



Inhibitory effect of aqueous spinach extract on degranulation of RBL-2H3 cells

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ABSTRACT

The inhibitory effect of an aqueous extract from spinach on degranulation of RBL-2H3 cells is herein reported. The extract significantly suppressed antigen-induced degranulation in a dose-dependent manner without affecting cell viability. Active substances in the extract were heat-stable and trypsin-resistant with molecular weights ranging from 500 Da to 14 kDa. The extract inhibited elevation of the intracellular Ca^{2+} concentration caused by stimulation by antigen, while not suppressing degranulation induced by a calcium ionophore A23187. Immunoblot analysis revealed that the inhibitory effect results from down-regulation of phosphorylation of both Syk kinase and phosphatidylinositol 3-kinase in the signalling pathways involved in degranulation caused by the antigen–antibody interaction. Taken together, these findings suggest that aqueous spinach extract has an anti-allergic activity that controls degranulation.

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1. Introduction

Allergy is mainly classified into four types, and the type I allergy is the most common allergic reaction associated with asthma and allergic dermatitis. The type I allergic reaction is provoked by cross-linkage of an antigen, referred to as allergen, such as cedar pollen to immunoglobulin E (IgE) bound on the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. The cross-linked FcεRI activates the Src family non-receptor tyrosine kinases Lyn and Fyn. Activation of Lyn induces phosphorylation of the Syk kinase which leads to Ca^{2+} mobilisation. As a result, chemical mediators such as histamine, eicosanoids, and inflammatory cytokines are released from intracellular granules, which is called degranulation (Kopeć, Panaszek, & Fal, 2006; Kraft & Kinet, 2007; Metcalfe, Peavy, & Gilfillan, 2009; Rivera & Gilfillan, 2006; Turner & Kinet, 1999). Released histamine induces an acute allergic

response, such as contraction of smooth muscle, vasodilation, and increased vascular permeability (White, 1999). Thus, mast cells play a crucial role in the type I allergic reaction, and prevention of degranulation is of great importance for the relief of allergic symptoms. Various food components have been reported to suppress degranulation in cellular systems, through inhibition of intracellular signalling pathways provoked by the cross-linkage between allergen and IgE on the FcεRI (Han, Park, Kim, & Jeong, 2009; Itoh et al., 2010).

Spinach, *Spinacia oleracea* Linn., is a vegetable rich in various nutrients, such as carotenes, vitamins, folic acid, and iron. Various biological functions based on these nutrients have been reported; lutein, a non-provitamin A carotenoid, has been, for example, strongly implicated as a protective agent against age-related macular degeneration and cataracts (Johnson, 2000; Riso, Brusamolino, Scafi, & Porrini, 2004). A glycolipid fraction from spinach has been reported to exert inhibitory effects on mammalian DNA polymerase activity and human cancer cell proliferation (Kuriyama et al., 2005; Maeda et al., 2005). So far, it has been reported that an aqueous spinach extract effectively ameliorates asthmatic symptoms in ovalbumin-challenged model mice, inducing a decrease in the CD4⁺ cell number and the levels of interleukin-4 and interleukin-13 in the lung (Heo et al., 2010). However, an anti-allergic activity of spinach based on inhibition of degranulation has yet to be reported. We herein report the inhibitory effect of an aqueous extract from spinach on antigen-induced degranulation using rat basophilic leukaemia RBL-2H3 cells and the properties of active substances in the extract. In addition, an intracellular mechanism of action of the spinach extract was also examined.

Abbreviations: BSA, bovine serum albumin; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's Modified Eagle Medium; DNP, dinitrophenol; FBS, foetal bovine serum; FcεRI, high-affinity IgE receptor; HRP, horseradish peroxidase; HSA, human serum albumin; IgE, immunoglobulin E; MWCO, molecular weight cut off; NaPB, sodium phosphate buffer; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; SNE, spinach NaPB extract; SD, standard deviation; TBS-T, Tris-buffered saline containing 0.1% Tween 20.

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2. Materials and methods

2.1. Reagents

RBL-2H3 cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, bovine serum albumin (BSA), mouse anti-dinitrophenol (DNP) monoclonal IgE, DNP-human serum albumin (HSA), a calcium ionophore A23187, and Triton X-100 were products of Sigma (St. Louis, MO). Aprotinin, Pefabloc SC, and a protease inhibitor cocktail were obtained from Roche Applied Science (Basle, Switzerland). A phosphatase inhibitor cocktail was purchased from Nacalai Tesque (Kyoto, Japan). Goat anti-actin antibody (sc-1616) and horseradish peroxidase (HRP)-labelled anti-goat IgG antibody (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphatidylinositol 3-kinase (PI3K) p85 antibody (#4257), anti-phosphorylated PI3K p85/p55 antibody (#4228), a B cell signalling antibody sampler kit (#9768) including antibodies against Syk, phosphorylated Syk, Lyn, phosphorylated Lyn, Btk, and phosphorylated Btk, a PLC γ antibody sampler kit (#3860) including antibodies against PLC γ 1, phosphorylated PLC γ 1, PLC γ 2, and phosphorylated PLC γ 2, and HRP-labelled anti-rabbit IgG antibody (#7074) were from Cell Signaling Technology (Danvers, MA).

2.2. Sample preparation

Freeze-dried spinach powder was suspended in 10 mM sodium phosphate buffer (NaPB; pH 7.4) at 0.1 g/mL at 4 °C overnight. After the suspension was centrifuged at 10,000 g at 4 °C for 20 min, the supernatant was filtrated through a 0.22- μ m membrane and used as the spinach NaPB extract (SNE). Protein concentration of SNE was determined by a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). To evaluate the effect of the dialysis treatment, SNE was dialysed using a dialysis membrane with molecular weight cut off (MWCO) of 14 kDa (Wako Pure Chemical Industries, Osaka, Japan) or with MWCO of 500 Da (Spectrum Laboratories, Rancho Dominguez, CA) against 10 mM NaPB at 4 °C overnight and used for the degranulation assay. To evaluate a heating effect, SNE was heated at 100 °C for 15 min and used for the degranulation assay. To evaluate the trypsin sensitivity, SNE (60 μ g/mL) treated with 50 or 500 μ g/mL of trypsin at 37 °C for 15 min was inactivated by the addition of 500 μ g/mL of a trypsin inhibitor (Wako Pure Chemical Industries) and then used for the degranulation assay.

2.3. β -Hexosaminidase release assay

The assay was performed by the method of Watanabe, Shinmoto, and Tsushida (2005) with some modifications. RBL-2H3 cells suspended in DMEM containing 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% foetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS,) were seeded into a 96-well cell culture plate (BD Falcon, Franklin Lakes, NJ) at 6.0×10^4 cells/well and cultured at 37 °C overnight under humidified 5% CO₂-95% air. The cells were next treated with anti-DNP monoclonal IgE at 50 ng/mL and incubated at 37 °C for 2 h. After washing the cells with a modified Tyrode's buffer (20 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA, pH 7.4) twice, anti-DNP IgE-sensitised cells were treated with 120 μ L of modified Tyrode's buffer containing various concentrations of SNE or 10 mM NaPB (control) and incubated at 37 °C for 10 min. Then, 10 μ L of DNP-HSA diluted in the modified Tyrode's buffer at 0.625 μ g/mL were added to each well and incubated at 37 °C for 30 min. After incubation, the supernatant from each was collected, and the cells

were sonicated in 130 μ L of the modified Tyrode's buffer containing 0.1% Triton X-100 for 5 s on ice. Both supernatant and cell lysate were transferred into a 96-well microplate at 50 μ L/well and incubated at 37 °C for 5 min. Then, 100 μ L of 3.3 mM *p*-nitrophenyl-2-acetamido-2-deoxy- β -d-glucopyranoside (Wako Pure Chemical Industries) dissolved in 0.1 M citrate buffer (pH 4.5) were added to each well and incubated at 37 °C for 25 min. The enzyme reaction was terminated by the addition of 100 μ L of 2 M glycine buffer (pH 10.4), and the absorbance was measured at 405 nm using a microplate reader (Model 550; Bio-Rad Laboratories). β -Hexosaminidase release rate was calculated as $100 \times [(A_{\text{supernatant}} - A_{\text{blank of supernatant}}) / ((A_{\text{supernatant}} - A_{\text{blank of supernatant}}) + (A_{\text{cell lysate}} - A_{\text{blank of cell lysate}}))]$, in which A is the absorbance of each well.

2.4. Measurement of cell viability

Cytotoxicity of SNE on RBL-2H3 cells was examined using a WST-8 assay kit (Kishida Chemical, Osaka, Japan) according to manufacturer's instructions. Anti-DNP IgE-sensitised cells were treated with various concentrations of SNE and stimulated by DNP-HSA as described above. After the cells were washed with phosphate-buffered saline (PBS, pH 7.4) once, 100 μ L of 10% FBS-DMEM containing 10 μ L of the WST-8 solution were added to each well of the cell culture plate and incubated for 30 min at 37 °C. Then, the absorbance was measured at 450 nm using a microplate reader.

2.5. Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i)

The [Ca²⁺]_i was measured using Calcium Kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's instructions. RBL-2H3 cells were seeded into a white 96-well culture plate and treated with anti-DNP IgE as described above. Then, the IgE-sensitised cells were washed with PBS twice and incubated with 100 μ L of Fluo-3 AM for 1 h. Treated cells were washed again with PBS and incubated with SNE (45 μ g/mL) or 10 mM NaPB at 37 °C for 10 min. Then, the cells were stimulated by the addition of 10 μ L of DNP-HSA diluted in the modified Tyrode's buffer at 0.625 μ g/mL, and the fluorescent intensity was immediately monitored with an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a microplate reader (SH-8000Lab; Corona Electric, Ibaraki, Japan).

2.6. A23187-induced degranulation assay

RBL-2H3 cells were seeded and treated with various concentrations of SNE as described above, except sensitisation by anti-DNP IgE. Ten microlitres of A23187 diluted in the modified Tyrode's buffer at 3 μ M were added to each well, and the cells were incubated at 37 °C for 30 min. Then, the β -hexosaminidase release rate was measured as described above.

2.7. Immunoblot analysis

RBL-2H3 cells were seeded into a 24-well culture plate at 2.5×10^5 cells/well and treated with anti-DNP IgE as described above. After the IgE-sensitised cells were washed with the modified Tyrode's buffer twice, 490 μ L of the modified Tyrode's buffer containing SNE (45 μ g/mL) or 10 mM NaPB as control were added to each well and incubated at 37 °C for 10 min. Then, the cells were stimulated by the addition of 10 μ L of DNP-HSA diluted in the modified Tyrode's buffer at 2.5 μ g/mL and further incubated for 10 min at 37 °C. After removing the added reagents, 30 μ L of a lysis buffer consisting of 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na₄P₂O₇, 2 μ g/mL of aprotinin, Pefabloc SC, a protease inhibitor cocktail, and a phosphatase inhibitor cocktail were added

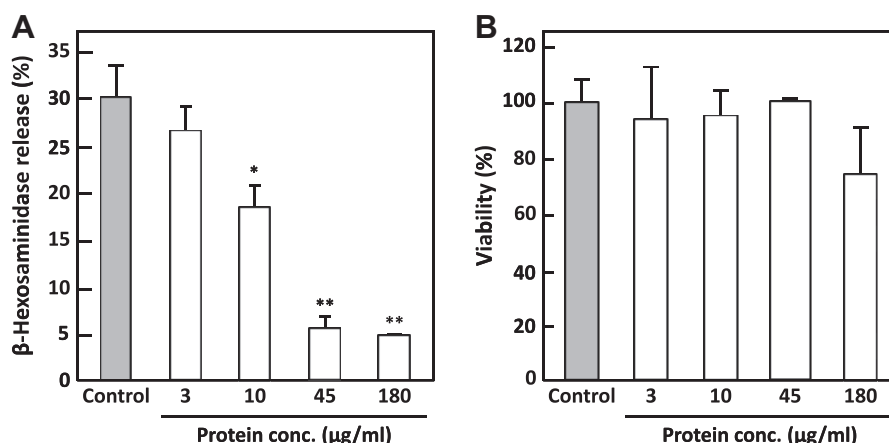


Fig. 1. Effects of SNE on degranulation and cell viability of RBL-2H3 cells. (A), Various protein concentrations of SNE or 10 mM NaPB as control were added to the culture medium of anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. Released β -hexosaminidase was used as a marker of degranulation. (B), Cell viability was measured using WST-8 solution after various concentrations of SNE or 10 mM NaPB as control were added to anti-DNP IgE-sensitised RBL-2H3 cells followed by stimulation by DNP-HSA. Data were represented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ against control.

to each well. After the cell lysates were centrifuged at 14,000g at 4 °C for 15 min, each supernatant was equalised to the same protein concentration. Proteins in a total cell lysate were then separated by SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, GE Healthcare, Amersham, UK). The membrane was blocked with 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with a primary antibody in 5% BSA-TBS-T at 4 °C overnight. After washing with TBS-T, the membrane was incubated with HRP-labelled anti-goat IgG antibody or HRP-labelled anti-rabbit IgG antibody in 5% skim milk-TBS-T at room temperature for 1 h. After washing with TBS-T, blots were developed by an ECL Western Blotting Detection Reagent (GE Healthcare) or ImmunoStar LD (Wako Pure Chemical Industries). Bands were visualised using a ChemiDoc XRS Plus apparatus (Bio-Rad Laboratories), and the chemiluminescent intensity was quantified using Quantity One software (Bio-Rad Laboratories).

2.8. Statistical analysis

Data obtained were expressed as the mean $\pm$ standard deviation (SD). Tukey's test was used to assess the statistical significance of the difference against control. Values with * $p < 0.05$ or ** $p < 0.01$ were considered to be statistically significant.

3. Results

3.1. Effect of SNE on degranulation of RBL-2H3 cells

An effect of SNE on degranulation of RBL-2H3 cells was first examined. SNE was added to culture medium of anti-DNP IgE-sensitised RBL-2H3 cells at various concentrations, and degranulation was induced by stimulation using DNP-HSA. As shown in Fig. 1A, degranulation was suppressed by the addition of SNE in a dose-dependent manner. The β -hexosaminidase release rate was nearly equal to that of control at the highest protein concentration of SNE. Significant differences in the β -hexosaminidase release rate were observed against control at 10 μ g/mL or higher protein concentrations of SNE, suggesting that SNE has a degranulation-suppressing activity on RBL-2H3 cells stimulated by antigen. A cell viability test showed that SNE was not cytotoxic within the tested concentrations, although the cell viability slightly decreased at the highest

protein concentration of SNE (Fig. 1B). From these results, SNE was used for further experiments at around 45 μ g/mL of protein concentration, showing a strong degranulation-suppressing activity without cytotoxicity.

3.2. Properties of active substances in SNE

To examine the molecular size of the active substances, SNE was subjected to a dialysis treatment and used for the degranulation assay. The results showed that SNE dialysed using a dialysis membrane with 14 kDa MWCO lost the degranulation-suppressing activity (Fig. 2A), whereas the activity remained with 500 Da MWCO (Fig. 2B), indicating that the molecular weight of the active substance in SNE is between 500 Da and 14 kDa.

To further investigate the properties of the active substance, SNE was heated at 100 °C for 15 min and used for the degranulation assay. As shown in Fig. 3A, there was little difference in the degranulation-suppressing activity between the heated and non-heated SNE, indicating that the active substance in SNE is stable to heat. Next, SNE was treated with trypsin and used for the degranulation assay. As shown in Fig. 3B, the trypsin treatment did not affect the inhibitory activity of SNE at all, even though the trypsin concentration increased, indicating that the active substance in SNE is trypsin-resistant.

3.3. Effect of SNE on $[Ca^{2+}]_i$

Ca^{2+} is one of major second messengers in intracellular signalling, and elevation of $[Ca^{2+}]_i$ is the critical process in degranulation. Thus, the degranulation-suppressing effect of SNE on $[Ca^{2+}]_i$ was examined using fluo-3 AM. As shown in Fig. 4A, $[Ca^{2+}]_i$ in RBL-2H3 cells rapidly increased and was sustained for 15 min after stimulation with antigen, while the elevation of $[Ca^{2+}]_i$ was suppressed in the presence of SNE, indicating that SNE would reduce degranulation by suppressing the elevation of $[Ca^{2+}]_i$ caused by stimulation of the antigen–antibody interaction. In addition, the degranulation-suppressing activity of SNE was examined under stimulation with calcium ionophore A23187 that carries Ca^{2+} into the cytosol across the plasma membrane. The result showed that SNE did not exert suppressive activity on degranulation induced by A23187 (Fig. 4B), indicating that SNE would be involved in an early stage of the signalling pathway specific to the antigen–antibody interaction.

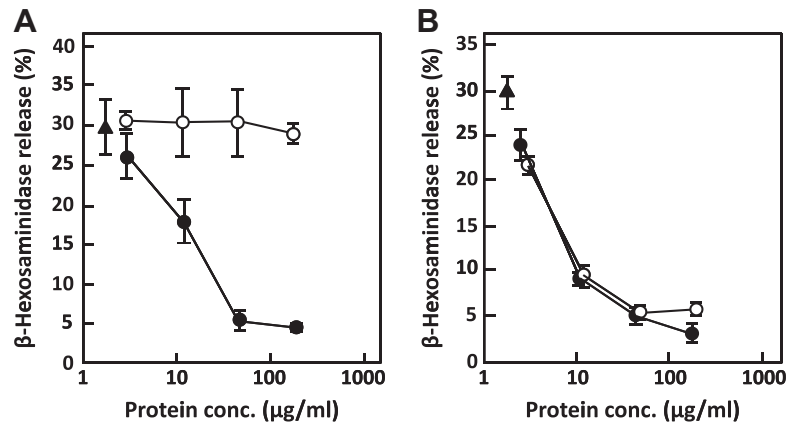


Fig. 2. Effect of dialysed SNE on degranulation of RBL-2H3 cells. (A), SNE was dialysed with a dialysis membrane of 14 kDa MWCO against 10 mM NaPB at 4 °C overnight. Then, various protein concentrations of dialysed (○) or non-dialysed (●) SNE or 10 mM NaPB (▲) as control were added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. (B), SNE was dialysed with a dialysis membrane of 500 Da MWCO against 10 mM NaPB at 4 °C overnight. Then, various concentrations of dialysed (○) or non-dialysed (●) SNE or 10 mM NaPB (▲) as control were added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. Released β -hexosaminidase was used as a marker of degranulation. Data were represented as the mean $\pm$ SD ($n = 3$).

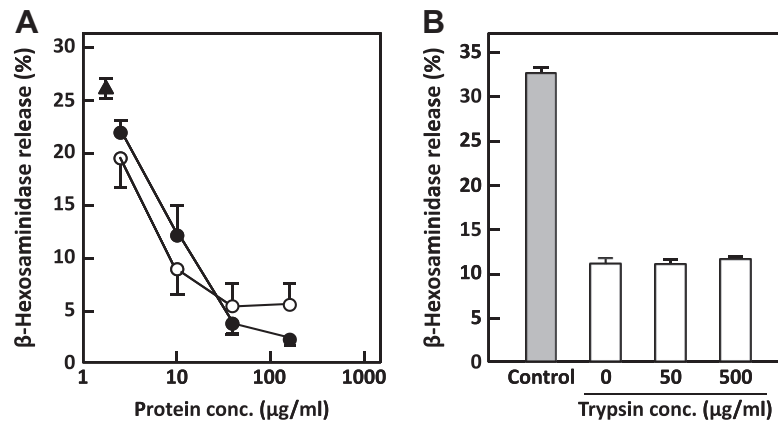


Fig. 3. Effects of heating and trypsin digestion of SNE on degranulation of RBL-2H3 cells. (A), Various protein concentrations of SNE were heated at 100 °C for 15 min or not heated. Then, heated (○) or non-heated (●) SNE or 10 mM NaPB (▲) as control were added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. (B), SNE at protein concentration of 60 μ g/mL was treated with 50 or 500 μ g/mL of trypsin for 15 min at 37 °C or not treated. Then, trypsin-treated or non-treated SNE or 10 mM NaPB as control was added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. Released β -hexosaminidase was used as a marker of degranulation. Data were represented as the mean $\pm$ SD ($n = 3$).

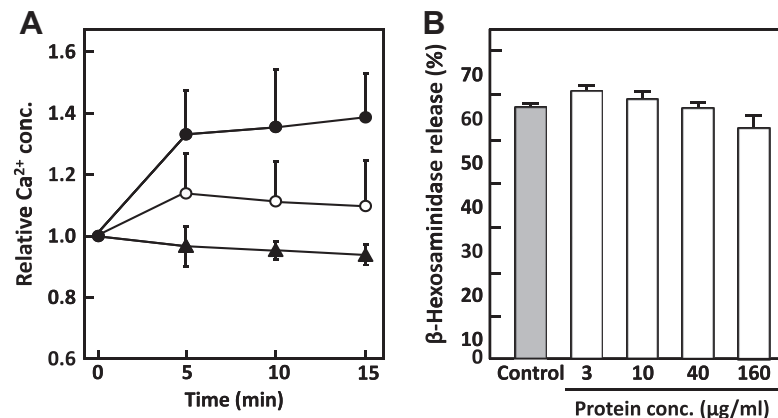


Fig. 4. Effect of SNE on $[Ca^{2+}]_i$ in RBL-2H3 cells. (A), $[Ca^{2+}]_i$ was measured by the Calcium Kit-Fluo-3. Anti-DNP IgE-sensitised cells were incubated with Fluo-3 AM for 1 h, and thereafter incubated with SNE (45 μ g/mL) or 10 mM NaPB for 10 min. Then, the treated cells were stimulated by DNP-HSA and the fluorescence intensity was measured. (●), non-SNE-treated cells stimulated with antigen; (○), SNE-treated cells stimulated with antigen; (▲), non-SNE-treated cells not stimulated with antigen. (B), Various protein concentrations of SNE or 10 mM NaPB as control were added to RBL-2H3 cells, and the treated cells were stimulated by the calcium ionophore A23187 for 30 min to induce degranulation. Released β -hexosaminidase was used as a marker of degranulation. Data were represented as the mean $\pm$ SD ($n = 3$).

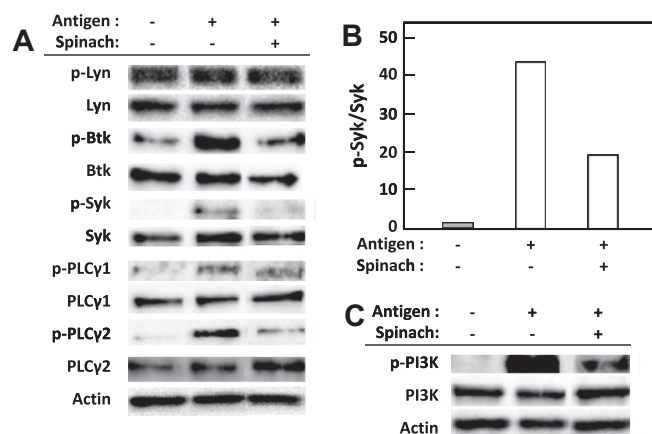


Fig. 5. Effect of SNE on the signalling pathways involved in degranulation of RBL-2H3 cells stimulated by antigen. (A) SNE was added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. Then, each cell lysate was subjected to immunoblot analysis. The p-Lyn, p-Btk, p-Syk, and p-PLC represent phosphorylated Lyn, phosphorylated Btk, phosphorylated Syk, and phosphorylated PLC, respectively. A representative blot from two independent experiments is shown. (B) The ratio of p-Syk/Syk in each cell lysate relative to that in the intact cells (antigen, –; spinach, –). (C) SNE was added to anti-DNP IgE-sensitised RBL-2H3 cells, and degranulation was induced by the addition of DNP-HSA. Then, each cell lysate was subjected to immunoblot analysis. The p-PI3K represents phosphorylated PI3K. A representative blot from two independent experiments is shown.

3.4. Effect of SNE on the signalling pathways involved in antigen-stimulated degranulation

Finally, we examined how SNE affects the signalling pathways involved in the antigen-stimulated degranulation by immunoblot analysis. As shown in Fig. 5A, the phosphorylation levels of Syk, Btk, and PLCγ1/2 locating downstream of the tyrosine kinase Lyn were suppressed by the addition of SNE, although SNE did not affect phosphorylation of Lyn that lies beneath the FcεRI receptor. Especially, the phosphorylation level of Syk was strongly suppressed by SNE (Fig. 5B). In addition, the phosphorylation level of PI3K was also decreased by the treatment of SNE (Fig. 5C). These results revealed that SNE suppresses antigen-induced degranulation in RBL-2H3 cells through downregulation of the signalling pathways involved in degranulation.

4. Discussion

In this study, we found that SNE suppresses the antigen-induced degranulation of RBL-2H3 cells. The properties of the active substance in SNE were suggested to be heat-stable and trypsin-resistant with a molecular weight between 500 Da and 14 kDa. Since an ethanolic extract from spinach did not suppress the antigen-induced degranulation of RBL-2H3 cells at all (data not shown), the active substances in SNE would not be phytochemicals, which are natural chemicals such as carotenoids and chlorophylls contained in plants (Johnson, 2000; Riso et al., 2004). Thus, the active substances in SNE are assumed to be peptides or water-soluble polysaccharides.

The mechanism underlying the inhibition of degranulation by SNE was investigated. It is known that $[Ca^{2+}]_i$ in mast cells and basophils rises through signalling after the cross-linkage of antigen to FcεRI through IgE for degranulation. From Fig. 4A, the antigen-induced degranulation was suppressed in the presence of SNE, although SNE did not exert inhibitory activity on the A23187-induced degranulation of RBL-2H3 cells. These results imply that SNE suppressed the elevation of $[Ca^{2+}]_i$ induced by intracellular

signalling pathways caused by the antigen–antibody interaction, but not the influx of extracellular Ca^{2+} .

The signalling pathways involved in degranulation initiate from phosphorylation of Src family non-receptor tyrosine kinases Lyn and Fyn after cross-linkage of antigen to FcεRI via IgE. The activated Lyn kinase next phosphorylates the β- and γ-chains of ITAMs, and the activated γ-ITAMs recruit and phosphorylate the Syk kinase (Kopeć et al., 2006; Kraft & Kinet, 2007; Metcalfe et al., 2009; Rivera & Gilfillan, 2006; Turner & Kinet, 1999). The activated Syk in turn phosphorylates the adaptor molecule LAT, and then the activated LAT forms a complex with other adapter proteins such as Grb2 and PLCγ1/2 (Saitoh et al., 2000). The phosphorylated PLCγ1/2 of the complex hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate, an inducer of intracellular Ca^{2+} mobilisation, and diacylglycerol that activates the protein kinase C (Cho, Woo, Yoon, & Kim, 2004; Kopeć et al., 2006). On the other hand, the Fyn kinase phosphorylates the adapter protein Gab2 (Gu et al., 2001), and then Gab2 phosphorylates PI3K that activates PLCγ1/2 by converting PIP2 to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Barker, Lujan, & Wilson, 1999; Kim, Rädinger, & Gilfillan, 2008; Koyasu, 2003). It has been reported that the Lyn-Syk-LAT pathway is calcium-dependent, while the Fyn-Gab2-PI3K pathway is calcium-independent (Nishida et al., 2005), because the calcium response was partially impaired in the Lyn-deficient mast cells, whereas there was a minimal effect in the Fyn-deficient mast cells (Parravicini et al., 2002).

As seen in the results of immunoblotting, SNE suppressed the phosphorylation levels of Syk and PLCγ1/2 (Fig. 5A), although not affecting the phosphorylation level of Lyn, suggesting a distinct involvement of SNE in the Lyn-Syk-LAT pathway. The results were consistent with the suppressed elevation of $[Ca^{2+}]_i$. Since SNE did not affect the phosphorylation of Lyn, SNE may suppress the phosphorylation of γ-ITAMs by inhibition of the enzymatic activity of Lyn or the activation of Syk by γ-ITAMs. There is another possibility that SNE is involved in upstream events, such as the cross-linkage of antigen to IgE and aggregation of FcεRI, since Lyn was phosphorylated even when the cells were not stimulated with antigen (Fig. 5A). In addition, SNE also remarkably suppressed the phosphorylation level of PI3K (Fig. 5C), indicating that SNE is also involved in the Fyn-Gab2-PI3K pathway. Btk, a Tec family kinase, is located downstream of Lyn and recruited to the plasma membrane by binding to PIP3 converted from PIP2 by PI3K (Kraft & Kinet, 2007; Turner & Kinet, 1999). Then, Btk activates PLCγ1/2 in the LAT complex, leading to Ca^{2+} mobilisation and the activation of protein kinase C (Turner & Kinet, 1999). For this reason, it is thought that the decreased phosphorylation level of Btk is due to the suppressed enzymatic activity of Lyn or suppressed phosphorylation of PI3K. On the other hand, phosphorylation of Gab2 has been reported to require Lyn and Syk (Yu, Lowell, Neel, & Gu, 2006). In addition, it has been reported that PI3K is also activated not only by Fyn but also by Syk (Kraft & Kinet, 2007). The decreased phosphorylation level of PI3K may result from the suppressed activation of Syk. Moreover, there are several reports that the Lyn-Syk-LAT and Fyn-Gab2-PI3K pathways are synergistically involved in degranulation (Nishida et al., 2005; Parravicini et al., 2002). Further investigations will be required to clarify the mechanism underlying the inhibitory activity of SNE on degranulation of RBL-2H3 cells.

5. Conclusions

SNE suppressed the antigen-induced degranulation in RBL-2H3 cells. The active substance in SNE was expected to be heat-stable and trypsin-resistant with molecular weight ranging from 500 Da to 14 kDa. In addition, the inhibitory effect of SNE was shown to

result from suppressed Ca^{2+} mobilisation through the downregulated phosphorylation of Syk and PI3K. Taken together, these findings suggest that SNE has an anti-allergy effect that controls degranulation.

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