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Inhibitory Effects of Whisky Congeners on IgE-Mediated Degranulation in Rat Basophilic Leukemia RBL-2H3 Cells and Passive Cutaneous Anaphylaxis Reaction in Mice

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Whisky is matured in oak casks. Many nonvolatile substances (whisky congeners, WC) seep from the oak cask during the maturing process. In this study, three antiallergic agents (syringaldehyde, SA; lyoniresinol, Lyo; and ellagic acid, EA) were isolated from WC. Treatment with SA, Lyo, and EA reduced the elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) and intracellular ROS production caused by FcεRI activation. The inhibitions of the elevation of $[Ca^{2+}]i$ and intracellular ROS production by SA and Lyo were mainly due to the suppression of the NADPH oxidase activity and scavenging of the produced radical, respectively. On the other hand, EA inactivated spleen tyrosine kinase and led to the inhibition of the elevation of $[Ca^{2+}]i$ and intracellular ROS production. Furthermore, it was found that WC strongly inhibited IgE binding to the FcεRIα chain, whereas SA, Lyo, and EA did not indicate this inhibitory effect. These results suggest that WC inhibits allergic reactions through multiple mechanisms. To disclose the in vivo effects of WC, SA, Lyo, and EA, these compounds were administered to type I allergic model mice, and the passive cutaneous anaphylaxis (PCA) reaction was measured. These compounds remarkably suppressed the PCA reaction. Taken together, these findings suggest that WC seemed to be beneficial to ameliorate allergic reactions.

KEYWORDS: Whisky congener; RBL-2H3; antiallergy; degranulation; NADPH oxidase

INTRODUCTION

In type I allergy, the binding of antigen to the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on the surface of mast cells and basophils is the first event leading to the release of chemical mediators such as histamine, arachidonic acid metabolites, and cytokines, which mainly causes asthmatic and inflammatory responses (*1*−*3*). Thus, mast cells were key effector cells in IgE-mediated immune responses. In recent studies, it has been reported that various natural products suppressed the degranulation in cellular systems (*4*−*6*). As the inhibition mechanisms, the suppression of FcεRI expression and the inhibition of intracellular signaling through FcεRI aggregation have been considered.

Whisky is matured in oak casks during the manufacturing process. The color, aroma, and flavor of matured whiskey are influenced by the nonvolatile substances (whisky congeners, WC) exuded from the oak cask. WC includes hundreds of flavor components such as ellagitannin, vanillin, protocatechuic acid, *p*-hydroxybenzoic acid, sinapinaldehyde, syringaldeyde, and coniferylaldeyde. Many studies have indicated that the byproducts eluted from oak casks in the maturing process contain various functional compounds that exhibit anti-inflammatory, antibacterial, antioxidative, and anticarcinogenesis activities (*7*−*11*). Ellagitannin, a typical constituent of alcoholic congeners, exerts similar functions. To date, WC has been shown to interfere with FcεRI−IgE binding, to inhibit melanogenesis, and to protect against ethanol-induced gastric mucosal damage (*12*−*14*). However, whether WC could ameliorate allergic reactions has not been examined.

In this study, we demonstrate the inhibitory effects of WC on antigen (Ag)-mediated degranulation in RBL-2H3 cells and identify the antiallergic compounds. Furthermore, we show that WC and the compounds inhibited type I allergy in model mice.

MATERIALS AND METHODS

Reagents and Materials. Mouse anti-dinitrophenol (DNP) monoclonal IgE was purchased from Yamasa (Tokyo, Japan). Quercetin was obtained from Cayman Chemical (Ann Arbor, MI). Disodium cromoglycate

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from ENZO Life Sciences (Plymouth Meeting, PA) was used. Vanillin, vanillic acid, *p*-hydroxybenzoic acid, and syringic acid were purchased from Extrasynthase (Lyon, France). Protocatechuic acid was obtained from LKT laboratories, Inc. (St. Paul, MN). Coniferylaldehyde and sinapinaldehyde from Sigma (St. Louis, MO) were used. *Rubus suavissimus* extract was provided by Suntory Holdings Limited. (Osaka, Japan). 25× Complete, a mixture of protease inhibitors, was from Roche (Penzberg, Germany). Phosphatase Inhibitor Cocktails 1 and 2 were from Sigma. Antibodies to p44/42 MAP kinase (ERK), phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK), SAPK/JNK (JNK), phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), p38 MAP kinase (p38), phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), Lyn, phospho-Lyn, $cPLA_2$, phospho-$cPLA_2$, phospho-PLCγ1, and phospho-PLCγ2 were from Cell Signaling Technology (Beverly, MA). Antibodies to Syk, $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody and ellagic acid were from Sigma. The FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, Molecular Immunology and Inflammation Branch). Anti-rabbit and -mouse antibodies conjugated with horseradish peroxidase (HRP) and the ECL chemiluminescence kit were obtained from GE Healthcare (Piscataway, NJ).

Isolation of Syringaldehyde (SA), Lyoniresinol (Lyo), and Ellagic Acid (EA) from Whisky Congeners (WC). The WC (7.91 g) were prepared from Whisky at the age of 12 years using freeze dehydration equipment. The powdered WC was subjected to column chromatography (Amberlite XAD-2) and eluted with a stepwise gradient with water and methanol. The methanol fraction was further separated by a Sephadex LH-20 column and eluted with methanol, to give fraction 1 (2436 mg), fraction 2 (846 mg), fraction 3 (110 mg), fraction 4 (148 mg), and fraction 4 (41 mg). These fractions were purified by preparative high-performance liquid chromatography, and the chemical structures were identified by ^1H and ^{13}C NMR and mass spectrometric analyses.

Cell Culture. RBL-2H3 cells were obtained from the Health Science Research Resource Bank (Tokyo, Japan). The cells were grown in Eagle's minimum essential medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

β-Hexosaminidase Release Assay. We used β-hexosaminidase as a marker of degranulation. RBL-2H3 cells (2×10^4 cells/well) were seeded onto 24-well plates and cultured for 1 h. Then, cells were treated with anti-DNP IgE at a concentration of 0.45 μg/mL and incubated for 24 h. After washing twice with siraganian buffer A (NaCl 119 mM, KCl 5 mM, $MgCl_2$ 0.4 mM, PIPES 25 mM, NaOH 40 mM, pH 7.2), 160 μL of siraganian buffer B (5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA) was added to each well. After incubation at 37 °C for 10 min, cells were treated with each sample at 37 °C for 30 min. Then, 20 μL of DNP-labeled bovine serum albumin (DNP−BSA) was added to culture medium at a concentration of 10 μg/mL. Ten minutes later, cells were put on ice for 10 min to terminate the reaction. Then, supernatants were harvested by centrifugation at 300*g* at 4 °C for 10 min. The supernatants (50 μL) were transferred into 96-well microplates and reacted with 50 μL of 0.1 M citrate buffer (pH 4.5) including 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (CB-PNAG) at 37 °C. One hour later, the reaction was terminated by adding stop buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured at 405 nm using the colorimetric microplate reader.

To evaluate β-hexosaminidase inhibitory activity, on 96-well microplates, each sample (5 μL) and conditioned medium prepared from Ag-stimulated RBL-2H3 cells (45 μL) were reacted with 50 μL of 0.1 M CB-PNAG at 37 °C for 1 h. After termination of the reaction, the absorbance at 405 nm was measured. The inhibition of degranulation was calculated as follows:

$$\begin{aligned}&\text{inhibition of degranulation (\%)}\\&= \{[1 - (A_{405\text{ nm of sample}} - A_{405\text{ nm negative control}})\\&/(A_{405\text{ nm positive control}} - A_{405\text{ nm negative control}})] \times 100\}\\&-\beta\text{-hexosaminidase inhibitory activity (\%)}\end{aligned}$$

$$\begin{aligned}&\beta\text{-hexosaminidase inhibitory activity (\%)}\\&= [1 - (A'_{405\text{ nm of sample}} - A'_{405\text{ nm negative control}})\\&/(A'_{405\text{ nm positive control}} - A'_{405\text{ nm negative control}})] \times 100\end{aligned}$$

Measurement of Intracellular Ca^{2+} Concentration. The intracellular Ca^{2+} level was determined with a Calcium Kit-Fluo 3 (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5×10^4 cells/well) were seeded into black-walled and black-bottomed, 96-well microplates and incubated for 1 h. After incubation, mouse monoclonal anti-DNP IgE was added to the cultured medium at a concentration of 0.45 μg/mL, and then the cells were incubated for 24 h. The IgE-sensitized cells were washed twice with PBS and incubated with 100 μL of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3) for 1 h. Treated cells were washed with PBS and incubated with 90 μL of loading buffer (Calcium Kit-Fluo 3) including 50 μM SA, Lyo, EA, or disodium cromoglycate (Dscg) for 30 min. Then, cells were stimulated by DNP−BSA (10 μg/mL), and the intracellular Ca^{2+} concentration was monitored by measuring the fluorescence with a fluorometric imaging plate reader (excitation, 490 nm; emission, 530 nm).

Measurement of Intracellular ROS Level by CM-H_2DCF-DA Fluorescent Probe. Intracellular ROS level was measured using 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA), which is a freely permeable fluorogenic tracer specific to detect ROS. CM-H_2DCF-DA is deacetylated by intracellular esterases to nonfluorescent 2′,7′-dichlorohydrofluorescein (DCFH), which is then oxidized into the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells (5×10^4 cells/well) were incubated with 10 μM CM-H_2DCF-DA for 30 min at 37 °C and washed twice with PBS to remove the excess of CM-H_2DCF-DA. The cells were incubated with 50 μM SA, Lyo, or EA for 30 min. Then, the cells were stimulated by DNP-BSA (10 μg/mL), and fluorescence was measured with excitation at 490 nm and emission at 530 nm using a fluorometric imaging plate reader.

Measurement of DPPH Radical Scavenging Activity. To measure antioxidant activity, a DPPH radical scavenging assay was carried out according to the previous method with a slight modification (*15*). Briefly, DPPH radical scavenging activity was measured in the reaction mixture containing 0.1 mL of 0.5 mM DPPH radical solution, 0.8 mL of 99% ethanol, and 0.1 mL of WC, SA, Lyo, or EA solution. The solution was rapidly mixed, and the scavenging capacity was measured with a colorimetric microplate reader at 517 nm. DPPH free radical scavenging ability (%) was calculated using the formula $[(A_{517\text{ nm of control}} - A_{517\text{ nm of sample}})/A_{517\text{ nm of control}}] \times 100$. Vitamin C (L-ascorbic acid), vitamin E (α-tocopherol), and Trolox were used as positive controls.

Immunofluorescence Staining. The cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were washed twice with PBS containing 10 mM glycine (PBS-G) and then treated with PBS containing 0.1% Triton X-100 (Sigma) (PBS-T) for 5 min at room temperature. Subsequently, the cells were blocked with 3% BSA for 10 min at room temperature. After incubation, treated cells were incubated with the primary antibody (anti-$p67^{phox}$), which was diluted with PBS-G for 1 h at room temperature. After washing with PBS-(−) containing 0.1% BSA, cells were incubated with secondary antibody (Alexa Fluor-488 rabbit IgG, Invitrogen) for 30 min at room temperature. The nuclei and cell membranes of treated cells were further stained with Hoechst33342 (Invitrogen) and Cell Mask Orange plasma membrane stain solution (Invitrogen) for 30 min. The cells were mounted with a drop of mounting medium (Dako cytometion fluorescent mounting medium, Dako, Carpinteria, CA) and then sealed with a coverslip. Photomicrographs of mounted cells were taken with a fluorescent microscope (KEYENCE BZ-8000, Osaka, Japan).

Immunoblot Analysis. Ag-stimulated RBL-2H3 cells with or without SA, Lyo, or EA treatment were washed twice with PBS and harvested. Cell lysates (20 μg) were resuspended in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) buffer containing 2% 2-mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE. The cytosolic and membrane fractions were also separated using a ProteoExtract subcellular proteome extraction kit (Merk KGaA, Darmstdt, Germany). Separated proteins were electroblotted onto a PVDF membrane (DuPont, Boston, MA). The membrane was blocked for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), washed three times with TPBS, and then incubated with each primary antibody, followed by incubation with a HRP-conjugated secondary antibody. After washing, proteins were detected using an ECL kit and a chemiluminescence detector (LAS-4000, Fujifilm, Tokyo, Japan).

FcεRI−IgE Binding Assay. To measure the inhibitory effect of WC, SA, Lyo, or EA on FcεRI−IgE binding, we constructed a FcεRI−IgE

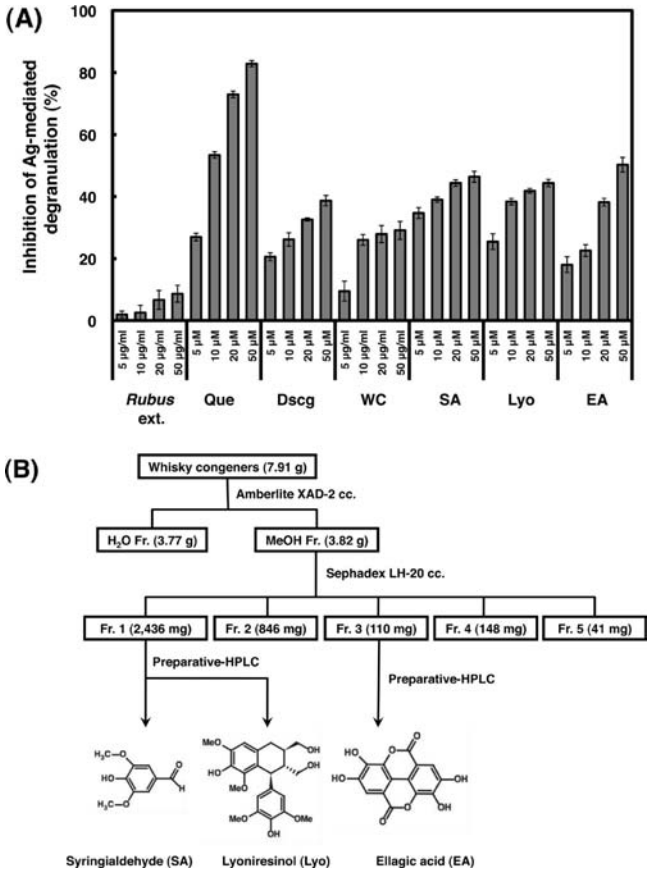

Figure 1. Inhibitory effects of WC, SA, Lyo, and EA on Ag-stimulated degranulation from rat basophilic leukemia RBL-2H3 cells. (**A**) IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence or absence of these compounds. *Rubus suavissimus* extract (*Rubus* ext.) has been reported as an antiallergic food material. Quercetin (Que) and disodium cromoglycate (Dscg) were used as antiallergic positive controls. Values are mean ± SEM ($n = 10$) of the inhibition of degranulation release. (**B**) The extraction procedure and chemical structures of antidegranulation substances isolated from WC are shown.

EIA system. Briefly, 0.15 μg/μL of human FcεRIα-chain recombinant protein (Abnova, Taipei, Taiwan) was conjugated in 50 mM carbonate buffer (pH 9.5) for 24 h at 4 °C. After incubation, each well was washed three times with TPBS and then 100 μL of human IgE solution (BioPort Corp., Lansing, MI) along with WC, SA, Lyo or EA was added. After reaction for 2 h, 100 μL of anti-human IgE−HRP solution (Bethyl Laboratories, Inc., Montgomery, TX) was added to each well. After incubation for 1 h at room temperature, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (Cell Signaling Technology) was added to each well, and the enzyme reaction was initiated. Ten minutes later, the reaction was terminated by adding stop buffer (0.5 M H_2SO_4/HCl). The absorbance was measured with a colorimetric microplate reader at 450 nm.

Competitive Degranulation Assay. To investigate the inhibitory effect of FcεRI−IgE binding by WC, we have constructed the competitive degranulation assay system. Briefly, RBL-2H3 cells were inoculated into 24-well plates (5×10^5 cells/mL, 400 μL/well; Nunc, Roskilde Denmark) and cultured for 12 h. The cells were washed twice by siraganian buffer A. After incubation at 37 °C for 10 min, the cells were treated with siraganian buffer B including mouse monoclonal anti-DNP IgE (final concentration = 0.45 μg/mL) plus WC, SA, Lyo, or EA at 37 °C for 1 min. Subsequently, 20 μL of DNP−BSA was added to the cultured medium at a concentration of 10 μg/mL, and then the cells were incubated for 5 min. To terminate the reaction, the treated cells were kept on ice for 10 min and then centrifuged at 300*g* at 4 °C for 10 min to obtain supernatants. The inhibitory effect was evaluated by measurement of β-hexosaminidase release. The assay system was described under Materials and Methods.

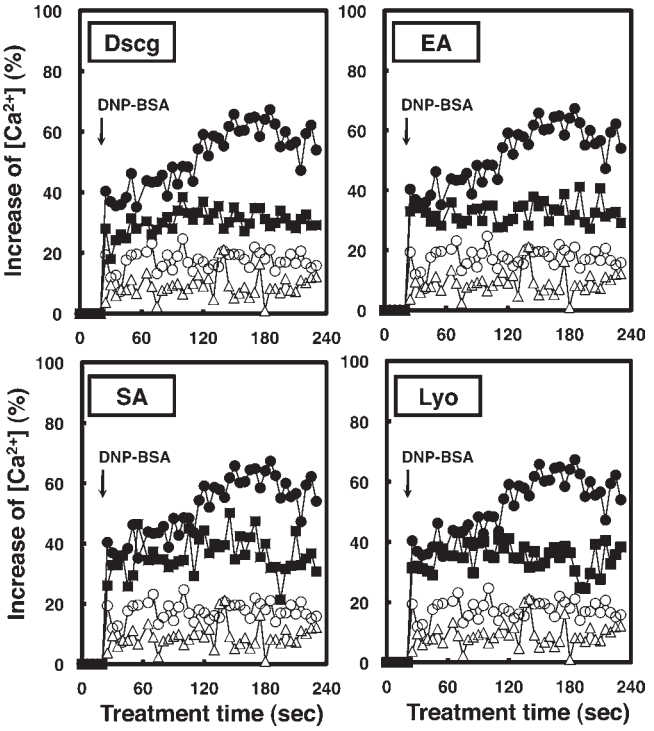

Figure 2. Suppression of elevation of $[Ca^{2+}]i$ by SA, Lyo, and EA in Ag-stimulated RBL-2H3 cells. Intracellular calcium levels were measured as described under Materials and Methods. Each value represents the mean with SEM ($n = 12$). →, antigen (DNP-BSA) challenge point; ●, Ag-stimulated cells; ○, Ag-nonchallenged cells; △, Ag plus EGTA-treated cells; ■, Ag plus SA-, Lyo-, EIA-, or Dscg-treated cells.

Animals and Diets. Four-week-old ICR male mice (Japan SLC, Ltd., Hamamatsu, Japan) were housed in an air-conditioned room (22 ± 2 °C) with a 12 h light and dark cycle (lighting from 7:00 a.m. to 7:00 p.m.). All of the mice were fed commercial CE-2 pellets (Clea Japan, Inc., Tokyo, Japan) and water ad libitum for 1 week to accustom them to the surroundings. This study was approved by the Gifu International Institute of Biotechnology Animal Use Committee, and the animals were maintained according to the guidelines of the Gifu International Institute of Biotechnology for the care of laboratory animals.

Measurement of Mouse Passive Cutaneous Anaphylaxis (PCA) Reaction. An IgE-induced passive cutaneous anaphylaxis reaction was measured according to the previous method of Choo et al. with a slight modification (*16*). The mice were injected intradermally with 10 μg of anti-DNP−BSA into two dorsal skin sites that had been shaved 24 h earlier. These sites were outlined with a water-insoluble marker. After 24 h, the mice were treated with 100 μL of corn oil including WC (1000 mg/kg of body weight), SA, Lyo, or EA (200 mg/kg of body weight) by oral administration. One hour after the administration, the mice received an injection of 200 μL of PBS containing 200 μg of DNP−BSA and 1% Evans blue (Sigma). One hour later, the mice were sacrificed, and their dorsal skin was removed. The skins (1 cm × 1 cm) were dissolved with 1 N KOH at 37 °C for 24 h, and the extravasated Evans blue dye was extracted with a mixture of acetone and 0.2 M phosphoric acid (13:5) from the solution. The amount of dye was determined colorimetrically at 620 nm.

Statistical Analysis. All data were analyzed first by one-way ANOVA and then by Fisher's multiple-range test. The differences among the means were considered to be significant at $p < 0.05$.

RESULTS

Determination of Chemical Structures of Active Compounds Isolated from WC. Prior to determination of chemical structures, we examined the effects of WC on Ag-stimulated β-hexosaminidase release from RBL-2H3 cells. As shown in **Figure 1A**, WC suppressed β-hexosaminidase release in a dose-dependent manner.

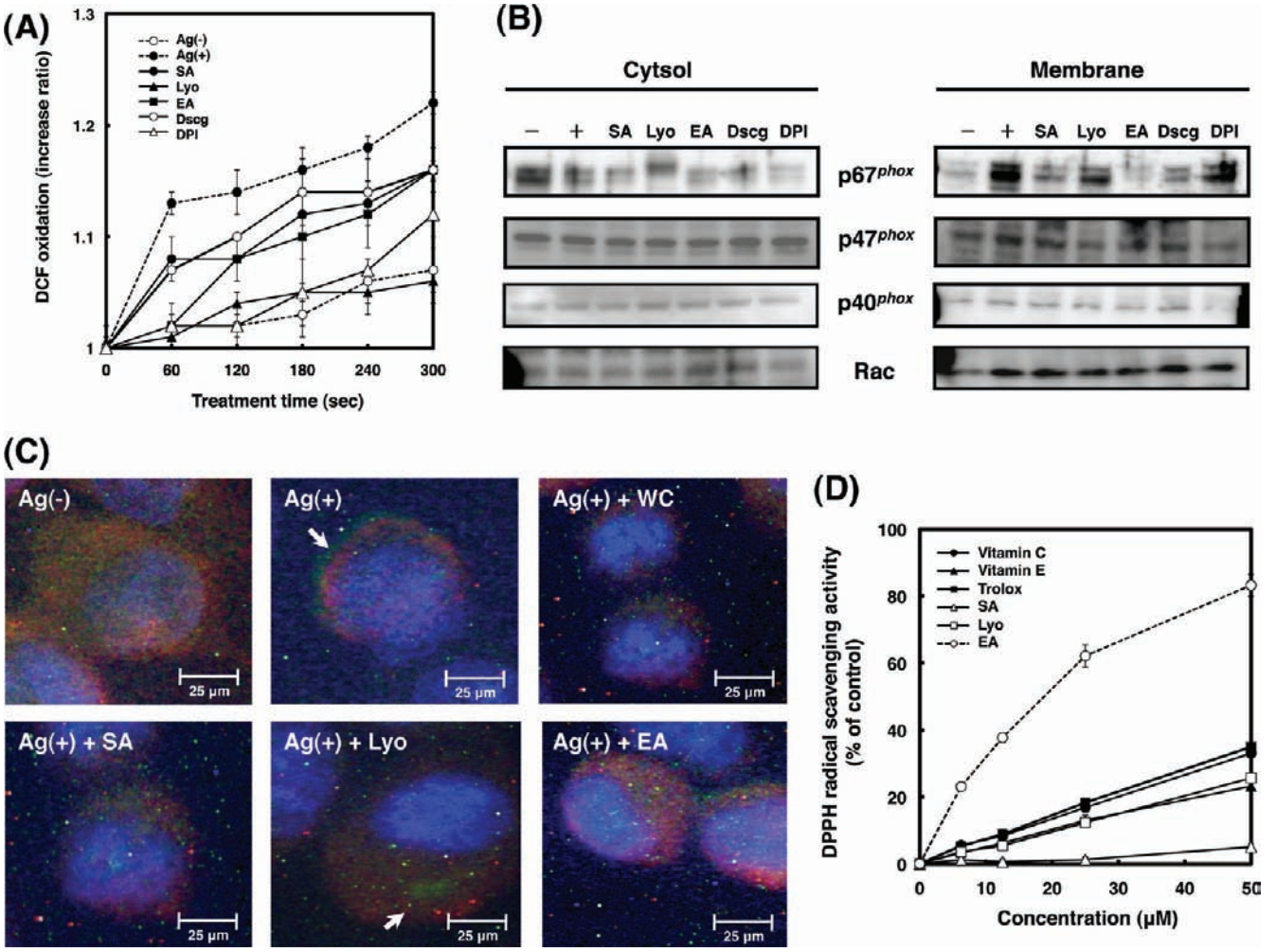

Figure 3. Effects of SA, Lyo, and EA on intracellular ROS production in Ag-stimulated RBL-2H3 cells. (**A**) Suppression effect of SA, Lyo, and EA (50 μM) on Ag-induced ROS production. Diphenyleneiodonium chloride (DPI; 200 nM) was used as a ROS production negative control. Values are mean ± SEM (n = 10). (**B**) Inhibitory effect of SA, Lyo, and Ella on translocation of cytosolic subunits of NADPH oxidase (p40^{phox}, p47^{phox}, p67^{phox}, and Rac) to the membrane. A representative blot from three independent experiments is shown. (**C**) Immunofluorescence analysis of the translocation to the membrane of p67^{phox} cytosolic subunits in Ag-stimulated RBL-2H3 cells. Blue (Hoechst33342), nuclei; red (Cell Mask Orange plasma membrane), cell membrane; green (Alexa Fluor-488 rabbit IgG), p67^{phox} cytosolic subunits. p67^{phox} cytosolic subunit was translocated by Ag stimulation (white arrow). Photographs represent three independent experiments. (**D**) Radical scavenging activities of SA, Lyo, and EA. Vitamins C and E and Trolox were used as positive control. All data are expressed as mean ± SEM (n = 12) of the inhibition of DPPH radical (% inhibition).

To determine active compounds, we subjected WC to several chromatographies and isolated three compounds: syringaldehyde (SA), lyoniresinol (Lyo), and ellagic acid (EA). The ^{1}H NMR, ^{13}C NMR, and FAB-MS data of these isolated compounds were in agreement with the literature values (*17*−*19*). The extraction procedure and the chemical structures of these compounds are shown in **Figure 1B**.

Syringaldehyde (*SA*)*:* $C_9H_{10}O_4$; ^{1}H NMR (500 MHz, $CDCl_3$), δ 9.82 (s, 1H), 7.15 (s, 2H), 6.05 (s, 1H), 3.97 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 190.9, 147.4, 140.9, 128.5, 106.8, 56.6; MS (FAB), m/z 183 $[M + H]^+$; matrix, magic bullet.

Lyoniresinol (*Lyo*)*:* $C_{22}H_{28}O_8$; ^{1}H NMR (500 MHz, $CDCl_3$), δ 6.45 (s, 1H), 6.34 (s, 2H), 5.37 (bs, 1H), 5.33 (bs, 1H), 4.01 (d, 1H, J = 7.6 Hz), 3.88 (s, 3H), 3.81 (dd, 1H, J = 11.5, 3.8 Hz), 3.80 (s, 6H), 3.77 (dd, 1H, J = 10.7, 4.6 Hz), 3.64 (dd, 1H, J = 10.7, 6.9 Hz), 3.58 (dd, 1H, J = 11.5, 6.1 Hz), 3.29 (s, 3H), 2.68 (dd, 1H, J = 15.3, 11.5 Hz), 2.59 (dd, 1H, J = 15.3, 3.8 Hz), 1.91 (m, 1H), 1.77 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 146.8, 146,2, 145.6, 138.4, 137.1, 132.9, 128.7, 125.3, 106.0, 105.4, 66.9, 64.0, 59.6, 56.5, 56.2, 49.6, 43.3, 40.5, 33.7; MS (FAB), m/z 421 $[M + H]^+$; matrix, magic bullet.

Ellagic acid (*EA*)*:* $C_{14}H_6O_8$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.53 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD), δ 160.3, 148.3, 140.0, 136.4, 112.9, 110.3, 108.0; MS (FAB), m/z 303 $[M + H]^+$; matrix, magic bullet.

Inhibition of Ag-Stimulated β-Hexosaminidase Release by SA, Lyo, and EA. To investigate the effects of SA, Lyo, and EA on degranulation, we measured the release of β-hexosaminidase from Ag-stimulated RBL-2H3 cells. Treatment with SA, Lyo, and EA inhibited the Ag-mediated degranulation, dose-dependently (**Figure 1A**). In the present study, *R. suavissimus* extract, quercetin (Que), and Dscg, well-known as antiallergic materials, were used as positive controls (*20*−*25*). We further examined the effects of other compounds including WC on Ag-stimulated β-hexosaminidase release from RBL-2H3 cells. The inhibitory effects of vanillin (V), vanillic acid (VA), syringic acid (SyA), sinapinaldehyde (SiA), and coniferylaldehyde (CA) on Ag-mediated degranulation were higher than those of SA, Lyo, and EA (Supporting Information Figure S1 and Table S1). WC components gradually increased during the maturing process. In particular, the variations in the contents of SA, Lyo, and gallic acid were remarkable. However, V, VA, protocatechuic acid

(PA), *p*-hydroxybenzoic acid (HbA), SyA, SiA, and CA were minor substances compared with SA, Lyo, and EA (Supporting Information Figure S2).

Inhibition of Ag-stimulated [Ca^{2+}]i Increase by SA, Lyo, and EA. To investigate the mechanism underlying the inhibition of degranulation by SA, Lyo, and EA, we first examined [Ca^{2+}]i levels following Ag stimulation of RBL-2H3 cells in the presence of these compounds. Upon Ag stimulation, the [Ca^{2+}]i level rapidly increased and sustained thereafter (**Figure 2**, ●). However, in the presence of these compounds, elevation of [Ca^{2+}]i was suppressed and these inhibitory effects were similar to that of Dscg (**Figure 2**, ■).

Effect of SA, Lyo, and EA on Ag-stimulated Intracellular ROS Production. We examined the production of intracellular ROS using a CM-H_2DCF-DA fluorescent probe. DCF oxidation gradually increased by Ag treatment (**Figure 3A**, ●, dotted line). In the presence of SA, Lyo, and EA, however, ROS production was significantly suppressed (**Figure 3A**). In particular, Lyo treatment completely blocked Ag-mediated ROS production.

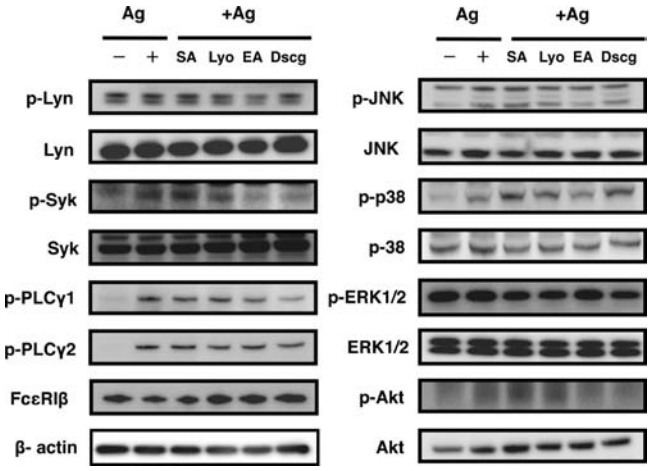

Figure 4. Effect of SA, Lyo, and EA on the signaling pathways in Ag-stimulated RBL-2H3 cells. A representative blot from three independent experiments is shown.

As NADPH oxidase was shown to be involved in Ag-stimulated ROS production, we examined the effects of SA, Lyo, and EA on the activation of NADPH oxidase by Western blot analysis. The levels of $p67^{phox}$ in membrane fractions were decreased by treatments with SA, Lyo, and EA (**Figure 3B**). Translocations to the membranes of other cytosolic subunits ($p40^{phox}$, $p47^{phox}$) and Rac were not changed by treatment with SA, Lyo, and EA. We further examined translocation to the membranes of $p67^{phox}$ cytosolic subunits by an immunofluorescence microscopic analysis. As shown in **Figure 3C**, $p67^{phox}$ cytosolic subunits were remarkably translocated to the membranes in Ag-stimulated RBL-2H3 cells. However, WC, SA, and EA treatments showed a tendency to suppress translocation to the membranes of $p67^{phox}$ cytosolic subunits in Ag-stimulated RBL-2H3 cells, whereas Lyo showed a marginal inhibitory effect (**Figure 3C**). Additionally, we examined the antioxidant activity of these compounds by DPPH radical scavenging assay. EA exhibited much stronger radical scavenging activity compared to other agents (**Figure 3D**).

Effect of SA, Lyo, and EA on Intracellular Signaling Pathways in Ag-Stimulated RBL-2H3 Cells. We investigated the early signaling events. FcεRI cross-linking on mast cells activates nonreceptor type protein tyrosine kinases such as Lyn and Syk (*26*, *27*). As shown in **Figure 4** (left panel), phosphorylation of Syk and PLCγ1/2 was slightly decreased by EA treatment, whereas other compounds did not affect activation of the Syk/PLCγs pathway. Next, we examined the effects of SA, Lyo, and EA on mitogen-activated protein kinases (MAPKs; ERK, JNK, p38). Ag stimulation induced phosphorylation of ERK and JNK, but not p38 MAP kinase (**Figure 4**, right panel). However, these compounds did not affect the phosphorylation of these kinases.

Suppressive Effect of WC on the Binding of IgE to FcεRI. To gain further insight into the mechanism underlying the inhibition of degranulation by WC, SA, Lyo, and EA, we examined the effect of these compounds on IgE binding to FcεRI. WC strongly suppressed FcεRI−IgE binding in a dose-dependent manner (**Figure 5**). However, SA, Lyo, and EA exhibited little or no effect. We further examined the effect of WC on FcεRI−IgE binding using a competitive degranulation assay system. Although all substances suppressed Ag-stimulated degranulation in RBL-2H3

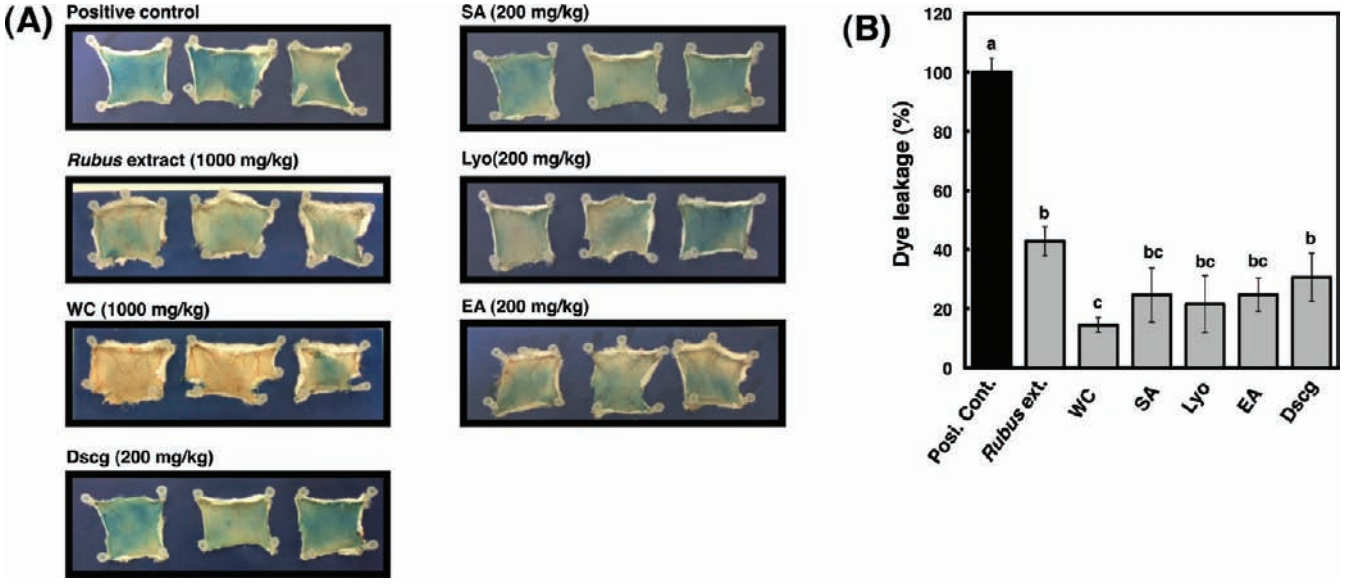

Figure 5. Inhibitory effects of WC on FcεRI−IgE binding. (**A**) Imaging of extravasated Evans blue on dorsal skin. Dscg was used as an antiallegic positive control. Photographs shown represent three independent experiments. (**B**) Amount of extravasated Evans blue dye from dorsal skin of mice treated with WC, SA, Lyo, and EA. Values are mean ± SEM ($n = 9$). Mean values with different letters are significantly different ($p < 0.05$, one-way analysis of variance followed by Fisher's multiple-range test).

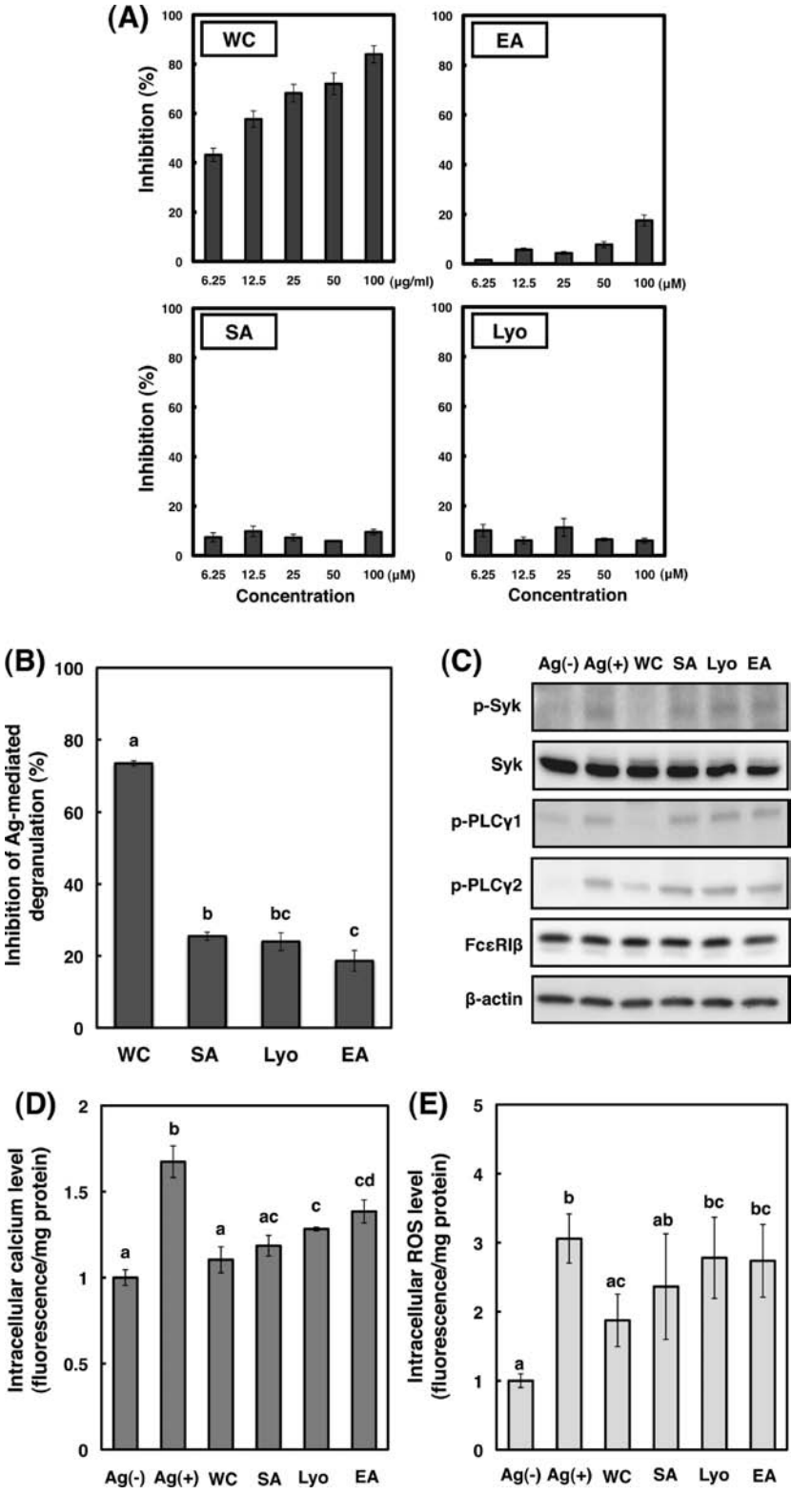

Figure 6. Effects of WC, SA, Lyo, and EA on mouse passive cutaneous anaphylaxis (PCA) reaction induced by IgE−antigen complex. (**A**) Inhibitory effect of FcεRI−IgE binding by WC, SA, Lyo, and EA. The percentage inhibition is expressed relative to the absence of these compounds as 100%. Values are mean ± SEM (n = 10). (**B**) Inhibitory effects of WC, SA, Lyo, and EA on Ag-stimulated IgE competitive degranulation. (**C**) Effect of WC, SA, Lyo, and EA on the signaling pathways in Ag-stimulated RBL-2H3 cells. A representative blot from three independent experiments is shown. (**D**) Suppression of elevation of $[Ca^{2+}]i$ by WC, SA, Lyo, and EA in Ag-stimulated RBL-2H3 cells. Data were obtained 2 min after Ag stimulation. (**E**) Suppression of intracellular ROS by WC, SA, Lyo, and EA in Ag-stimulated RBL-2H3 cells. Data were obtained 5 min after Ag stimulation. Mean values with different letters are significantly different ($p < 0.05$, one-way analysis of variance followed by Fisher's multiple-range test). Cells were treated with 50 μg/mL WC or 50 μM SA, Lyo, and EA in these experiments.

cells, WC strongly inhibited IgE competitive degranulation compared with SA, Lyo, and EA treatments (**Figure 5B**). From immunoblot analysis, the Syk activation by WC treatment was significantly decreased by the inhibition of FcεRI−IgE binding

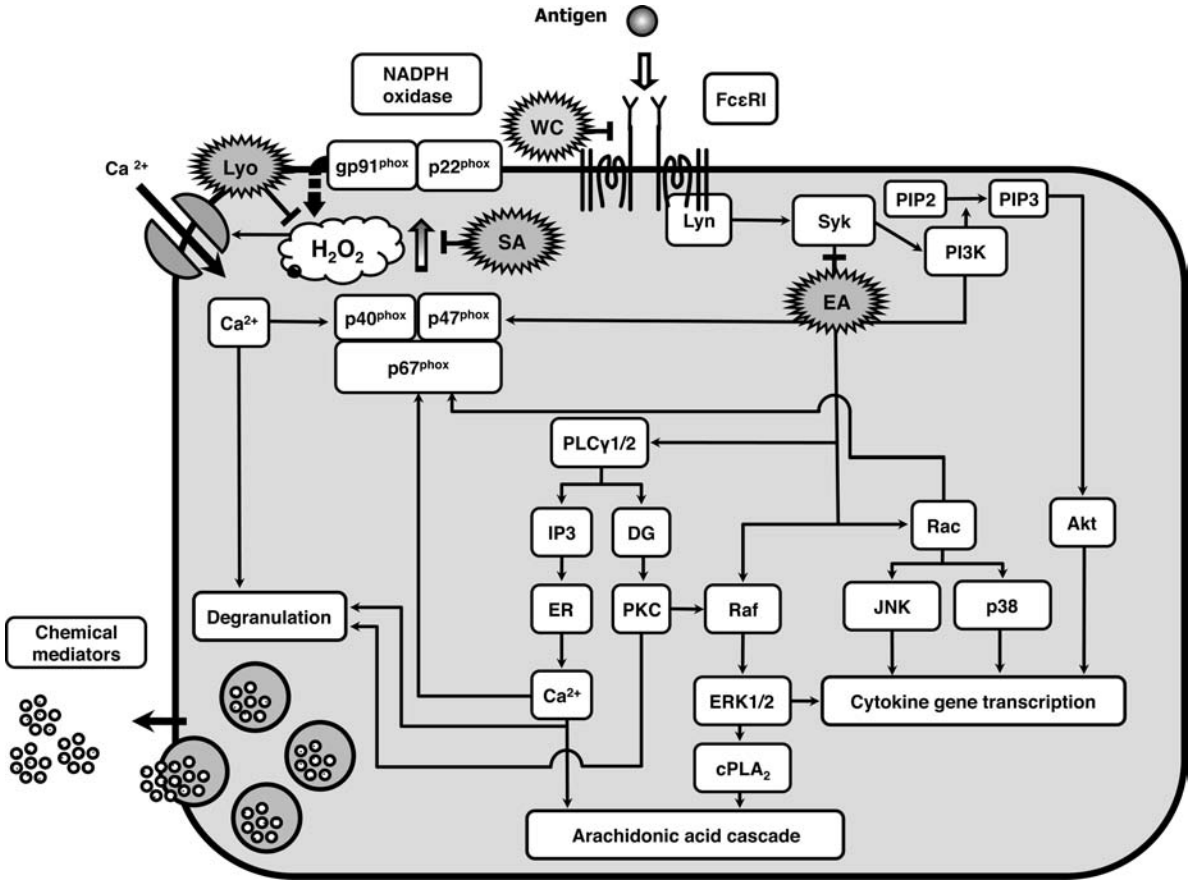

Figure 7. Scheme showing the inhibitory effects of WC, SA, Lyo, and EA on degranulation from RBL-2H3 cells. WC suppresses the IgE binding to FcεRI-α chain. SA attenuates intracellular ROS production through inhibition of NADPH oxidase activity. Lyo scavenges the produced ROS by Ag-stimulation, directly. EA suppresses Syk activation.

(**Figure 5C**). Thus, WC suppressed all events examined downstream of Syk such as the activation of PLCγ1/2, the elevation of $[Ca^{2+}]i$, and the production of intracellular ROS (**Figure 5C−E**).

Suppression of Mouse Passive Cutaneous Anaphylaxis (PCA) Reaction by WC, SA, Lyo, and EA Administration. To examine the effects of WC, SA, Lyo, and EA on type I allergy, we performed PCA reaction. Administrations of WC (1000 mg/kg of mouse body weight), SA, Lyo, and EA (200 mg/kg of mouse body weight) significantly suppressed mouse PCA reaction (**Figure 6A**,**B**). As shown in **Figure 6A**, the suppression of PCA reaction by these compounds was greater than that caused by *Rubus* extract administration.

DISCUSSION

In this study, we found that WC inhibits IgE-mediated allergic reactions through multiple mechanisms (**Figure 7**).

Ag-mediated aggregation of FcεRI on mast cells leads to the activation of Syk/PLCγs/PKC signaling pathways. Activated Syk induces $[Ca^{2+}]i$ elevation and activates PKC. Recent studies also reported that Syk-deficient mast cells completely abrogated degranulation, the elevation of $[Ca^{2+}]i$, and the activation of ERK and JNK (*27*, *28*). Yoshimaru et al. have reported that endogeneous ROS acts as a Ca^{2+} regulator (*29*). The ROS production induced by Ag stimulation is most likely through NADPH oxidase, because ROS production is abolished by pretreatment with DPI, a potent inhibitor for the enzyme (*30*,*31*). NADPH oxidase is an enzyme complex composed of membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$), cytosolic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), and a monomeric GTP binding protein of Rho family Rac2 (*32*). In the activation of phagocytic NADPH oxidase, the cytosolic subunits are translocated to the membrane, initiating the production of superoxide (*33*). As shown in **Figure 3B**,**C**, the levels of $p67^{phox}$ in membranes by SA and EA treatment for 10 min were decreased compared with Ag-stimulated RBL-2H3 cells. The $p67^{phox}$ subunit is necessary for the induction of electron transport through $gp91^{phox}$ (*34*). Therefore, it is likely that the suppression of ROS production by SA and EA was mediated by the inhibition of translocation of $p67^{phox}$ subunit to the membrane. In the present study, EA remarkably suppressed the activation of Syk, which acts upstream of NADPH oxidase. Thus, it was considered that the inhibition of translocation of $p67^{phox}$ subunits by EA treatment was mainly due to Syk inactivation. Conversely, the suppression of intracellular ROS production and $[Ca^{2+}]i$ elevation by SA treatment was ascribed to the direct inhibition of translocation of $p67^{phox}$ subunits. It was considered that the inhibition of intracellular ROS production by Lyo was due to scavenging of the produced ROS (O_2^-) by its antioxidant activity, because it had no effect on Ag-stimulated signal transduction and NADPH oxidase inhibition.

It is also of interest to note that WC significantly suppressed the FcεRI−IgE binding, whereas SA, Lyo, and EA did not affect it. We further confirmed the inhibition of degranulation, Syk activation, $[Ca^{2+}]i$ elevation, and intracellular ROS production by WC treatment. Thus, the inhibition of FcεRI−IgE binding by WC was validated by these experiments. Although we could suggest the inhibition of FcεRI−IgE binding by WC, we could not identify its suppressive compound from WC. To date, Nakagawa has reported that castalagin isolated from WC has a strong inhibitory activity toward FcεRI−IgE binding (*12*). However, castalagin is a

very minor component compared with SA, Lyo, and EA in WC. Other minor compounds such as V, VA, PA, HbA, SyA, SiA, and CoA may suppress the FcεRI−IgE binding. We need further study to disclose the mechanism and the active compound underlying the inhibition of FcεRI−IgE binding by WC. Our results in this study suggested that WC inhibits the allergic reaction through multiple mechanisms. Thus, to ameliorate type I allergy, the intake of WC would be more effective than the intake of pure materials isolated from WC, such as SA, Lyo, EA, and castalagin.

In summary, WC was found to suppress Ag-mediated degranulation in RBL-2H3 cells and type I allergy reaction in a mouse model, suggesting that WC would be an effective agent for type I allergy.

ABBREVIATIONS USED

Ag, antigen; BSA, bovine serum albumin; $[Ca^{2+}]i$, intracellular free calcium concentration; CM-H_2DCF-DA, 5- (and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorohydrofluorescein; DNP, dinitrophenol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DPI, diphenyleneiodonium chloride; Dscg, disodium cromoglycate; EA, ellagic acid; ERK, extracellular signal-regulated kinase; EGTA, *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; EIA, enzyme immunoassay; FcεRI, high-affinity IgE receptor; HRP, horseradish peroxidase; IgE, immunoglobulin E; Lyo, lyoniresinol; SAPK/JNK, stress-activated protein kinase/c-jun-N-terminal kinase; Lyn, Src family protein kinase; MAPK, mitogen-activated protein kinase; PCA, passive cutaneous anaphylaxis; PKC, protein kinase C; cPLA_2, cytosolic phospholipase A_2; PLC, phospholipase C; PVDF, polyvinylidene fluoride; SA, syringaldehyde; Syk, spleen tyrosine kinase; TMB, 3,3′,5,5′-tetramethylbenzidine; ROS, reactive oxygen species. WC, whiskey congeners.

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Supporting Information Available: Figures S1 and S2 and Table S1.This material is available free of charge via the Internet at http://pubs.acs.org.

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