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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.

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, Scalfi, & 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; $[Ca^{2+}]i$, 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 PLCγ antibody sampler kit (#3860) including antibodies against PLCγ1, phosphorylated PLCγ1, PLCγ2, and phosphorylated PLCγ2, 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-μm 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 μg/mL) treated with 50 or 500 μg/mL of trypsin at 37 °C for 15 min was inactivated by the addition of 500 μg/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 μg/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% CO_2-95% 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 $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% BSA, pH 7.4) twice, anti-DNP IgE-sensitised cells were treated with 120 μL 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 μL of DNP-HSA diluted in the modified Tyrode's buffer at 0.625 μg/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 μL 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 μL/well and incubated at 37 °C for 5 min. Then, 100 μL 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 μL 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 μL of 10% FBS-DMEM containing 10 μL 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 Ca^{2+} concentration ($[Ca^{2+}]i$)

The $[Ca^{2+}]i$ 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 μL of Fluo-3 AM for 1 h. Treated cells were washed again with PBS and incubated with SNE (45 μg/mL) or 10 mM NaPB at 37 °C for 10 min. Then, the cells were stimulated by the addition of 10 μL of DNP-HSA diluted in the modified Tyrode's buffer at 0.625 μg/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 μM 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 μL of the modified Tyrode's buffer containing SNE (45 μg/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 μL of DNP-HSA diluted in the modified Tyrode's buffer at 2.5 μg/mL and further incubated for 10 min at 37 °C. After removing the added reagents, 30 μL of a lysis buffer consisting of 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 2 μg/mL of aprotinin, Pefabloc SC, a protease inhibitor cocktail, and a phosphatase inhibitor cocktail were added