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Inhibitory effects of flavonoids isolated from *Fragaria ananassa* Duch on IgE-mediated degranulation in rat basophilic leukemia RBL-2H3

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ABSTRACT

We isolated the 4 kinds of flavonoids from strawberry 'Nohime' and examined the effect of these flavonoids on the degranulation in RBL-2H3 cells. The flavonoids were found to suppress the degranulation from Ag-stimulated RBL-2H3 cells to different extents. To disclose the inhibitory mechanism of degranulation by flavonoids, we examined their effects on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) and the intracellular signaling pathway such as Lyn, Syk, and PLCγs. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) was elevated by FcεRI activation, but these flavonoid treatments reduced the elevation of $[Ca^{2+}]i$ by suppressing Ca^{2+} influx. Kaempferol strongly suppressed the activation of Syk and PLCγs. It was thus suggested that suppression of Ag-stimulated degranulation by the flavonoids is mainly due to suppression of $[Ca^{2+}]i$ elevation and Syk activation. These results suggested that strawberry would be of some ameliorative benefit for the allergic symptoms.

1. Introduction

Type I allergy reaction (anaphylactic type, e.g., Anaphylactic shock) is initiated from the binding of antigen (Ag) to the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. As results of the Ag-binding, mast cell immediately released chemical mediators such as histamine, arachidonic acid metabolites, proteases, serotonin, and heparin from intragranular.[1–3] Thus, mast cell plays a crucial role in the allergic reaction.

Strawberry (*Fragaria ananassa* Duch) is frequently eaten over the world and many kinds are maintained. In this study, we used strawberry 'Nohime', which was developed for forcing culture in Gifu Prefectural Agricultural Technology Center. 'Nohime' was selected from the cross between 'Aiberry' and 'Nyoho' in 1988 to obtain the characteristics such as large size and good taste, and was released in 1995. Recently, it has been reported that strawberry has anti-tumor,[4] anti-diabetes,[5] anti-oxidant,[6] and anti-obesity activity.[7] In the present study, we have isolated four kinds of flavonoids from strawberry 'Nohime' and determined their chemical structures. Moreover, we investigated the effects of these flavonoids on intracellular signaling pathways leading to degranulation in Ag-mediated rat basophilic leukemia RBL-2H3 cells. The strawberry-flavonoids significantly suppressed the activation of Syk and elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$). Thus, these compounds would be beneficial candidates for the anti-allergic agents.

2. Results

2.1. Determination of chemical structures of flavonoids

Prior to determination of chemical structures, we first examined the effects of the strawberry extract, which was obtained by extraction with 50% ethanol, on Ag-stimulated β-hexosaminidase

Abbreviations: Ag, antigen; A23187, calcimycin, calcium ionophore; CM-H_2DCF-DA, 5-(and-6-)-carboxy-2′,7′-dichlorodihidrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorohydrofluorescein; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DPI, diphenyleneiodonium chloride; ERK, extracellular signal-regulated kinase; EGCG, (−)-epigallocatechin gallate; EGTA, *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; FcεRI, high affinity IgE receptor; IgE, immunoglobulin E; SAPK/JNK, stress-activated protein kinase/c-jun-N-terminal kinase; Lyn, Src family protein kinase; MAPK, mitogen-activated protein kinase; PVDF, polyvinylidene fluoride; Syk, Syk/Zap-70 family protein kinase, spleen tyrosine kinase; PTLC, preparative thin layer chromatography; ROS, reactive oxygen species.

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release from RBL-2H3cells. As shown in Figure 1A, the extract suppressed the Ag-induced β-hexosaminidase release. To determine the active compound, we have subjected the 50% ethanol extract to chromatography and isolated 4 flavonoids; chrysin (CH), kaempferol (KF), *trans*-tiliroside (TL) and 7-*O*-*trans*-cinnamoylchrysin (CC) from strawberry 'Nohime'.[8,9] Their chemical structures are shown in Figure 1B.

2.2. Inhibition of Ag-stimulated β-hexosaminidase release by strawberry-flavonoids

To investigate the effects of the flavonoids on degranulation, we measured the release of β-hexosaminidase from Ag-stimulated RBL-2H3 cells. Chrysin (CH), kaempferol (KF), and *trans*-tiliroside (TL) treatments strongly inhibited the Ag-mediated degranulation, and these inhibitory effects were almost similar to that of ellagic aid (EA). On the other hand, the inhibitory potency of 7-*O*-*trans*-cinnamoylchrysin (CC) was much lower compared to other flavonoids (Fig. 2A). A calcium ionophore A23187-induced β-hexosaminidase release was also inhibited by these flavonoids (Fig. 2B). Thus, the inhibitory effect of degranulation by flavonoids was considered to be mainly due to the inhibition of Ca^{2+}-dependent degranulation process.

2.3. Inhibition of $[Ca^{2+}]i$ increase by strawberry-flavonoids

To gain further insight into the mechanism underlying inhibition of degranulation by CH, KF, TL, and CC, we examined intracellular Ca^{2+} levels following Ag-stimulation of RBL-2H3 cells in the presence of these flavonoids. Upon Ag-stimulation of FcεRI-activated of the cells, the $[Ca^{2+}]i$ level rapidly increased and retained sustained levels thereafter (Fig. 3, filled circle). However, the elevations of $[Ca^{2+}]i$ were significantly suppressed by flavonoids (Fig. 3, filled square). Especially, CH and KF exerted dramatic inhibitory effect on the $[Ca^{2+}]i$ elevation.

2.4. Effect of strawberry-flavonoids on Ag-mediated intracellular ROS production

We examined the production of intracellular ROS by using a CM-H_2DCF-DA fluorescent probe. DCF oxidation was gradually increased by Ag-treatment (Fig. 4, filled triangle). In the presence of TL and CC,

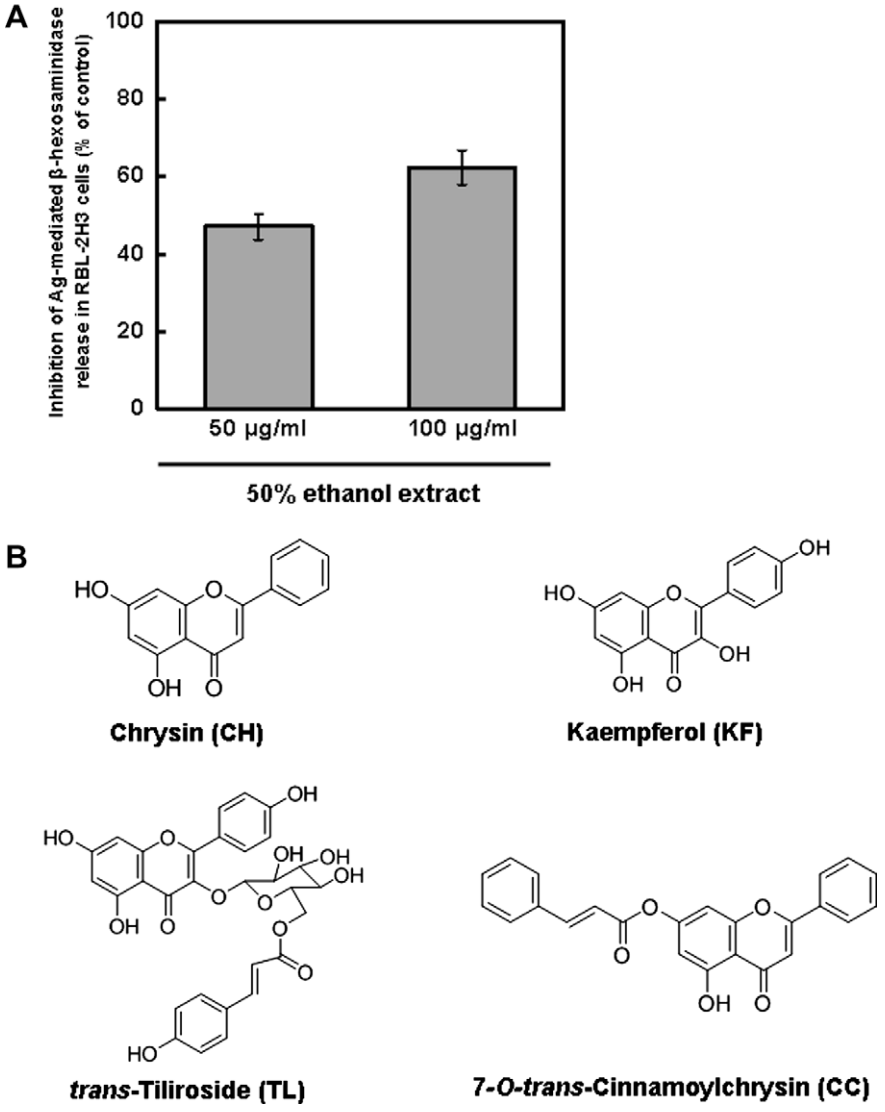

Figure 1. Inhibitory effects of the 50% ethanol extract of strawberry on Ag-stimulated degranulation from rat basophilic leukemia RBL-2H3 cells. (A) IgE-sensitized RBL-2H3 cells were stimulated with DNP–BSA in the presence of the 50% ethanol extract of strawberry (50 or 100 μg/ml); (B) chemical structures of flavonoids isolated from the extract of strawberry.

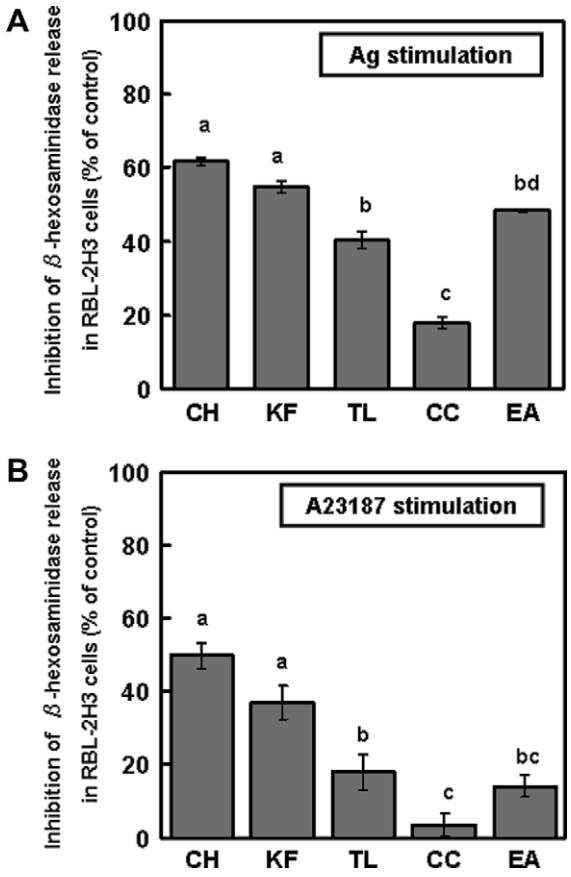

Figure 2. Inhibitory effects of the strawberry-flavonoids on Ag-stimulated degranulation from rat basophilic leukemia RBL-2H3 cells. (A) IgE-sensitized RBL-2H3 cells were stimulated with DNP–BSA in the presence of each flavonoid. As a marker of degranulation, we measured the release of β-hexosaminidase; (B) RBL-2H3 cells were stimulated with A23187 is in the presence of each flavonoid. Values are mean ± SEM ($n = 9$) of the inhibition of degranulation release. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance followed by Fisher's-multiple range test). Chrysin (CH), kaempferol (KF), *trans*-tiliroside (TL), 7-*O*-*trans*-cinnamoylchrysin (CC) and ellagic acid (EA).

the ROS production was suppressed to different extents (Fig. 4). As DPI was shown to prevent the ROS production evoked by Ag-stimulation, it was considered that ROS was generated via NADPH oxidase. Furthermore, we examined the anti-oxidant activity of these flavonoids by DPPH radical-scavenging assay. Unlike the typical antioxidants (EGCG, Vitamin C, and -E), these strawberry-flavonoids did not exhibit radical-scavenging activity (Table 1).

2.5. Effect of strawberry-flavonoids on intracellular signaling pathways in Ag-stimulated RBL-2H3 cells

We investigated the early signaling events. FcεRI cross-linking on mast cells has been known to activate non-receptor type protein tyrosine kinases such as Lyn and Syk.[10,11] We examined activation (phosphorylation) of Lyn, Syk, and PLC-γs which plays important roles in degranulation process. As shown in Figure 5 (left panel), phosphorylations of Syk and PLCγ1/2 were markedly decreased by kaemoferol treatment, while other flavonoids did not affect the activation of Syk/PLCγs pathway.

Next, we examined the effects of flavonoids on mitogen-activated protein kinases (MAPKs; ERK, JNK, p38). Ag-stimulation induced phosphorylation of ERK and JNK but not p38 MAP kinase (Fig. 5; right panel). However, their phosphorylations were slightly suppressed by KF, TL, and CC-treatment. On the other hand, CH did not affect. Moreover, phosphorylation of cytosolic phospholipase A_2 (cPLA$_2$), which has been known to be phosphorylated by ERK, is suppressed KF, TL, and CC-treatment.

3. Discussion

It has been considered that the inhibitory effect of degranulation by the strawberry extract was mainly due to ellagic acid.[12] In the current study, we have isolated four kinds of flavonoids from strawberry 'Nohime' which show the inhibitory effect on degranulation in RBL-2H3 cells. Chrysin, kaempferol and *trans*-tiliroside exhibited an inhibitory activity of Ag-stimulated degranulation which were nearly equal to that of ellagic acid.

Interestingly, these flavonoids were found to suppress the elevation of [Ca^{2+}]i mediated by influx from extracellular medium. Several studies have reported that endogenous ROS plays as a Ca^{2+} regulator.[13–16] The intracellular ROS production was observed to occur after Ag-stimulation and its source was thought to be most likely due to the action of a NADPH oxidase because of the abolished ROS production by DPI, a potent inhibitor for the enzyme.[17,18] Although CH and KF-treatment strongly suppressed the elevation of [Ca^{2+}]i, intracellular ROS production was not affected by the same treatment. Thus, the intracellular ROS production was thought not to be associated with Ca^{2+} influx. Recent studies reported that Syk-deficient mast cells completely abrogated degranulation, [Ca^{2+}]i elevation, and activation of the ERK and JNK.[19,20] Similarly, inhibition of Syk by KF-treatment was accompanied with inhibition of those events. Cytosolic phospholipase A_2 (cPLA$_2$) is activated by the increase of [Ca^{2+}]i elevation and ERK1/2 activation in Ag-mediated stimulation of mast cells.[21–24] In present study, it was suggested that cPLA$_2$ phosphorylation suppressed by KF, TL and CC-treatment was mainly due to the suppression of [Ca^{2+}]i elevation and ERK activation. Especially, KF remarkably suppressed Syk activation. Thus, it was suggested that the suppression of phosphorylation of ERK and cPLA$_2$ by KF was also due to Syk inactivation. Although CH suppressed the [Ca^{2+}]i elevation most strongly among the isolated four kinds of flavonoids and slightly suppressed ERK activation, cPLA$_2$ phosphorylation was not inactivated. Further study should be needed to clarify the discrepancy of cPLA$_2$ suppression mechanism by CH and other flavonoids will be needed.

In conclusion, the flavonoids isolated from strawberry 'Nohime', exerted an inhibitory effect on Ag-stimulated degranulation by suppressing of Ca^{2+} influx and Syk activation (Fig. 6). These results, suggested that strawberry would be of some benefit for prevention or improvement of type I allergy.

4. Materials and methods

4.1. Isolation of flavonoids and their chemical structures

Strawberries (7.5 kg; Nohime) were squeezed and separated into juice and residual substance. The juice was concentrated under reduced pressure, partitioned with ethyl acetate, then further separated by a Sephadex LH-20 column (50 × 5 cm) eluted with methanol, to give Fr. 1 (1.15 g) and Fr. 2 (250 mg).

The Fr. 1 was separated by a silica gel column (50 × 3 cm) eluted with $CHCl_3$–MeOH (10:1–0:1; v/v) and 4 fractions (Fr.1-1 to Fr. 1-4) were obtained. Fr. 1-2 (365 mg) was further purified by PTLC (1 mm layer thickness) using $CHCl_3$–MeOH (5:1) to give chrysin (3.5 mg; CH). Fr. 2 was purified by a silica gel column (50x3 cm) eluted with $CHCl_3$–MeOH (15:2 to 5:3; v/v) in turn, to give 3 fractions (Fr.2-1 to Fr. 2-3). Fr. 2-1 was further purified by PTLC using $CHCl_3$–MeOH (5:1) to give 7-*O*-cinnamoylchrysin (3.3 mg; CC) and kaempferol (1.2 mg; KF). The residual substance

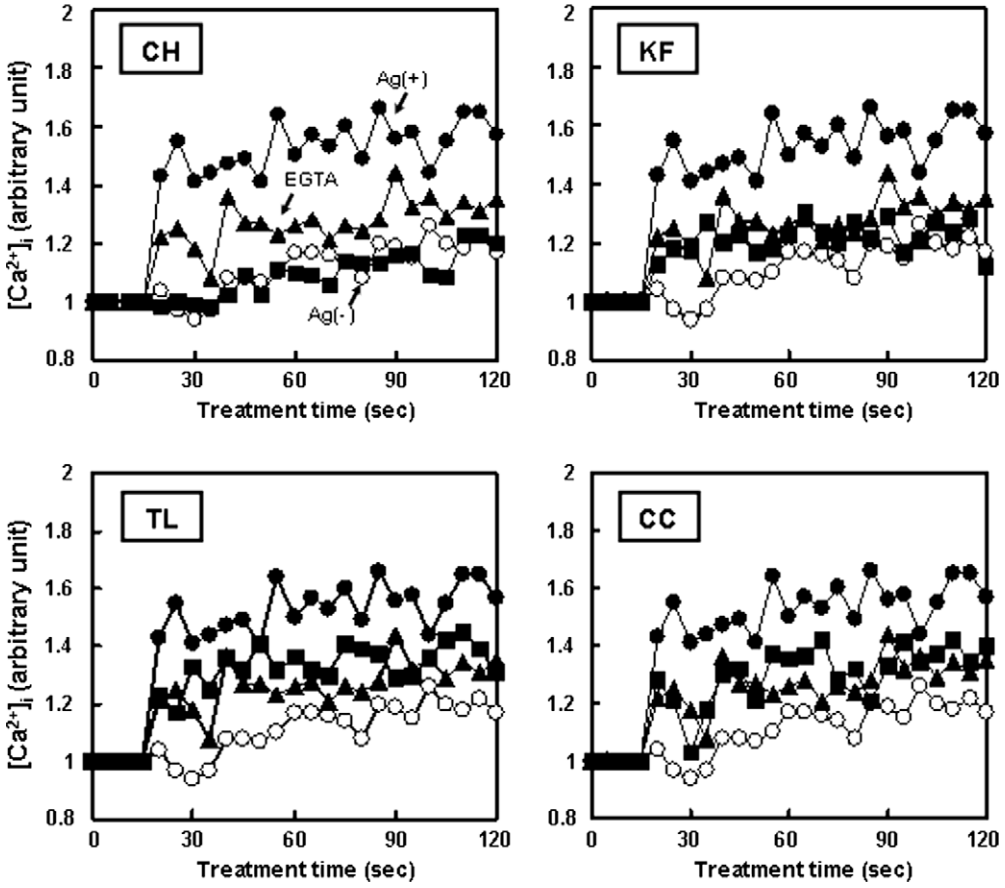

Figure 3. Suppression of the elevation of $[Ca^{2+}]i$ by the strawberry-flavonoids in Ag-stimulated RBL-2H3 cells. Ag-stimulated RBL-2H3 cells were stimulated with either DNP–BSA in loading buffer, DNP–BSA in loading buffer including strawberry-flavonoids, or DNP–BSA in calcium-free loading buffer including EGTA (1 mM; selective Ca^{2+} chelating reagent) for indicated periods. Intracellular calcium was measured as described in Section 4. Each value represents the mean with SEM ($n = 12$). ●: Ag-stimulated cells, ○: Ag-nontreated cells, ▲: Ag plus EGTA-treated cells, ■: Ag plus each flavonoid-treated cells. chrysin (CH), kaempferol (KF), *trans*-tiliroside (TL) and 7-*O*-*trans*-cinnamoylchrysin (CC).

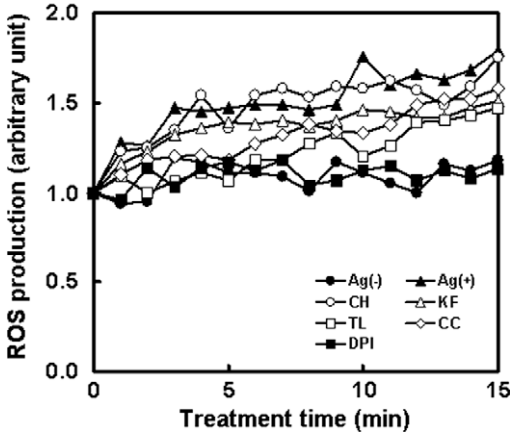

Figure 4. Effect of the strawberry-flavonoids on the intracellular ROS production in Ag-stimulated RBL-2H3 cells. CM-H_2DCF-DA-loaded cells were incubated with flavonoids (50 μM) for 30 min. CM-H_2DCF-DA treated cells were stimulated by DNP–BSA (10 μg/ml). ROS-mediated DCF oxidation was measured using a microplate fluorometer as described in Section 4. chrysin (CH), kaempferol (KF), *trans*-tiliroside (TL) and 7-*O*-*trans*-cinnamoylchrysin (CC).

extracted with 0.5% TFA in MeOH. The filtrate was concentrated under reduced pressure, to give MeOH extract (70.1 g), then subjected to Amberlite XAD-7 column eluted with distilled H_2O and MeOH. The MeOH fraction was concentrated, then purified by a Sephadex LH-20 column (50 × 5 cm) eluted with MeOH, to give 5 fractions (Fr. 3-1 to Fr. 3-5). Fr. 3-3 (427 mg) was further purified by PTLC using $CHCl_3$/EtOAc/MeOH (2:2:1) to give kaempferol (2.2 mg; KF). Also Fr. 3-4 was further purified by PTLC using $CHCl_3$–MeOH (10:3) to give *trans*-tiliroside (10.9 mg; TL).

Table 1
Anti-oxidant activity of flavonoids isolated from strawberry

	Anti-oxidant activity (ED_{50} value)
Chrysin	100 μm<
Kaempferol	100 μm<
trans-Tiliroside	100 μm<
7-O-*trans*-Cinnamoylchrysin	100 μm<
EGCG	7 μm
Vitamin C	18 μm
Vitamin E	78 μm

DPPH radicals-scavenging activity was measured in the reaction mixture containing 0.5 mM DPPH radical solution (0.1 ml), 99% ethanol (0.8 ml), and sample solution (0.1 ml). The scavenging activity was determined by monitoring the decrease in absorbance at 517 nm.

4.1.1. Chrysin (CH)

Yellow powder. IR (KBr): 3382, 1615 cm^{-1}. ^1H NMR (600 MHz, acetone-d_6): δ 8.04 (dd, 2H, $J = 7.8$, 1.9 Hz), 7.59–7.56 (m, 3H), 6.77 (s, 1H), 6.56 (d, 1H, $J = 1.8$ Hz), 6.25 (d, 1H, $J = 1.8$ Hz). ^{13}C NMR (150 MHz, acetone-d_6): δ 182.3, 164.3, 163.8, 162.5, 158.1, 131.9, 131.4, 129.2, 126.4, 105.5, 105.4, 99.1, 94.1. MS (FAB): $m/z = 255$ $[M+H]^+$ matrix: magic bullet.

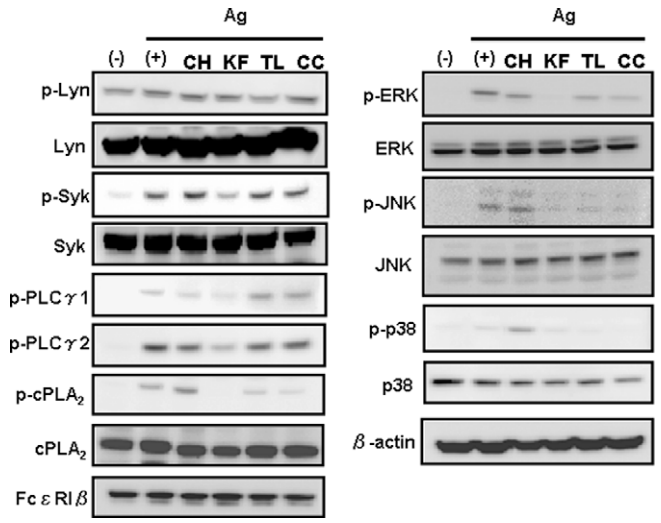

Figure 5. Effect of strawberry-flavonoids on the signaling pathway in Ag-stimulated RBL-2H3 cells. The strawberry-flavonoids-treated cells were stimulated with DNP–BSA for 10 min and were resuspended in RIPA buffer. Twenty micrograms of protein of each cell lysate was separated by SDS–PAGE and electroblotted onto a PVDF membrane for Western blotting. chrysin (CH), kaempferol (KF), *trans*-tiliroside (TL) and 7-*O*-*trans*-cinnamoylchrysin (CC).

4.1.2. 7-*O*-cinnamoylchrysin (CC)

White powder. IR (KBr): 3409, 1665, 1625 cm^{-1}. ^1H NMR (600 MHz, chloroform-d_1): δ 7.91 (d, 1H, J = 15.8 Hz), 7.89 (d, 2H, J = 7.5 Hz), 7.61–7.59 (m, 2H), 7.56–7.51 (m, 3H), 7.45–7.42 (m, 3H), 6.95 (d, 1H, J = 1.8 Hz), 6.74 (s, 1H), 6.66 (d, 1H, J = 1.8 Hz), 6.62 (d, 1H, J = 15.8 Hz). ^{13}C NMR (150 MHz, chloroform-d_1): δ 182.9, 164.8, 164.4, 162.0, 156.8, 156.2, 147.8, 133.9, 132.2, 131.1, 131.0, 129.2, 129.1, 128.5, 126.5, 116.5, 109.0, 106.2, 105.6, 101.1. MS (FAB): m/z = 385 $[M+H]^+$ matrix: magic bullet.

4.1.3. Kaempferol (KF)

Yellow powder. IR (KBr): 3435, 1660 cm^{-1}. ^1H NMR (600 MHz, acetone-d_6): δ 7.98 (d, 2H, J = 8.3 Hz), 6.96 (d, 2H, J = 8.3 Hz), 6.34 (d, 1H, J = 2.0 Hz), 6.27 (d, 1H, J = 2.0 Hz). ^{13}C NMR (150 MHz, acetone-d_6): δ 175.8, 164.3, 161.5, 159.4, 157.0, 146.2, 135.8, 129.6, 122.5, 115.5, 103.3, 98.4, 93.7. MS (FAB): m/z = 287 $[M+H]^+$ matrix: magic bullet.

4.1.4. *trans*-Tiliroside (TL)

Yellow powder. IR (KBr): 3433, 1682, 1609 cm^{-1}. ^1H NMR (600 MHz, methanol-d_4): δ 7.97 (d, 2H, J = 8.9 Hz), 7.38 (d, 1H, J = 15.8 Hz), 7.28 (d, 2H, J = 8.3 Hz), 6.79 (d, 2H, J = 8.9 Hz), 6.76 (d, 2H, J = 8.3 Hz), 6.26 (d, 1H, J = 2.1 Hz), 6.10 (d, 1H, J = 2.1 Hz), 6.05 (d, 1H, J = 15.8 Hz), 5.20 (d, 1H, J = 7.6 Hz), 4.29 (dd, 1H, J = 11.6, 2.0 Hz), 4.16 (dd, 1H, J = 11.6, 2.0 Hz), 3.47–3.40 (m, 3H), 3.31–3.29 (m, 1H). ^{13}C NMR (150 MHz, methanol-d_4): δ 178.0, 167.4, 165.0, 161.6, 160.1, 159.8, 157.9, 157.0, 145.2, 133.8, 130.8, 129.8, 125.7, 121.3, 115.4, 114.6, 113.3, 104.1, 102.6, 98.7, 93.5, 76.0, 74.4, 74.3, 70.3, 62.9. MS (FAB): m/z = 595 $[M+H]^+$ matrix: glycerin.

4.2. Reagents and materials

Monoclonal mouse IgE anti-dinitrophenol (DNP) was purchased from Yamasa Co. Ltd (Tokyo, Japan). The 25 × Complete®, a mixture of protease inhibitors was from Roche (Penzberg, Germany). The phosphatase Inhibitor Cocktail® 1 and 2 was from Sigma (St. Louis, Mo, USA). The antibodies to anti-rat p44/42 MAP Kinase (ERK), anti- rat phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK), anti- rat SAPK/JNK (JNK), anti-rat phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), anti- rat p38 MAP kinase (p38), anti-rat phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), anti-rat Lyn, anti-rat phospho-Lyn, anti-rat $cPLA_2$, anti-rat phosphor-$cPLA_2$, anti-rat phosphor-PLCγ1, anti-rat phosphor-PLCγ2, and anti-phosphotyrosine (pTyr) were from Cell Signaling Technology (MA, USA). The antibody to anti-rat Syk was from Santa Cruz Biotechnology (CA, USA). The antibody to anti-rat ß-actin and ellagic acid were from Sigma. FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, molecular immunology and inflammation branch). Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from GE Healthcare Sci. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, England).

4.3. Cell culture

RBL-2H3 cells were obtained from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium (Gibco, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of stereptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

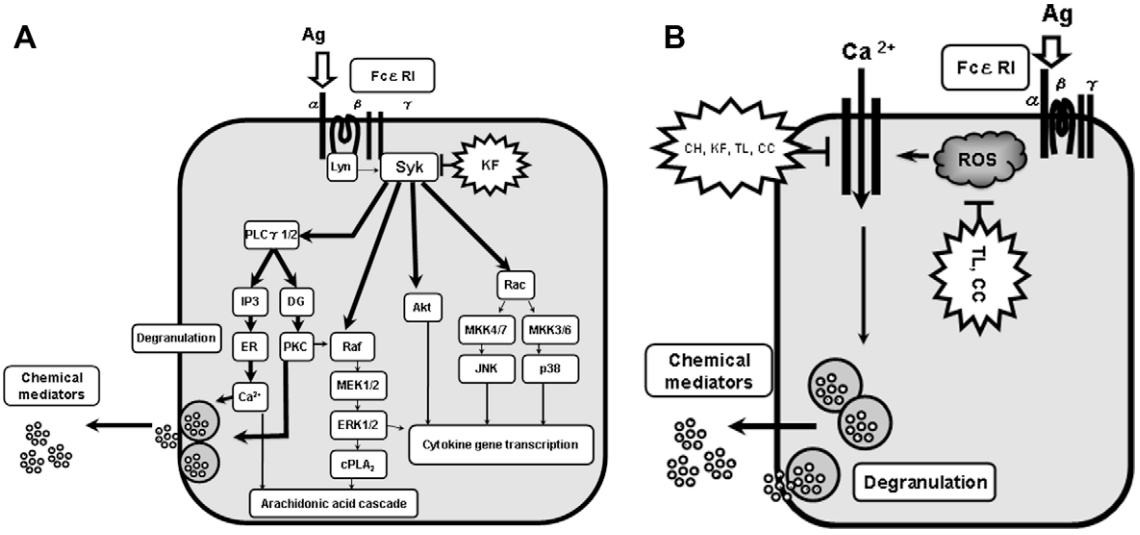

Figure 6. A scheme showing the inhibitory effect on degranulation by the strawberry-flavonoids in RBL-2H3 cells. (A) Strawberry-flavonoids were suppressed Syk activation; (B) Strawberry-flavonoids were suppressed the intracellular ROS production and Ca^{2+} influx.

4.4. β-Hexosaminidase release assay

As a marker of degranulation, we measured the release of β-hexosaminidase as described previous report.[25]

4.5. Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} level was determined with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5×10^4 cells/well) were seeded into 94-black walled-bottom clear micro plates and incubated for 1 h. After incubation, monoclonal mouse IgE anti DNP was added to cultured medium at a concentration of 0.45 μg/ml and 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. The treated cells were washed with PBS and incubated with 90 μl of loading buffer (Calcium Kit-Fluo 3™) including 50 μM of flavonoids for 30 min. Intracellular Ca^{2+} concentration monitored by measuring the fluorescence with a fluorometric imaging plate reader (excitation; 490 nm, emission; 530 nm).

4.6. Measurement of intracellular ROS level by CM-H_2DCF-DA fluorescent probe

Intracellular ROS level was measured by using CM-H_2DCF-DA, which is a fluorogenic freely permeable tracer specific for ROS assessment. It is deacetylated by intracellular esterases to the non-fluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS. The 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. CM-H_2DCF-DA-loaded cells were incubated with 50 μM flavonoids for 30 min. CM-H_2DCF-DA-flavonoids treated cells were stimulated by DNP–BSA (10 μg/ml) and then measured with excitation at 490 nm and emission at 530 nm with a fluorometric imaging plate reader.

4.7. Measurement of DPPH radical-scavenging activity

To measure anti-oxidant activity, a DPPH radical-scavenging assay was carried out according to the previous method with a slight modification.[26]

Briefly, DPPH radical-scavenging activity was measured in the reaction mixture containing 0.5 mM DPPH radical solution 0.1 ml, 99% ethanol 0.8 ml, and 0.1 ml of flavonoids solutions (50 μM). The solution was rapidly mixed and the scavenging capacity was measured by monitoring the decrease in absorbance at 517 nm. Vitamin C (L-ascorbic acid) and vitamin E (α-tocopherol), and EGCG were used as positive controls.

4.8. Immunoprecipitation and immunoblot analysis

For immunoblot analysis, Ag-treated RBL-2H3 cells with flavonoids or without flavonoids were washed twice with PBS and harvested. These cell lysates were prepared as following to previous report.[25] These cell lysates were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer containing 2% 2-mercaptoethanol, and boiled for 5 min and subjected to Western blot analysis. After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with appropriate primary antibody. The membrane was then washed three times with TPBS, incubated further with horseradish peroxidase-conjugated secondary antibody at room temperature, and washed three times with TPBS. Proteins were detected with enhanced ECL kit and chemiluminescence detector (LAS-4000, Fujifilm, Japan).

4.9. Statistical analysis

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

5. Conclusions

In this study, we have demonstrated that flavonoids isolated from *F. ananassa* Duch., significantly suppressed the degranulation in Ag-mediated activation of FcεRI in RBL-2H3 cells. It was thus suggested that inhibition of degranulation by the flavonoids was mainly due to the inhibition of $[Ca^{2+}]$i elevation and Syk inactivation. Thus, strawberry-flavonoids would be an effective agent for anti-allergy.

Acknowledgments

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