

Nothofagin suppresses mast cell-mediated allergic inflammation

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ABSTRACT

Mast cells play a major role in immunoglobulin E-mediated allergic inflammation, which is involved in asthma, atopic dermatitis, and allergic rhinitis. Nothofagin has been shown to ameliorate various inflammatory responses such as the septic response and vascular inflammation. In this study, we assessed the inhibitory effect of nothofagin on allergic inflammation using cultured/isolated mast cells and an anaphylaxis mouse model. Nothofagin treatment prevented histamine and β -hexosaminidase release by reducing the influx of calcium into the cytosol in a concentration-dependent manner. Nothofagin also inhibited the gene expression and secretion of pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-4 by downregulating the phosphorylation of Lyn, Syk, Akt and nuclear translocation of nuclear factor- κ B. To confirm these effects of nothofagin *in vivo*, we used a passive cutaneous anaphylaxis mouse model. Topical administration of nothofagin suppressed local pigmentation and ear thickness. Taken together, these results suggest nothofagin as a potential candidate for the treatment of mast cell-involved allergic inflammatory diseases.

1. Introduction

Allergic inflammation is a typical characteristic of various immune diseases, including anaphylaxis, allergic rhinitis, and atopic dermatitis [1,2]. These diseases occur after repeated exposure to a specific allergen such as pollen, dust, mites, metals, and foods. In the pathogenesis of these diseases, mast cells induce allergic inflammation by releasing various pre-formed mediators, lipid mediators, chemokines, and cytokines [3,4]. Among these mediators, histamine is a major factor causing vasodilation and increased vascular permeability, subsequently leading to the development of hypothermia and recruitment of leukocytes in the acute allergic response [5]. Therefore, suppressing mast cell activation is a critical factor for effectively managing allergic inflammation [6].

The activation of mast cells depends on the cross-linking of antigen-

immunoglobulin (Ig) E complexes with Fc ϵ RI located on the surface of mast cells. The complex aggregates and induces the secretion of diverse substances such as histamines, proteases, and chemotactic factors, as well as the *de novo* synthesis of various cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-4, IL-6, and transforming growth factor- β . These cytokines play important roles in the inflammatory reaction with the enhancement of T cell activation and B cell survival [4].

Natural products are regarded as abundant sources of new drug candidates and their pharmacological usefulness has been proven through decades of research [7]. In addition, drugs derived from natural products are known to induce less side effects than many synthetic drugs [8]. Therefore, finding and revealing the unique effects of natural products in organisms still have pharmacological significance. *Aspalathus linearis* (Fabaceae), commonly known as rooibos, is a plant that is uniquely grown in South Africa and has been listed as a medicinal plant

Abbreviations: DNP, dinitrophenyl; HSA, human serum albumin; IgE, immunoglobulin E; IL, interleukin; PCA, passive cutaneous anaphylaxis; RPMCs, rat peritoneal mast cells; TNF, tumor necrosis factor

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based mostly on anecdotal evidence [9]. Various studies have demonstrated its medicinal effects [10–12] and active components, especially dihydrochalcones [13]. Nothofagin, one of the dihydrochalcones, is present in large amounts in unprocessed rooibos tea [14], and has been reported to have several biological effects such as ameliorating septic responses, vascular inflammation, and anti-thrombotic activities through inhibiting nuclear factor (NF)- κ B [15–18]. In the present study, we tested the hypothesis that nothofagin suppresses mast cell-mediated allergic inflammation by reducing NF- κ B activation.

2. Materials and methods

2.1. Reagents and cell culture

o-Phthaldialdehyde, dimethyl sulfoxide (DMSO), anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), 4-nitrophenyl N-acetyl- β -D-glucosaminide, dexamethasone, and histodenz were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nothofagin was purchased from Wuhan ChemFaces Biochemical (purity $\geq$ 98%, Wuhan, China). RBL-2H3 cells and rat peritoneal mast cells (RPMCs) were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum and 100 units/mL of antibiotics (Gibco).

2.2. Animals

Male Sprague-Dawley (SD) rats (total $n = 3$) weighing 240–280 g (8 weeks old) and male Imprinting Control Region (ICR) mice (total $n = 20$) weighing 35–40 g (6 weeks old) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). All animals had ad libitum access to standard rodent chow and filtered water during the study. The animals were housed at a density of 5 per cage in a laminar air flow room maintained at a temperature of $22 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 5\%$, and a 12-h light:dark cycle throughout the study. The care and treatment of the animals were conducted in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

2.3. Cell viability

Cell viability was measured using an MTT assay kit (WelGENE, Seoul, Korea) as previously described [19]. In brief, RBL-2H3 cells were seeded at 5×10^4 cells/well in 96-well plates in serum-free media, treated with various concentrations of nothofagin, and then incubated with 1 mg/mL MTT reagent at 37°C . After 2 h, 100 μL DMSO was added to each well to dissolve the formazan crystal by-products in the cells. The absorbance levels were measured at 570 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Cell viability was converted into a percentage compared with that of the control wells.

2.4. β -Hexosaminidase assay

β -hexosaminidase was measured as previously described [2]. In brief, RBL-2H3 cells (5×10^5 cells/well in 12-well plates) were sensitized with anti-DNP IgE (50 ng/mL); the cells were pretreated with or without each concentration of nothofagin for 1 h. After washing with phosphate-buffered saline (PBS), the cells were stimulated with DNP-HSA (100 ng/mL). After 4 h incubation, the medium was collected, the remaining cells were lysed with 0.5% Triton X-100, and the supernatants were centrifuged for 5 min at 4°C at 150 g. The supernatants (40 μL) were transferred to a 96-well plate and incubated with an equal volume of substrate solution (1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide in 0.1 M citrate buffer, pH 4.5) at 37°C . After 1 h incubation,

200 μL of the stop solution (0.1 M Na_2CO_3 – NaHCO_3 , pH 10) was added to each well. The absorbance was measured at 405 nm using a spectrophotometer, and the amount of β -hexosaminidase release was calculated by the following formula:

$$\% \text{ Degranulation} = [\text{OD}_{\text{culture media}} / (\text{OD}_{\text{culture media}} + \text{OD}_{\text{cell lysate}})] \times 100$$

2.5. Histamine assay

Anti-DNP IgE (50 ng/mL)-sensitized RBL-2H3 cells (5×10^5 cells/well in 12-well plates) were washed with PBS and pretreated with or without various concentrations of nothofagin for 1 h. The cells were then stimulated with DNP-HSA (100 ng/mL) for 4 h. Isolated RPMCs were seeded into 24-well plates with anti-DNP IgE (50 ng/mL). After 24 h, the cells were washed with PBS, pre-treated with nothofagin for 1 h, and then stimulated with DNP-HSA (100 ng/mL) for 30 min. After the stimulation, the medium was collected and centrifuged at 150 g for 5 min at 4°C . To precipitate extra protein, the supernatant was transferred to a 1.5 mL Eppendorf tube, 0.1 M HCl and 60% perchloric acid were added, and it was centrifuged again. After transferring the remaining supernatant, 5 M NaCl, 5 M NaOH, and *n*-butanol were added, and the solution was vortexed and centrifuged at 12,000 g. The upper layer was transferred to another tube to which 0.1 M HCl and *n*-heptane were added, and then centrifuged at 12,000 g. The histamine content in the bottom layer was measured using the *o*-phthaldialdehyde spectrofluorometric procedure as previously described [20]. The fluorescence intensity was detected at an excitation wavelength of 380 nm and an emission wavelength of 440 nm using a fluorescence plate reader (Molecular Devices).

2.6. Preparation of RPMCs

RPMCs were isolated from SD rats as previously described [19]. In brief, the rats were euthanized with CO_2 and injected with 20 mL of Tyrode's buffer A (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO_3 , 2.7 mM KCl, 0.3 mM NaH_2PO_4 , and 0.1% gelatin) into the peritoneal cavity. The abdomen was gently massaged for 120 s. The peritoneal cavity was carefully opened, and the fluid containing the peritoneal cells was collected with a Pasteur pipette. The cells were collected after centrifugation at 150g for 10 min at room temperature (25°C) and then resuspended in 1 mL of Tyrode's buffer A. To separate the mast cells from the other major rat peritoneal cells, macrophages and small lymphocytes, the peritoneal cells suspended in Tyrode's buffer A were layered on 2 mL of a 0.235 g/mL histodenz solution and centrifuged at 400 g for 15 min at room temperature. The cells at the buffer–histodenz interface were discarded, and the cells in the pellet were washed and resuspended. The mast cell preparations had a purity of approximately 95% as determined by toluidine blue staining, and more than 97% of the cells were viable based on trypan blue staining.

2.7. Intracellular calcium levels

The concentration of intracellular calcium was measured using the fluorescent indicator Fluo-3/AM (Invitrogen, Carlsbad, CA, USA). Anti-DNP IgE (100 ng/mL)-sensitized RBL-2H3 cells (2×10^4 cells/well in 96-well plates) were preincubated with Fluo-3/AM (5 μM) for 1 h at 37°C . The cells were treated with or without nothofagin for 1 h after washing three times with PBS and then were stimulated with DNP-HSA (100 ng/mL). The fluorescence intensity was measured using a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 510 nm as previously described [5]. The intracellular calcium levels were compared to those of untreated control cells set to 1 relative absorbance unit.

2.8. Quantitative real-time polymerase chain reaction (qPCR)

Prior to the isolation of total cellular RNA, anti-DNP IgE (50 ng/mL)-sensitized RBL-2H3 cells (5×10^5 cells/well in 12-well plates) were pretreated with nothofagin for 1 h and then stimulated with DNP-HSA (100 ng/mL) for 1 h. Total RNA was isolated using an RNAiso Plus kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using Maxime RT Premix (iNtRON Biotech, Sungnam, Korea). qPCR was performed using a Thermal Cycler Dice TP850 system (Takara Bio) according to the manufacturer's protocol. In brief, 1.5 μ L of cDNA (150 ng), 1 μ L of each of the forward and reverse primers (0.4 μ M), 12.5 μ L of SYBR Premix Ex Taq (Takara Bio), and 9 μ L of dH₂O were mixed together to obtain a final 25 μ L reaction mixture in each reaction tube. The conditions for amplification of DNA were 95 °C for 30 s, 45 cycles of 95 °C for 5 s, and 60 °C for 30 s. A melting curve analysis was conducted after amplification, and relative quantification of mRNA expression levels was performed using TP850 software; β -actin was used as the internal control. The relative transcription levels of the mRNAs were measured according to the $2^{-\Delta\Delta C_q}$ method [21]. The primer sequences used were as follows: TNF- α (F 5'-TCC CAA ATG GGC TCC CTC TC-3', R 5'-AAA TGG CAA ACC GGC TGA CG-3'), IL-4 (F 5'-TGC ACC GAG ATG TTT GTA CCA GA-3', R 5'-TTG CGA AGC ACC CTG GAA G-3'), IL-6 (F 5'-CAG ATT GTT TTC TGA CAG TG-3', R 5'-CAG GGA GAT CTT GGA AAT GA-3') and β -actin (F 5'-GAA GCT GTG CTA TGT TGC CCT AGA-3', R 5'-GTA CTC CTG CTT GCT GAT CCA CAT-3').

2.9. Enzyme-linked immunosorbent assay (ELISA)

TNF- α , IL-4 and IL-6 levels were measured using an ELISA kit (BD Biosciences, San Diego, CA, USA) in a 96-well immunoplate (Nunc, Rochester, NY, USA) as previously described [22]. Anti-DNP IgE (100 ng/mL)-sensitized RBL-2H3 cells (5×10^5 cells/well in 12-well plates) were pretreated with nothofagin for 1 h after washing with PBS and were then stimulated with DNP-HSA (100 ng/mL) for 6 h. After terminating the reaction, the absorbance intensity was detected using a microplate reader at a wavelength of 450 nm.

2.10. Western blot

Nuclear and cytosolic proteins were extracted as previously described [2]. After measuring equal amounts of cellular, nuclear, or total protein with a spectrophotometer, the proteins were electrophoresed using 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with a primary antibody against the target protein and then with anti-IgG horseradish peroxidase-conjugated secondary antibody. The following antibodies were used: NF- κ B (sc-109, rabbit polyclonal, 1:1000), I κ B α (sc-371, rabbit polyclonal, 1:1000), β -actin (sc-8432, mouse monoclonal, 1:1000), lamin B1 (sc-6217, goat polyclonal, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-Lyn (#2731, Tyr507, rabbit polyclonal, 1:1000), phospho-Syk (#2711, Tyr525/526, rabbit polyclonal, 1:1000), phospho-Akt (#9271, Ser473, rabbit polyclonal, 1:1000), Lyn (#2732, rabbit polyclonal, 1:1000), Syk (#2712, rabbit polyclonal, 1:1000), and Akt (#9272, rabbit polyclonal, 1:1000) (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive protein bands were visualized using a chemiluminescent substrate (Thermo Scientific).

2.11. Reporter assay

The transcriptional activity of NF- κ B was measured as previously mentioned [23]. For transient transfection, cells were seeded at a density of 1.5×10^5 in a 24-well plate. After the containing medium was changed with Opti-MEM (Gibco) medium, the cells were transfected with an expression vector containing an NF- κ B luciferase

reporter construct (pNF- κ B-LUC, plasmid containing an NF- κ B-binding site, Stratagene, Grand Island, NY, USA) in antibiotics-free media containing 2 μ L of the Lipofectamine 2000 reagent (Invitrogen) and then incubated at 37 °C for 12 h. After the incubation, the medium was replaced with serum-free medium, and the cells were subsequently stimulated with DNP-HSA. The cells were lysed and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA).

2.12. IgE-mediated PCA model

An IgE-mediated PCA model was established as previously described [5]. For sensitization, the ear skins of mice ($n = 5$ /group) were intradermally injected with anti-DNP IgE (0.5 μ g/site) for 48 h. Nothofagin was topically administered at a dose of 100 ng/ear 2 h before the intravenous injection of DNP-HSA (1 μ g/mouse) in 4% Evans blue at a 1:1 mixture. After 30 min, the mice were euthanized and both ears were collected for measurement of the pigment dye. The amount of dye was extracted with 1 mL of 1 M KOH and 4 mL of an acetone and phosphoric acid (5:13) mixture and determined using a spectrophotometer. The absorbance intensity was detected by spectrophotometry at 620 nm.

2.13. Statistical analysis

Statistical analyses were performed using the Prism statistical software program (GraphPad Software, Inc., La Jolla, CA, USA). Treatment effects were analyzed using one way of analysis of variance followed by Tukey's post test; $p < 0.05$ indicated statistical significance. Each data point represents the mean $\pm$ SEM of three independent experiments.

3. Results

3.1. Effects of nothofagin on the degranulation of mast cells

Upon cross-linking of Fc ϵ RI-bound IgE molecules by a multivalent antigen, the IgE receptors aggregate, which causes the mast cells to release pre-formed mediators such as histamine and β -hexosaminidase [3]. The mast cell-like cell line RBL-2H3 and RPMCs were used for *in vitro* study. For the degranulation assay, each cell was sensitized with anti-DNP IgE and challenged by DNP-HSA. The molecular structure of nothofagin is shown in Fig. 1A. Dexamethasone was used as a positive control drug. Histamine is regarded as a representative mediator in the allergic response and is an important marker of degranulation [24]. The DNP-HSA-challenged RBL-2H3 and RPMCs were released more than double the amount of histamine compared to that released by the unstimulated group. However, nothofagin pre-treatment (RBL-2H3: 1–10 μ M, RPMCs: 0.1–10 μ M) decreased the level of histamine release (Fig. 1B and C). Moreover, β -hexosaminidase, another marker of mast cell degranulation, was also suppressed by pre-treatment with nothofagin (0.1–10 μ M) in RBL-2H3 (Fig. 1D).

Calcium ion functions as a secondary messenger, and its influx to the cytoplasm is essential for mast cell degranulation [25]. To determine the effect of nothofagin on the cytosolic calcium level, intracellular calcium levels were measured using the fluorescent indicator Fluo-3/AM. BAPTA-AM, an intracellular calcium chelator, was used as a positive control. Intracellular calcium levels were increased by the DNP-HSA challenge but downregulated by nothofagin pre-treatment (Fig. 1E). In addition, the MTT assay was performed to detect the potential cytotoxicity of nothofagin. Nothofagin showed no cytotoxicity up to 10 μ M during 24 h of treatment (Fig. 1F).

3.2. Effects of nothofagin on the expression of pro-inflammatory cytokines

Pro-inflammatory cytokines such as TNF- α , IL-4 and IL-6 are known to cause various inflammatory responses such as recruitment and

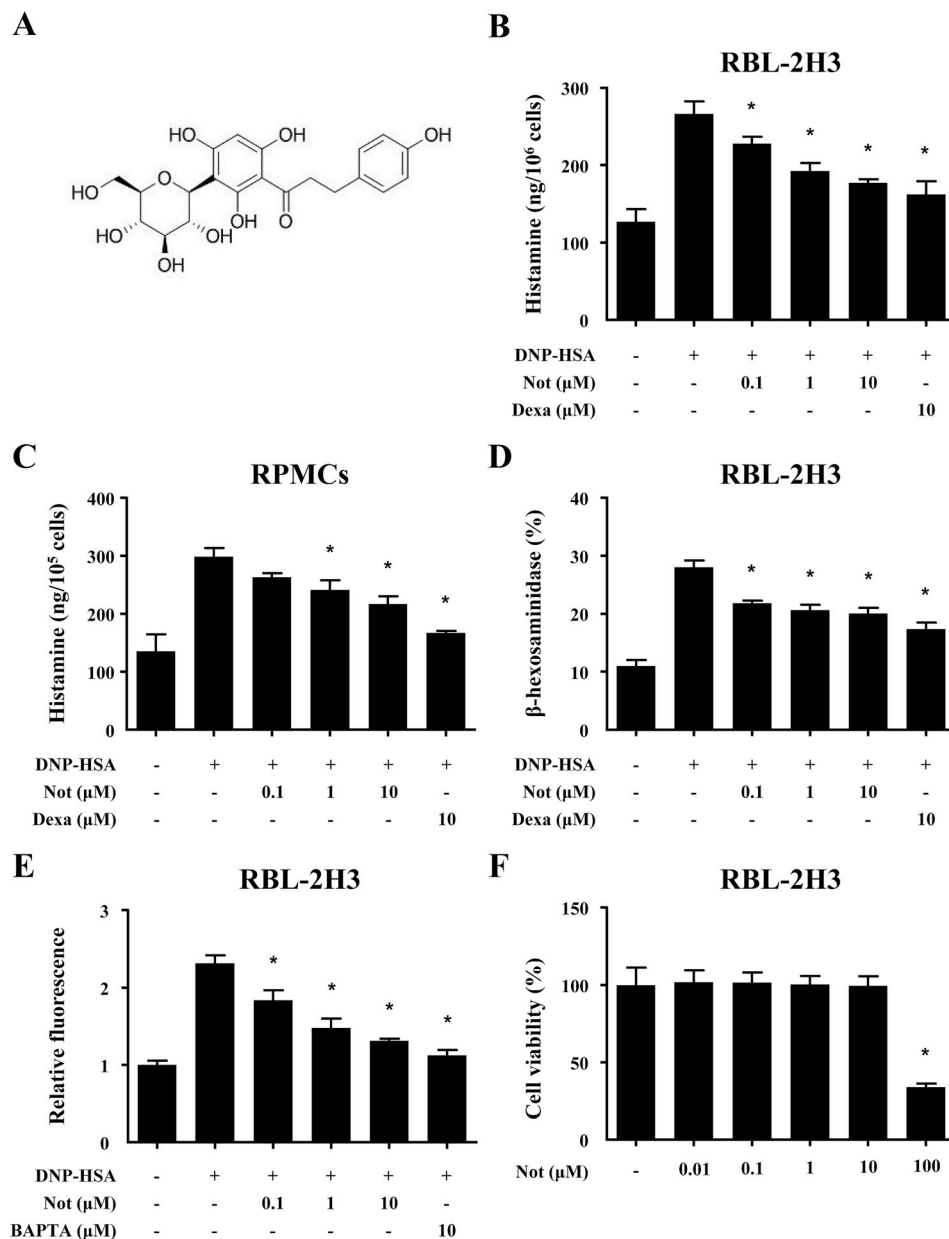


Fig. 1. Effects of nothofagin on mast cell degranulation. (A) Chemical structure of nothofagin. (B, C) In RBL-2H3 and RPMCs, histamine levels were measured using a spectrophotometric technique with *o*-phthaldialdehyde. (D) β -Hexosaminidase levels were measured in RBL-2H3 using a spectrophotometer. (E) Following sensitization, RBL-2H3 were pre-incubated with Fluo-3/AM, and intracellular calcium was detected using a fluorescence plate reader; BAPTA-AM was used as a positive control. (F) After the cells were incubated for 24 h, the absorbance levels were detected using a spectrophotometer. *Significantly different at $p < 0.05$. Not: nothofagin, Dexa: dexamethasone.

activation of immune cells, as well as contribute to the tissue remodeling in chronic inflammation [24]. We performed qPCR and ELISA to measure the transcriptional and translational levels of cytokines, respectively, upon nothofagin treatment. The expression levels of TNF- α , IL-4 and IL-6 were increased by DNP-HSA challenge. However, pre-treatment with nothofagin suppressed the expression of these pro-inflammatory cytokines (TNF- α : 0.1–10 μ M; IL-4: 10 μ M, IL-6: 1–10 μ M). Similarly, the production of cytokines were downregulated by nothofagin pre-treatment (TNF- α : 1–10 μ M; IL-4: 0.1–10 μ M, IL-6: 1–10 μ M) (Fig. 2A).

To identify the mechanisms by which nothofagin inhibits pro-inflammatory cytokine production, we investigated the effect of nothofagin on intracellular signaling molecules. The signal, which starts from the aggregation of antigen–IgE complexes bound to Fc ϵ RI, leads to the phosphorylation of Src family protein tyrosine kinases such as Lyn and Syk. This activation subsequently induces the phosphorylation of Akt, resulting in the degradation of I κ B α and the ultimate nuclear translocation of NF- κ B [26]. Our results showed that the phosphorylation of Lyn, Syk, and Akt was markedly suppressed by pre-treatment of DNP-HSA-stimulated RBL-2H3 with nothofagin (Fig. 2B). Once NF- κ B

translocates into the nucleus, it functions as a transcription factor by binding to the DNA template and can thus induce the expression of various cytokines such as TNF- α , IL-4 and IL-6 [27]. Thus, we performed Western blot analysis to measure the inhibitory effect of nothofagin on the activation of these intracellular signal proteins. Furthermore, we also measured the activity of NF- κ B with a reporter gene assay by inserting a DNA vector containing the NF- κ B-binding site. The results of these assays clearly showed that the nuclear translocation and activity of NF- κ B were both significantly suppressed by pre-treatment of nothofagin (Fig. 2C).

3.3. Effects of nothofagin on PCA

PCA is a well-characterized animal model of a local allergic reaction [28]. After intradermal injection of anti-DNP IgE (0.5 μ g/site) in the ears of mice, they received intravenous injection of a DNP-HSA (1 μ g/mouse) and 4% Evans blue (1:1) mixture. After antigen challenge, a blue spot was detected at the sensitized site due to a marked increase in the vascular permeability of the ears. When nothofagin was topically administered to the mice, this vascular permeability was attenuated, as

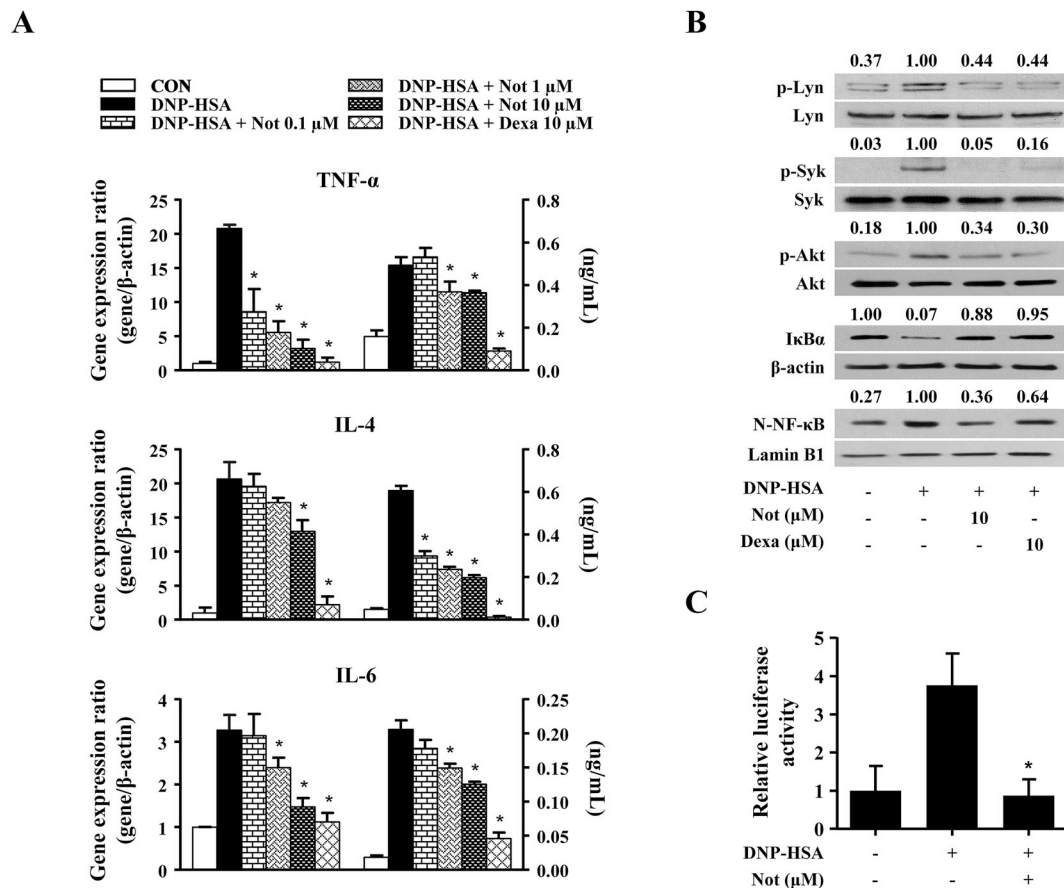


Fig. 2. Effects of nothofagin on inflammatory cytokines and signal molecules. (A) The gene expression levels of pro-inflammatory cytokines were determined with qPCR. The secretion of pro-inflammatory cytokines was measured by ELISA. Each data point represents the mean $\pm$ SEM of three independent experiments. (B) NF- κ B translocation, I κ B α degradation, and the activation of signal molecules were assayed by Western blot (N-: nuclear, p-: phosphorylated). β -actin and lamin B1 were used as loading controls. The bands shown are representative of three independent experiments. The band intensity was digitized and normalized to the relative ratio. (C) Cells were transiently transfected with the NF- κ B-luciferase reporter construct gene and then incubated with or without nothofagin, and finally stimulated with anti-DNP IgE and DNP-HSA. NF- κ B-dependent transcriptional activity was determined by a reporter gene assay. *Significant difference at $p < 0.05$. Not: nothofagin, Dexa: dexamethasone.

indicated by a decrease in the extent of blue staining of the ear (Fig. 3A). Further, we measured the ear thickness and absorbance level of Evans blue that infiltrated the ear tissue. The ear thickness and pigment absorbance were both increased by antigen injection. Nothofagin and dexamethasone appeared to show similar suppressive effects on ear thickness and pigment absorbance at the same dose (Fig. 3B and C).

4. Discussion

IgE-mediated mast cell degranulation is a major response against an allergens, contributing to the development of allergic inflammation [3]. Repeated allergen exposure enhances allergic responses and activates mast cells, which can be implicated in the pathophysiology of many diseases, including allergy, asthma, and anaphylaxis. Therefore, it is necessary to inhibit the excessive activation and degranulation of mast cells to prevent the progress of allergic inflammation for benefits to human health. In this study, we demonstrated that nothofagin could suppress IgE-mediated mast cell degranulation both *in vitro* and *in vivo*.

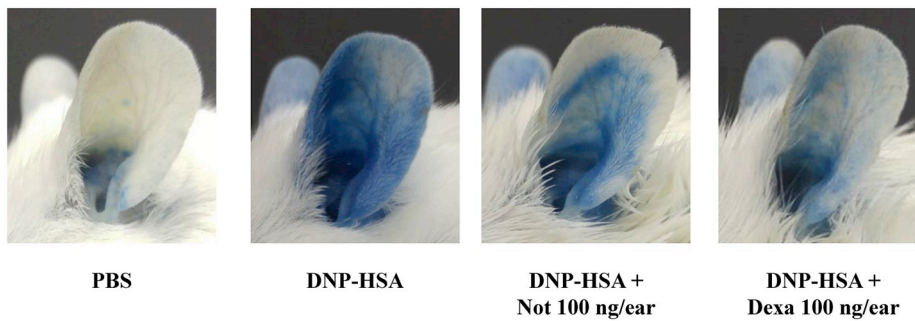
The mechanisms of IgE-mediated mast cell degranulation are well understood. Initially, IgE attaches to Fc ϵ RI on the mast cell surface. After the IgE-Fc ϵ RI complexes combine with the allergen, the complexes are aggregated and signal transduction starts in the cytoplasm. Activated mast cells release pre-formed mediators and produce newly synthesized cytokines, which lead to the development of allergic diseases [29]. We used two types of mast cells: RBL-2H3 and RPMCs. RBL-

2H3 is one of the most popular mast cell lines and is known as a particularly good histamine releaser compared with other RBL sublines [30]. Despite several issues related to the reliability of RBL-2H3 cells, they are still considered suitable for demonstrating IgE-mediated mast cell degranulation [31]; RPMCs, primary cultured mast cells, were additionally used to verify the reliability of RBL-2H3.

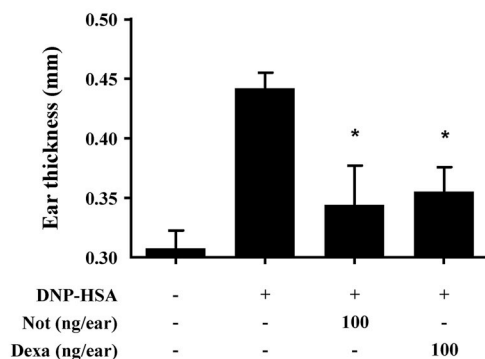
Among the mediators that mast cells release, histamine is regarded as a representative degranulation marker with various impacts such as vascular permeability, vasodilation, and smooth muscle contraction [32]. In addition, mast cells are considered to be the major histamine-producing cell types. Therefore, downregulating histamine production is key to ameliorate the systemic changes caused by allergen exposure; another important marker is β -hexosaminidase, which is a lysosomal enzyme released from mast cells [3,33]. Our results showed that nothofagin inhibited mast cell degranulation.

Furthermore, we evaluated cytosolic calcium levels with or without nothofagin. It is well accepted that calcium ion functions as a secondary messenger, and that intracellular calcium levels increase in the face of IgE-mediated mast cell degranulation [25,34]. After antigen-IgE-induced cross-linking and aggregation of Fc ϵ RI on the cell surface, PLC γ is activated. Activated PLC γ then hydrolyzes phosphatidylinositol-4,5-bisphosphate to form soluble inositol-1,4,5-trisphosphate (IP $_3$) and membrane-bound diacylglycerol [35]. IP $_3$ binds to its receptor on the endoplasmic reticulum and triggers transient calcium influx into the cytoplasm, which provides positive feedback to cause more calcium ion influx from outside the cell membrane. Various reports have shown that

A



B



C

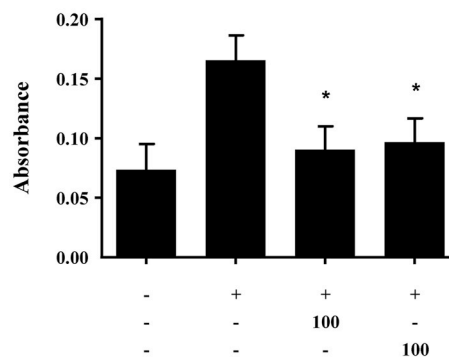


Fig. 3. Effects of nothofagin on IgE-mediated passive cutaneous anaphylaxis. (A) The ear skin of mice ($n = 5/\text{group}$) was sensitized with an intradermal injection of anti-DNP IgE (0.5 mg/site) for 48 h. Nothofagin was topically administered at a dose of 100 ng/ear 2 h before the intravenous injection of a DNP-HSA and 4% Evans blue (1:1) mixture. Thirty minutes later, the pictures were taken. (B) Ear thickness was measured with a dial thickness gauge. (C) After the thickness of both ears was measured, the ears were melted. The dye was detected using a spectrophotometer. *Significantly different at $p < 0.05$. Not: nothofagin, Dexa: dexamethasone.

intracellular calcium functions as an important secondary messenger to trigger histamine release [36,37]. We found that nothofagin inhibited the degranulation of mast cells and intracellular calcium increase in a concentration-dependent manner. Therefore, the suppressive effects of nothofagin might be caused by its ability to reduce the intracellular calcium level.

The increase of intracellular calcium not only affects degranulation but also is directly proportional to the gene expression of pro-inflammatory cytokines [26]. Indeed, the release of pro-inflammatory cytokines such as TNF- α , IL-4 and IL-6 is an integral component of the progression to chronic allergic inflammation [4]; TNF- α is a pro-inflammatory cytokine that plays one of the most important roles to promote inflammation, and stimulates vasodilation and edema formation, immune cell proliferation, migration, and reactive oxygen/nitrogen species generation [38]. TNF- α also induces eosinophil survival, thereby contributing to the development of chronic inflammation [2]; IL-4 induces an IgE class switch on B cells and leads to Th2 cell polarization by stimulating the maturation of naïve T cells [3]; IL-6 drives disease progression or supports the maintenance of immunological reactions [39]. Recently, IL-6 has been reported its priming effect in IgE-mediated mast cell activation [40]. Although IL-6 secretion is strictly managed by physiological mechanisms, dysregulated or excessive secretion of IL-6 functions a pathological role on chronic inflammation [41]. In the present study, we found that nothofagin inhibited the gene expression and production of TNF- α , IL-4 and IL-6 in a concentration-dependent manner. From these results, we suggest that nothofagin suppresses the release of pre-formed and newly synthesized inflammatory mediators.

The release of various cytokines is caused by the activation of transcription factors such as NF- κ B, which is involved in the transcription of many cytokine-related genes, including TNF- α , IL-4 and IL-6 [24]. In the inactive condition, NF- κ B is present in the cytosol where it interacts with I κ B. After being triggered with Fc ϵ RI cross-linking of an IgE-antigen complex, ITAMs phosphorylate Src family kinases such as

Lyn and Syk. Activated Lyn and Syk after ITAM binding then phosphorylate several adaptor molecules and enzymes, which lead to activating Akt. The phosphorylated IKK complex by activated Akt induces the degradation of I κ B combined with NF- κ B, which releases NF- κ B to move into the nucleus and leads to the *de novo* synthesis of cytokines, ultimately inducing late-phase allergic inflammation. Thus, we measured the activation and nuclear translocation of NF- κ B, the major transcription factor regulating gene expression on mast cells [42]. Our results showed that nothofagin significantly suppressed NF- κ B activation and its translocation, as well as activation of upstream signal proteins such as p-Lyn, p-Syk, and p-Akt. These results indicate that nothofagin inhibits mast cell activation by interrupting the Fc ϵ RI signaling pathway.

The IgE-mediated PCA model is a well-characterized animal model that effectively mimics the allergic reaction [2]. Mast cells are mostly found in close contact with the external environment, such as in airways, the intestine, and the skin [43]. Intradermal injection of IgE promotes local allergic conditions exposed to a specific allergen in skin tissue. After challenge with the DNP-HSA and Evans blue mixture, enhanced vascular permeability by histamine appeared based on an increase of pigment infiltration and ear thickness; however, these effects were attenuated by the topical administration of nothofagin. These results suggest that nothofagin suppresses the allergic reaction through inhibiting IgE-mediated mast cell activation.

In conclusion, nothofagin reduced the degranulation of mast cells via regulating intracellular calcium levels. The transcription and translation of pro-inflammatory cytokines were also downregulated by nothofagin through inhibition of NF- κ B activity. The attenuation of Lyn, Syk, and Akt, acting downstream of the Fc ϵ RI signaling pathway, led to a reduction of the transcription and translation of pro-inflammatory cytokines. Furthermore, nothofagin reduced the vascular permeability and plasma extravasation in local anaphylaxis. Taken together, our study demonstrates that nothofagin could be a potential therapeutic candidate for mast cell-mediated allergic inflammation, which warrants

further exploration.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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