1 activation increased via PMACI was inhibited by α -PN. Treatment with α -PN (10 μ g/ml) alone had no effect on activation of RIP2, IKK- β , or caspase-1 (Fig. 7B–F).

3.7. Clinical symptoms and IgE levels were decreased by post-treatment of α -PN

To examine the regulatory effect caused by post-treatment of α -PN in the AR mouse model, we sensitized mice on days 1, 5, and 14 by intraperitoneal injections of OVA. Thereafter, α -PN (10 mg/kg) was administrated orally 1 h after the intranasal OVA challenge from days 15 to 24. Post-treatment with α -PN 1 h after OVA challenge also resulted in a significant reduction of clinical symptoms and IgE levels (Fig. 8, $P < 0.05$). Clinical symptoms and IgE levels revealed significant differences between the OVA-sensitized animals treated with α -PN and the non-sensitized animals.

4. Discussion

In this study, we demonstrated that α -PN inhibited the allergic and inflammatory reaction in the AR mice model.

AR is characterized by immediate type I hypersensitive inflammatory reactions occurring due to interaction between IgE and environmental factors. The IgE-dependent AR cascade is characterized by induction phase, early-phase, and late-phase reactions. Each phase is characterized by the production and interplay of various cell-derived mediators. During the induction phase, Th cells produce a variety of cytokines, such as IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-12, IL-13, IL-19, IFN- γ , TNF- α , TNF- β , and granulocyte-macrophage colony-stimulating factor [23]. IL-4 and IL-13 play a role in the activation of B cells to produce IgE [23]. Subsequently, the late-phase reaction is induced by various mediators derived from inflammatory cells, including eosinophils, neutrophils, T cells, macrophages, dendritic cells, and endothelial cells [24]. Structural changes of nasal mucosa occur during this phase. The allergic response in nasal mucosa challenged with OVA is characterized by common symptoms of AR and the production of inflammatory cytokines such as IL-1 β , IL-6, and TNF- α [19]. In the current study, α -PN caused a significant reduction in clinical symptoms and inflammatory cytokine levels, such as TNF- α and IL-6 in the AR animal model. The levels of IL-1 β were not significantly decreased in this model. However, α -PN significantly suppressed IL-1 β production and caspase-1 activation in the PMACI-stimulated HMC-1 cells. The inflammatory cells (mast cells, eosinophils, macrophages, and neutrophils) were infiltrated into

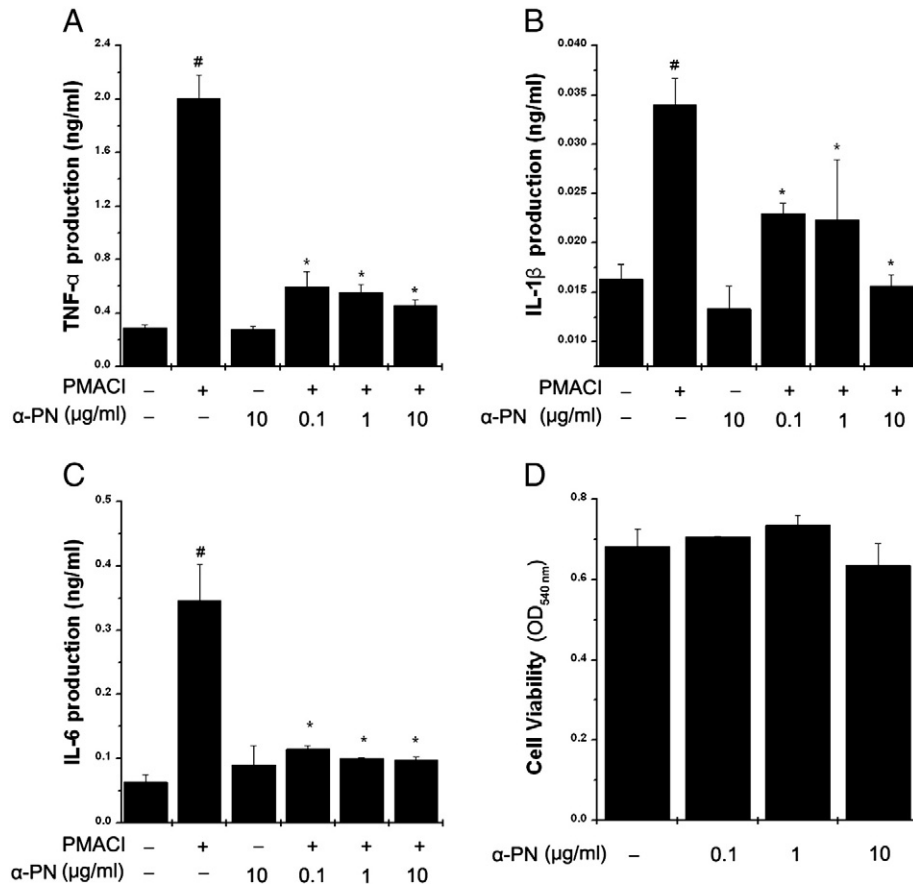


Fig. 5. α -PN reduced production of pro-inflammatory cytokines. HMC-1 cells (3×10^5) were treated with α -PN (0.1, 1, and 10 μ g/ml) for 1 h and then stimulated by PMACI for 8 h. (A–C) Production of cytokines in the supernatant was measured using the ELISA method. (D) MTT assay. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. # $P < 0.05$; significantly different from unstimulated cells. * $P < 0.05$; significantly different from PMACI-stimulated cells. PMACI, PMA plus A23187.

the nasal mucosa of AR mice and also produced various inflammatory cytokines including IL-1 β . Therefore, we postulate that α -PN inhibits mast cell-mediated inflammatory reactions. However, further study is needed to elucidate the effect and mechanism of α -PN with respect to various other immune cells.

AR patients are characterized by the influx of a variety of immune cells, including mast cells, eosinophils, and basophils in the epithelium of the nasal mucosa [25]. Eosinophils, innate effector cells, are closely

linked to allergic inflammatory conditions, since eosinophil granule protein causes the development of rhinitis and chronic rhinosinusitis [26,27]. Mast cells play a key role in the induction and maintenance of eosinophilic inflammation [28]. Infiltration of these cells into injury lesions is associated with expression of chemokines in mucosal epithelial cells. MIP-2 and ICAM-1 are well documented chemokines in the late allergic response [29,30]. In this study, we observed that α -PN inhibited the levels of inflammation-related protein and infiltration of

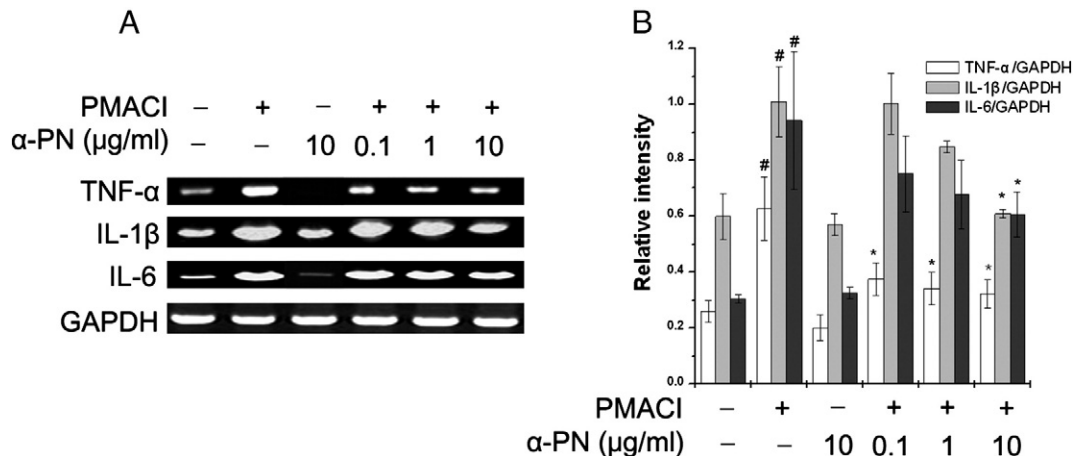


Fig. 6. Effect of α -PN on mRNA expression of pro-inflammatory cytokines. (A) α -PN reduced the mRNA expression of pro-inflammatory cytokines. HMC-1 cells (3×10^6) were treated with α -PN (0.1, 1, and 10 μ g/ml) for 1 h and then stimulated by PMACI for 6 h. TNF- α , IL-1 β , and IL-6 mRNA expressions were analyzed by RT-PCR. (B) The levels of mRNA expression were quantified by densitometry. Expression was normalized to GAPDH expression. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. # $P < 0.05$; significantly different from unstimulated cells. * $P < 0.05$; significantly different from PMACI-stimulated cells. PMACI, PMA plus A23187.

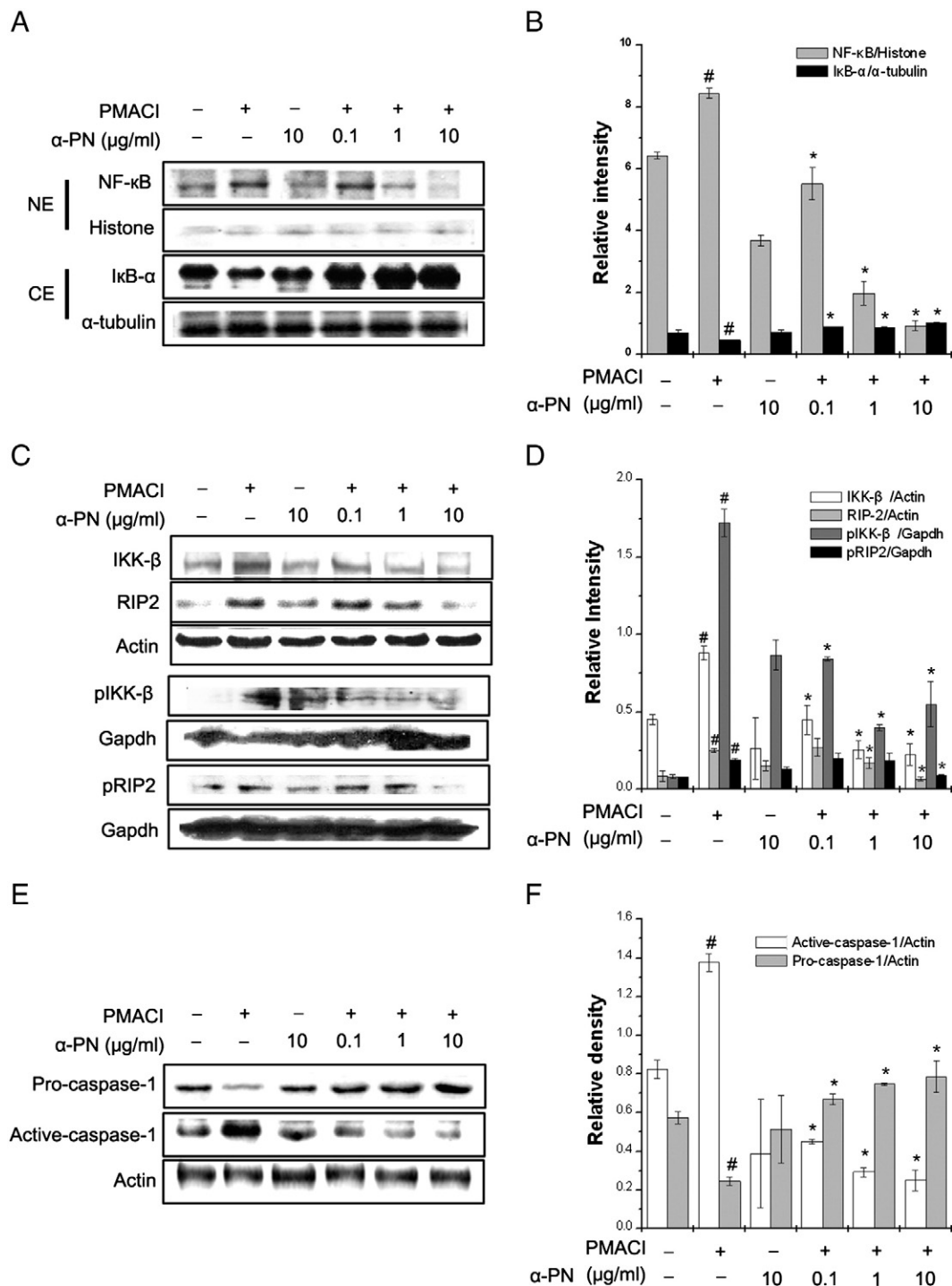


Fig. 7. α-PN reduced the activation of NF-κB, and IKK-β, RIP2, and caspase-1. (A) HMC-1 cells (3×10^6) were treated with α-PN (0.1, 1, and 10 μg/ml) for 1 h and then stimulated by PMACI for 2 h. NF-κB and degradation of IκB-α were determined by Western blot analysis. (B) RIP2, pRIP2, IKK-β, and p-IKK-β protein expression were evaluated by Western blot analysis. (C) The levels of caspase-1 were assayed by Western blot analysis. (D–E) The levels of protein expression were quantified by densitometry. Expression was normalized to histone, tubulin, or actin expression. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. [#] $P < 0.05$; significantly different from unstimulated cells. ^{*} $P < 0.05$; significantly different from PMACI-stimulated cells. PMACI, PMA plus A23187.

inflammatory cells. An anti-allergic effect by α-PN was associated with a reduction in the number of infiltrated inflammatory cells. Therefore, the present study with α-PN provides affirmative proof of a possible therapeutic application of this agent in allergic inflammatory diseases.

HMC-1 cells, derived from a patient with mast cell leukemia, exhibit many characteristics of human mast cells, such as the expression of

histamine, tryptase, heparin, and a similar cell surface antigen-profile [31,32]. Protein kinase C (PKC) leads to the activation of many signal pathways involved in allergic inflammation. PMA, a potent PKC activator, plays an important role in the activation of PKC in mast cells. A23187, calcium ionophore, was used as an activator of HMC-1 cells [33]. In mast cells, PMACI induced an increase in cytosolic calcium

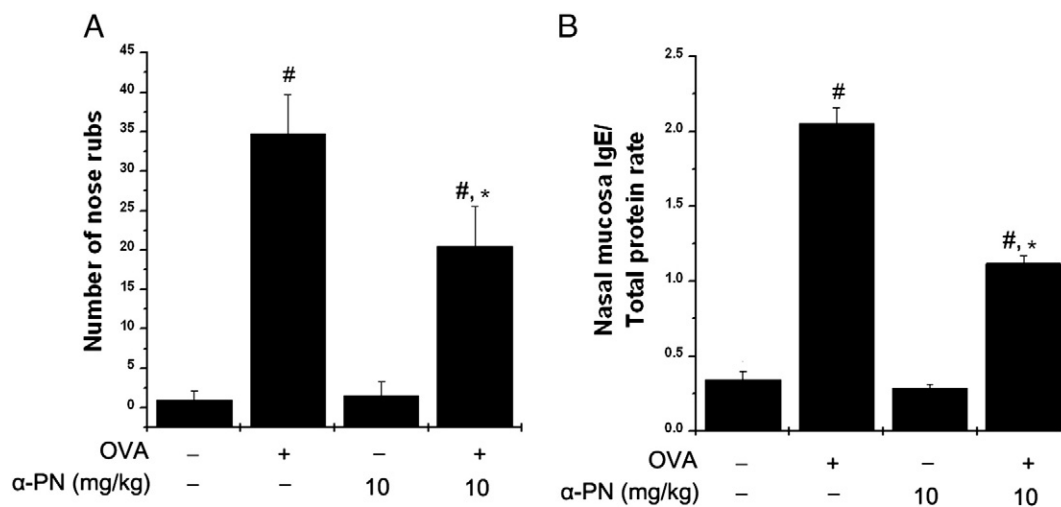


Fig. 8. Effects of α -PN on nasal rub scoring and IgE production in the AR animal model. Mice received α -PN 1 h after OVA challenge for 10 days by oral administration. (A) The rub scoring was counted. (B) OVA-specific IgE levels in nasal mucosa tissues were measured using the ELISA method. Data are represented as the mean $\pm$ S.E.M. with $n = 5$ mice per group. # $P < 0.05$; significantly different from OVA-unsensitized mice. * $P < 0.05$; significantly different from OVA-sensitized mice.

concentration, which leads to mast cell activation [34]. Activated mast cells produce several proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6. In this study, stimulation with PMACI led to increased production and mRNA expression of proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6 in HMC-1 cells. However, treatment with α -PN resulted in decreased production and mRNA expression of these cytokines. A previous study also reported that α -PN inhibited pancreatitis by reducing proinflammatory cytokines, including IL-1 β , IL-6, and TNF- α [17]. Therefore, we can deduce that α -PN exerts a strong anti-allergic effect through the regulation of proinflammatory cytokines.

The NF- κ B/I κ B signal pathway has been reported to induce mRNA expression of pro-inflammatory cytokines in human mast cells [35]. Therefore, suppression of NF- κ B activation has been related with the inhibition of inflammation [36,37]. Rufino et al. reported that α -PN inhibited IL-1 β -induced inflammation through the inhibition of I κ B- α phosphorylation and NF- κ B activation in C28-I2 chondrocytic cells [18]. RIP2 and IKK complexes are essential for NF- κ B activation and RIP2 is an upstream activator of NF- κ B and caspase-1 [38,39]. RIP2 activated by proinflammatory stimuli promotes caspase-1 activation and recruits the I κ B kinase (IKK) complex through the direct interaction of its intermediate domain with IKK- β , leading to activation of the NF- κ B [40]. Our previous studies showed that PMACI induced the expression of RIP2 and IKK- β and that increased expression was decreased by anti-inflammatory drugs [25,41]. Gomisin has an anti-inflammatory effect by inhibiting the expression and phosphorylation of IKK- β and expression of RIP2 [39]. In this study, we showed that α -PN suppressed the activation of NF- κ B, RIP2, IKK- β , and caspase-1 induced by PMACI. Thus, we can speculate that α -PN has an anti-inflammatory effect by blocking the inflammatory signal pathway in the PMACI-stimulated mast cells.

In conclusion, while α -PN has yet to be examined extensively with regard to its effect on AR reactions and the mechanism involved in such an effect, we observed for the first time that α -PN dramatically inhibited the symptoms of AR by blocking the NF- κ B/I κ B- α /RIP2/IKK- β /caspase-1 signal cascade. Overall, the present study provides evidence indicating that α -PN can be beneficial as a compound in the treatment of inflammatory and allergic diseases.

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