Prev. Nutr. Food Sci. 2018;23(1):30-34
https://doi.org/10.3746/pnf.2018.23.1.30
pISSN 2287-1098 · eISSN 2287-8602

Suppressive Effects of *Vaccinium angustifolium* Root Extract via Down-Regulation of Activation of Syk, Lyn, and NF-κB in FcεRI-Mediated Allergic Reactions

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ABSTRACT: *Vaccinium angustifolium*, reported as the lowbush blueberry, has a rich polyphenolic content with which biological activities have been closely associated. In this study, the effects of *V. angustifolium* root extract (VAE) on the anti-FcεRI α chain antibody (CRA-1)-induced FcεRI-mediated signaling factors, protein tyrosine kinases (PTK), Lyn, Syk, and nuclear factor kappa-B cells (NF-κB) in KU812F cells were investigated. The total phenolic content of VAE was found to be 170±1.9 mg gallic acid equivalents/g. Western blot analysis revealed that VAE dose-dependently inhibited FcεRI-mediated phosphorylation of PTK involving Lyn and Syk. Evaluation of intracellular reactive oxygen species (ROS) by spectrofluorometric analysis using 2'7'-dichlorofluorescin-diacetate revealed that they were reduced by VAE in a dose-dependent manner. Moreover, VAE reduced the levels of β-hexosaminidase released from CRA-1-stimulated KU812F cells. It was identified that VAE suppressed CRA-1-induced activation of NF-κB by Western blot analysis. Our results show that VAE may contribute to the inhibition of allergic actions via inactivation of basophils through the inhibition of β-hexosaminidase release and ROS production, which occurs as a result of inhibition of PTK, Syk, Lyn, and NF-κB.

Keywords: *Vaccinium angustifolium*, FcεRI, Syk, Lyn, NF-κB

INTRODUCTION

Blueberries are flowering plants in the genus of *Vaccinium*. Many species of blueberries have various beneficial properties. Among *Vaccinium*, the wild blueberry (*Vaccinium angustifolium*) has consistently shown higher levels of phenolics and anthocyanins, and demonstrated a wide variety of health-relevant bioactivities including anti-cancer, anti-diabetic, anti-hypertensive, anti-inflammatory effects, and protection against chronic diseases (1-8). We previously reported that *V. angustifolium* root extract (VAE) inhibited A23187 and phorbol myristate acetate (PMA)-induced degranulation via down-regulation of protein kinases C (PKC) translocation (9). Moreover, the expression of FcεRI, a high affinity IgE receptor, was down-regulated by VAE (10). However, the regulation of protein tyrosine kinases (PTK) and nuclear factor kappa-B (NF-κB) expression by VAE has not been examined.

The high affinity IgE receptor, FcεRI, plays a crucial role in IgE-mediated allergic reactions, and it is expressed on the surface of effector cells such as basophils and mast cells (11,12). Binding of allergen and IgE antibody complexes to FcεRI causes the activation of a signaling cascade, which triggers the elevation of intracellular calcium levels and the secretion of various inflammatory mediators from activated basophils and mast cells, and causes allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (13,14). We previously reported that VAE inhibited FcεRI-mediated calcium influx and degranulation (10). Degranulation of mast cells and basophils is induced by various stimuli such as calcium ionophore, antigens, and anti-FcεRI α chain antibody (CRA-1), which is accompanied by production of reactive oxygen species (ROS). Moreover, ROS generation depended on the activation of PTK such as Lyn and Syk, and PI3K in FcεRI-signaling (15). Activation of the signaling cascade after cross-linking of FcεRI-bound IgE antibody with allergens determines the interaction of FcεRI with Src kinases, Lyn and subsequent activation of Syk, other tyrosine kinases, and mitogen-activated protein kinases (MAPK) such as extracellular regulated kinases (ERK)-1/2, c-jun N-terminal kinase (JNK), and p38 MAPK (16-19). Moreover, NF-κB activation is regulated by MAPK and contribute to the expression of inflammatory mediators in allergic re-

Received 11 October 2017; Accepted 7 December 2017; Published online 31 March 2018

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actions (17). We previously reported that VAE negatively regulated degranulation through inhibition of PKC translocation and FcεRI expression through inhibition of ERK-1 activation in human basophilic KU812F cells (18, 19).

To identify the suppressive molecular activities of VAE on FcεRI-mediated allergic reactions, we evaluated the regulation of FcεRI-mediated PTK involving Syk and Lyn, and NF-κB activities in anti-human FcεRI α chain antibody, in CRA-1-stimulated KU812F cells.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT, USA). CRA-1 was acquired from Kyokuto (Tokyo, Japan). Antibiotics and antimycotics were purchased from Gibco BRL (Gaithersburg, MD, USA). Protease inhibitor cocktail was obtained from Roche Diagnostics GmbH (Penzberg, Germany). β-Actin, anti-phosphorylated Syk, Lyn, and NF-κB, and horseradish peroxidase (HRP)-conjugated secondary antibody were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescence detection reagents were acquired from Perkin Elmer (Waltham, MA, USA), and polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). 2'7'-dichlorofluorescin-diacetate (DCF-DA) was obtained from Sigma Chemicals (St. Louis, MO, USA). Protease inhibitor cocktail was purchased from Roche (Penzberg, Germany). Enhanced chemiluminescence detection reagents were procured from Perkin Elmer.

Cell culture, treatment, and stimulation

The human basophilic KU812F cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% heat-inactivated FBS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3～4 days. KU812F cells were treated with various concentrations of VAE in FBS-free RPMI-1640, and were induced by CRA-1.

Extract preparation

The *V. angustifolium* roots were obtained from Quebec, Canada, and the dried. For extraction, 10 volumes of methanol was added to the powered *V. angustifolium* roots. The supernatant of the mixture was condensed in a vacuum, and lyophilized. The VAE was stored at −20°C in dimethyl sulfoxide.

Total phenolic content (TPC) assay

The TPC of the VAE was assayed using the Folin-Ciocalteau method, with slight modifications (20). A 20 μL aliquot of the extract was added to 100 μL Folin-Ciocalteau reagent and 300 μL 20% Na_2CO_3 solution, and distilled water was added to a final volume of 2 mL. After 2 h, the absorbance was measured at 765 nm, and the concentration of TPC expressed as gallic acid equivalents (GAE) was determined using a calibration curve with gallic acid as a standard polyphenol.

Intracellular ROS analysis

The intracellular ROS activity was measured by the ROS-specific fluorescent probe, DCF-DA (21). Cells were pretreated with VAE for 24 h, and then stimulated with CRA-1 for 30 min. The cells were treated with DCF-DA for 30 min, and the absorbance was measured at 485 nm for excitation wavelength and at 528 nm for emission wavelength.

β-Hexosaminidase release assay

The β-hexosaminidase activity in the supernatant of treated and stimulated cells was determined spectrophotometrically. Briefly, the sample was aliquoted and 100 μL of 2 μM NP-GlcNAc (in 0.4 M citrate and 0.2 M phosphate buffer, pH 4.5) was added. The color was formed, after which the reaction was finished by adding 200 μL of 0.2 M glycine-NaOH, pH 10.7, and the absorbance was measured at 405 nm. The cells were subsequently lysed with 0.1% Triton X-100, after which the β-hexosaminidase activity of the VAE was measured.

Western blot analysis

Phosphorylation of Lyn and Syk, and NF-κB was examined by Western blot analysis. Briefly, induced cells were lysed in cell lysis buffer containing 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM ethylenediaminetetraacetic acid, and a protease inhibitor cocktail. The proteins were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked with 10% skim in plain buffer (50 mM Tris-HCl, pH 7.5, 34 mM NaCl, and 0.001% Tween 20). The membrane was incubated with primary antibodies followed by anti-HRP conjugated secondary antibodies. Then, the chemoreactive proteins were visualized by enhanced detection reagents according to the manufacturer's instructions, and the membrane was then exposed to X-ray film, after which it was quantified.

Statistical analysis

All experiments were carried out independently in triplicate. The data were presented as the mean±standard deviation (SD). Statistical differences between the control

and VAE were resolved by a Student's *t*-test using the statistical software, SPSS (version 12.0; SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effects of VAE on FcεRI-mediated PTK, Syk, and Lyn activation

V. angustifolium contains many polyphenolic compounds, that can be extracted with methanol, which is the most suitable solvent for plant extraction. In a previous study, the TPC of VAE was 1,700±1.9 mg GAE/g (9,10). Human basophilic KU812F cells express a high affinity for the IgE receptor, and they are therefore used as a cell line in FcεRI expression research (22). We previously found that VAE exerted no cytotoxicity at ≤20 μg/mL (data not shown) (9,10). Therefore, the VAE concentrations of 1∼20 μg/mL were selected for further experiments.

Mast cells and basophils are major allergic and immune effector cells in FcεRI-mediated allergic reactions. A high affinity IgE receptor, FcεRI, expressed on the surface of mast cells and basophils, has an important role in IgE-mediated allergic reactions (23,24). The transcriptional signaling results in the activation by Fc receptors in basophils and mast cells have been extensively characterized, and the initial FcεRI stimulation activates a transcriptional signaling cascade that involves activation of PTK such as Syk and Lyn and MAPK such as ERK 1/2, p38, and JNK (25,26). We previously reported that VAE suppressed FcεRI expression through inhibition of ERK-1 phosphorylation (10). However, the effects of VAE on the regulation of FcεRI-mediated PTK such as Syk and Lyn in FcεRI-mediated allergic responses have not been characterized. Therefore, in this study, KU812F cells were pretreated with VAE for 24 h, and then stimulated with CRA-1 for 30 min. Western blot analysis showed that activation of Syk and Lyn was profoundly and dose-dependently inhibited by VAE (Fig. 1).

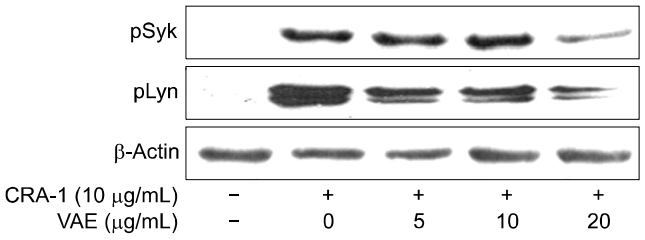

Fig. 1. Effects of *Vaccinium angustifolium* root extract (VAE) on FcεRI-mediated PTK phosphorylation. Cells were treated with various concentrations of VAE and stimulated with anti-FcεRI α chain antibody (CRA-1). The cellular lysates were obtained, and the expression of Syk, Lyn, and β-actin was analyzed by Western blot analysis using corresponding antibodies. The results presented are representative of three independent experiments.

Effects on FcεRI-mediated ROS production

Activation of PTK, Lyn, and Syk stimulates ROS production (27). To examine the effects of VAE on FcεRI-mediated ROS production, KU812F cells were pretreated with VAE for 24 h, and then stimulated with CRA-1 for 30 min. VAE concentration dependently inhibited CRA-1-induced ROS production (Fig. 2). The produced ROS participate in the regulation of calcium mobilization. We previously reported that VAE negatively regulated FcεRI-mediated intracellular calcium levels (10). The degranulation process depends on increases in cytosolic calcium concentrations, and ROS play a crucial role in FcεRI-dependent signaling, which results in degranulation of mast cells and basophils (15). The histamine content and β-hexosaminidase activity are powerful markers of allergic disorders such asthma, atopic dermatitis, and rhinitis. In a previous study, we found that VAE inhibited FcεRI-mediated histamine release (data not shown) (10).

Effects on FcεRI-mediated β-hexosaminidase release

Degranulation of effector cells such as mast cells and basophils through FcεRI-cross linking or various stimuli leads to release of histamine and β-hexosaminidase, which causes the symptoms of allergic disorders (23-29). To assess the suppressive effects of inflammatory mediator secretion, KU812F cells were treated with VAE at 0, 1, 5, 10, and 20 μg/mL for 24 h, and then stimulated with CRA-1 for 30 min. VAE suppressed CRA-1-induced β-hexosaminidase release in a dose-dependent manner (Fig. 3). The results demonstrate that the secretion of inflammatory mediators from activated KU812F cells was negatively regulated by VAE, and it showed that VAE sup-

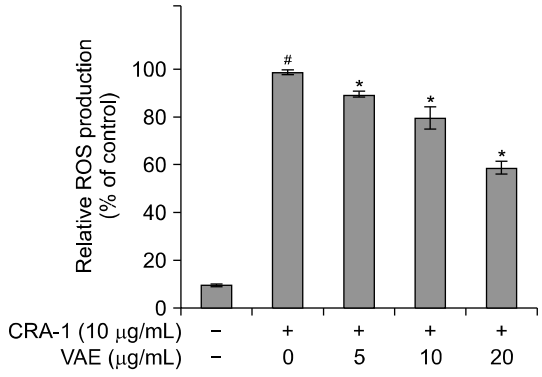

Fig. 2. Effects on FcεRI-mediated reactive oxygen species (ROS) production. Cells pretreated with *Vaccinium angustifolium* (VAE) were stimulated with anti-FcεRI α chain antibody (CRA-1). ROS levels were measured by 2'7'-dichlorofluorescin-diacetate by fluorescence analysis. Data are mean±SD of three independent experiments. #P<0.05 indicates a significant difference between negative control and positive control. *P<0.05 indicates significant differences from the CRA-1 treated group.

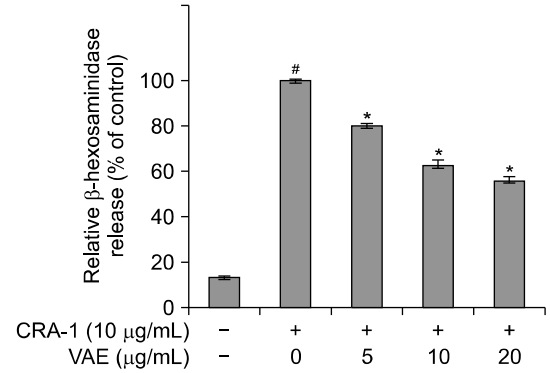

Fig. 3. Effects on FcεRI-mediated β-hexosaminidase release. Cells treated with *Vaccinium angustifolium* root extract (VAE) were stimulated with anti-FcεRI α chain antibody (CRA-1), and then β-hexosaminidase content was measured. Data are mean ±SD of three independent experiments. $^{\#}P<0.05$ indicates a significant difference between negative control and positive control. $^{*}P<0.05$ indicates significant differences from the CRA-1 treated group.

presses basophils degranulation.

VAE inhibits FcεRI-mediated NF-κB activation

The activation of MAPK and NF-κB is intimately associated with the expression of inflammatory mediators. MAPK involving ERK-1, p38, and JNK are important signal transcription factors that mediate cellular reactions including the allergic response, and are key signaling molecules in cell growth, development, differentiation, inflammation, and apoptosis (16-18). Moreover, MAPK have been investigated as rational targets for drug design for the treatment of allergic diseases. Activated MAPK regulate the transcriptional activity of many genes involved in the maintenance of cellular homeostasis. We previously reported that VAE down-regulated FcεRI-mediated phosphorylation of ERK-1 (10). NF-κB plays a crucial role in allergic reactions, and it is a transcriptional factor required for the expression of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor-necrosis factor-α (28). To examine the effects of NF-κB activation, cells were treated with VAE at various concentrations, and then stimulated with CRA-1 for 30 min. Western blot analysis revealed that VAE down-regulated the activation of NF-κB in a dose-dependent manner (Fig. 4). We previously reported that VAE showed potent inhibition of degranulation through down-regulation of PKC translocation in A23187 and PMA stimulated KU812F cells (9). Moreover, VAE inhibited FcεRI expression via suppression of ERK-1 activation in FcεRI-mediated KU812F cells (10).

In the present study, we showed that VAE negatively regulated degranulation via inhibition of the activation of Lyn, Syk, and NF-κB. Further studies investigating the molecular mechanism of FcεRI α chain gene regulation by multiple transcription factors are needed.

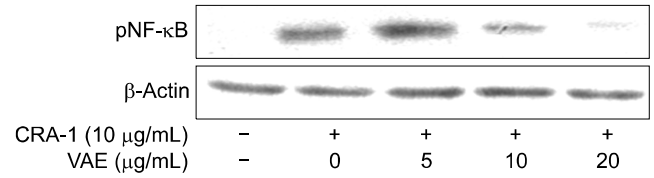

Fig. 4. Effects on FcεRI-mediated nuclear factor kappa-B cells (NF-κB) activation. Cells pretreated with *Vaccinium angustifolium* root extract (VAE) were stimulated with anti-FcεRI α chain antibody (CRA-1), and whole cell lysates were prepared, and the expression of NF-κB and β-actin was detected by Western blot analysis using corresponding antibodies. The results presented are representative of three independent experiments.

Nevertheless, the results of this study suggest that the effects of VAE occur via down-regulation of FcεRI-mediated allergic reactions, which contributes to its therapeutic activity. Accordingly, VAE may be useful for protection against allergic disorders.

AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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