



Short communication

Inhibitory effects of flavonoid glycosides isolated from the peel of Japanese persimmon (*Diospyros kaki Fuyu*) on antigen-stimulated degranulation in rat basophilic leukaemia RBL-2H3 cells

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ABSTRACT

We found that two distinct flavonoid glycosides isolated from the peel of Japanese persimmon (*Diospyros kaki Fuyu*), isoquercitrin (Isq) and hyperin (Hyp), are capable of inhibiting antigen-stimulated degranulation in rat basophilic leukaemia RBL-2H3 cells. In order to elucidate the underlying mechanisms, we examined effects of Isq and Hyp on cellular responses induced by antigen stimulation. Treatment with both Isq and Hyp markedly inhibited antigen-stimulated elevation of intracellular free Ca^{2+} concentration and reactive oxygen species (ROS). Isq and Hyp did not affect NADPH oxidase (NOX) activity, but they possessed DPPH radical-scavenging activity similar to that of epigallocatechin gallate, a potent anti-oxidant. Finally, Isq and Hyp showed little or no effects on Ag-stimulated Syk activation or phosphorylation of signalling molecules. These results indicate that inhibition of antigen-stimulated degranulation by Isq and Hyp is mainly due to suppression of intracellular Ca^{2+} elevation, which is caused by direct scavenging of ROS that are generated by NOX. Our findings suggest that Isq and Hyp, isolated from the peel of persimmon, would be beneficial for alleviating symptoms of type I allergy.

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1. Introduction

Type I allergic reaction, including anaphylactic shock, is initiated by the binding of antigen (Ag) to IgE molecules bound to the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. The interaction causes aggregation of FcεRI receptors, which induces a variety of cellular responses, including mobilisation of Ca^{2+} , production of reactive oxygen species (ROS) and activation of signal transduction pathways, resulting in degranula-

tion, cytokine production and leukotriene secretion (Beaven & Metzger, 1993; Mekori & Metcalfe, 2000; Turner & Kinet, 1999).

Persimmon (*Diospyros kaki Fuyu*) is mainly raised and consumed in the far east of Asia. There are many different species of persimmons. The extract of persimmon leaves, “kaki-yo”, has been used as a traditional medicine in Japan for the treatment of hypertension. Recent reports have demonstrated that the extracts prepared from leaves, peels and flesh of persimmon have anti-tumour (Khanal, Oh, Thuong, Cho, & Choi, 2010), anti-hyperlipidemia (Lee, Cho, & Yokozawa, 2008a), anti-diabetes (Lee, Kim, Cho, & Yokozawa, 2007), anti-oxidant (Chen, Fan, Yue, Wu, & Li, 2008; Lee, Cho, & Yokozawa, 2008b), and neuroprotective activities (Bei et al., 2009). In our previous study, we isolated two distinct flavonoid glycosides from the peel of persimmon, which were shown to inhibit melanogenesis in B16 mouse melanoma cells (Ohguchi et al., 2010).

In this study, we demonstrated the inhibitory effects of flavonoid glycosides, isolated from the peel of persimmon, on antigen-stimulated degranulation in rat basophilic leukaemia RBL-2H3 cells and investigated the underlying mechanisms.

Abbreviations: Ag, antigen; BSA, bovine serum albumin; CM-H₂DCF-DA, 5-(and 6-)carboxy-2',7'-dichlorodihydrofluorescein diacetate; DNP, dinitrophenol; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DPI, diphenyleneiodonium chloride; EGCG, (–)-epigallocatechin gallate; ERK, extracellular signal-regulated kinase; FcεRI, high affinity IgE receptor; HRP, horseradish peroxidase; Hyp, hyperin; IgE, immunoglobulin E; Isq, isoquercitrin; Lyn, MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC, phospholipase C; Que, quercetin; ROS, reactive oxygen species; Rut, rutin; SAPK/JNK, stress-activated protein kinase/ c-jun-N terminal kinase; Src, family protein kinase; Syk, spleen tyrosine kinase.

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2. Materials and methods

2.1. Isolation of flavonoid glycosides

Isoquercitrin (Isq) and hyperin (Hyp) were isolated from the peel of persimmon (*D. kaki Fuyu*), as described previously (Fig. 1, Ohguchi et al., 2010).

2.2. Reagents and materials

Mouse anti-dinitrophenol (DNP) monoclonal IgE was purchased from Yamasa (Tokyo, Japan). The 25×Complete®, a mixture of protease inhibitors, was from Roche (Penzberg, Germany). The phosphatase Inhibitor Cocktail® reagents 1 and 2 were from Sigma (MO, USA). The antibodies to Akt, phospho-Akt, 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, cPLA₂, phospho-cPLA₂, phospho-PLCγ1, phospho-PLCγ2, and phospho-Syk were from Cell Signaling Technology (MA, USA). The antibody to Syk, gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox} and Rac were from Santa Cruz Biotechnology (CA, USA). Anti-β-actin antibody and epigallocatechin gallate (EGCG) were from Sigma (MO, USA). The FcεRIβ antibody was kindly provided by Dr. Juan Rivera (NIH, Molecular Immunology and Inflammation

Branch). Anti-rabbit and – mouse antibodies conjugated with horseradish peroxidase and the ECL chemiluminescence kit were obtained from GE Healthcare (NJ, USA).

2.3. 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 foetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

2.4. Degranulation assay

2.4.1. β-Hexosaminidase release assay

β-Hexosaminidase was used as a marker of degranulation. RBL-2H3 cells (2 × 10⁴ 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 (119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 25 mM PIPES, 40 mM NaOH, pH 7.2), 160 µl of Siraganian buffer B (5.6 mM glucose, 1 mM CaCl₂, and 0.1% BSA) were 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-labelled bovine serum albumin (DNP-BSA) were added to the

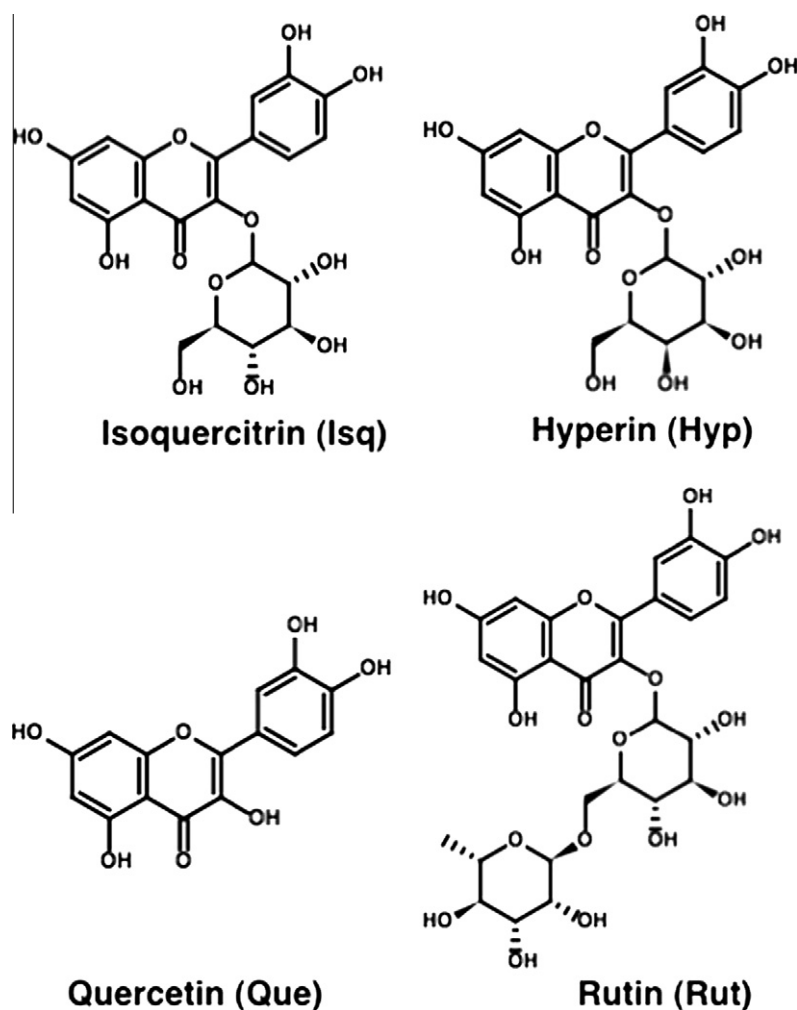


Fig. 1. The chemical structures of two major flavonoid glycosides isolated from Japanese persimmon peel; isoquercitrin (Isq) and hyperin (Hyp) are shown, along with quercetin (Que) and rutin (Rut).

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 300g 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 Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured at 405 nm, using the colorimetric microplate reader.

2.4.2. β-Hexosaminidase inhibitory activity

On 96-well microplates, samples (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 terminating the reaction, the absorbance at 405 nm was measured. The inhibition of degranulation was calculated as follows:

$$\text{Inhibition of degranulation (\%)} = \left[1 - \frac{(A_{405\text{nm of sample}} - A_{405\text{nm negative control}})}{(A_{405\text{nm positive control}} - A_{405\text{nm negative control}})} \right] \times 100 - \beta\text{-hexosaminidase inhibitory activity, \%}$$

β-Hexosaminidase inhibitory activity %.

$$= \left[1 - \frac{(A'_{405\text{nm of sample}} - A'_{405\text{nm negative control}})}{(A'_{405\text{nm positive control}} - A'_{405\text{nm negative control}})} \right] \times 100.$$

2.5. Measurement of intracellular Ca²⁺ concentrations

Intracellular Ca²⁺ levels were determined with Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5 × 10⁴ cells/well) were seeded onto 96-well microplates and incubated for 1 h. Then, cells were treated with anti-DNP IgE (0.45 µg/ml) and incubated for 24 h. After washing twice with PBS, 100 µl of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3™) were added to the culture medium. One hour later, cells were washed with PBS and incubated in 90 µl of loading buffer containing 50 µM concentrations of sample. After incubation for 30 min, cells were stimulated by DNP-BSA (10 µg/ml) and the fluorescence was measured using the fluorometric imaging plate reader (excitation; 490 nm, emission; 530 nm).

2.6. Measurement of intracellular ROS levels

Intracellular ROS levels were measured by using CM-H₂DCF-DA. IgE-sensitized RBL-2H3 cells (5 × 10⁴ cells/well) were incubated with 10 µM CM-H₂DCF-DA for 30 min at 37 °C. After washing twice, cells were incubated with 50 µM concentrations of sample for 30 min. Then, cells were stimulated by DNP-BSA (10 µg/ml) and the fluorescence was measured using the fluorometric imaging plate reader (excitation; 490 nm, emission; 530 nm).

2.7. Measurement of DPPH radical-scavenging activity

To measure *in vitro* anti-oxidant activity, the DPPH radical-scavenging assay was carried out, as described previously (Itoh, Umekawa, & Furuichi, 2005). Briefly, 0.1 ml of 0.5 mM DPPH radical solution, 0.8 ml of 99% ethanol and 0.1 ml of sample were rapidly mixed and the decrease in absorbance at 517 nm was monitored. DPPH free radical-scavenging activity (%) was calculated using the following formula: $[(A_{517\text{nm of control}} - A_{517\text{nm of sample}}) / A_{517\text{nm of control}}] \times 100$. Epigallocatechin gallate (EGCG), a potent anti-oxidant, was used as a positive control.

2.8. Immunoblot analysis

Cell lysates were prepared as described previously (Itoh, Ohguchi, Iinuma, Nozawa, & Akao, 2008). The cytosolic and membrane fractions were separated using the ProteoExtract subcellular proteome extraction kit (Merck KGaA, Darmstadt, Germany). Protein samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto PVDF membranes. After blocking for 1 h in 5% non-fat milk, the membrane was incubated with a primary antibody at 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Immunoreactive proteins were detected with the enhanced ECL kit and the chemiluminescence detector (LAS-4000, Fujifilm, Japan).

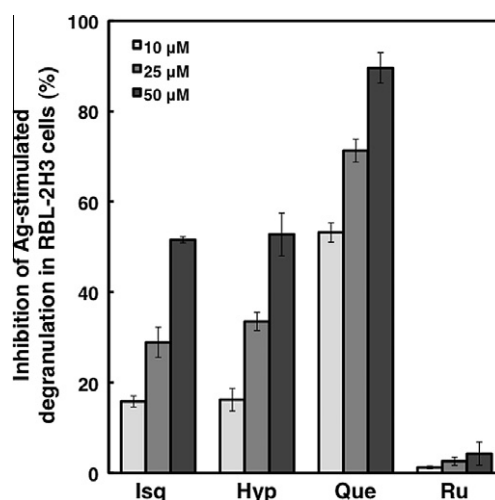


Fig. 2. Effects of Isq and Hyp on Ag-stimulated degranulation in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with Isq, Hyp, Que or Rut (10, 25 or 50 µM) and stimulated with antigen (DNP-BSA), followed by β-hexosaminidase release assays. Que and Rut were used as reference materials. Values are means ± SEM (*n* = 10).

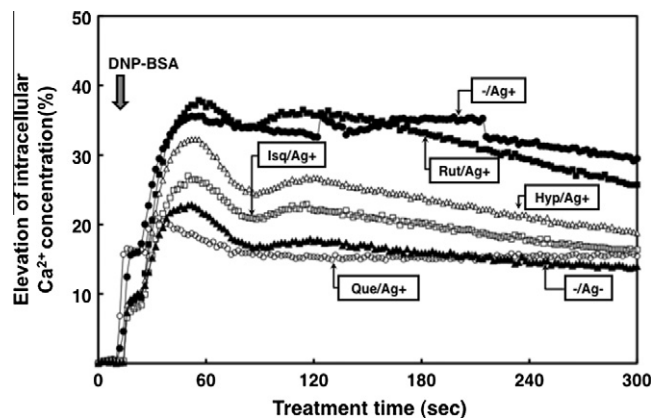


Fig. 3. Effects of Isq and Hyp on Ag-stimulated intracellular Ca²⁺ elevation in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated without or with 50 µM of Isq, Hyp, Que or Rut for 30 min. Then, cells were stimulated without or with DNP-BSA, followed by measurement of intracellular Ca²⁺ levels. Values are means ± SEM (*n* = 12). Arrow: time at which DNP-BSA was challenged, filled circle: Ag-treated cells, filled triangle: non-Ag-treated cells, open square: Ag- and Isq-treated cells, open triangle: Ag- and Hyp-treated cells, open circle: Ag- and Que-treated cells, filled square: Ag- and Rut-treated cells.

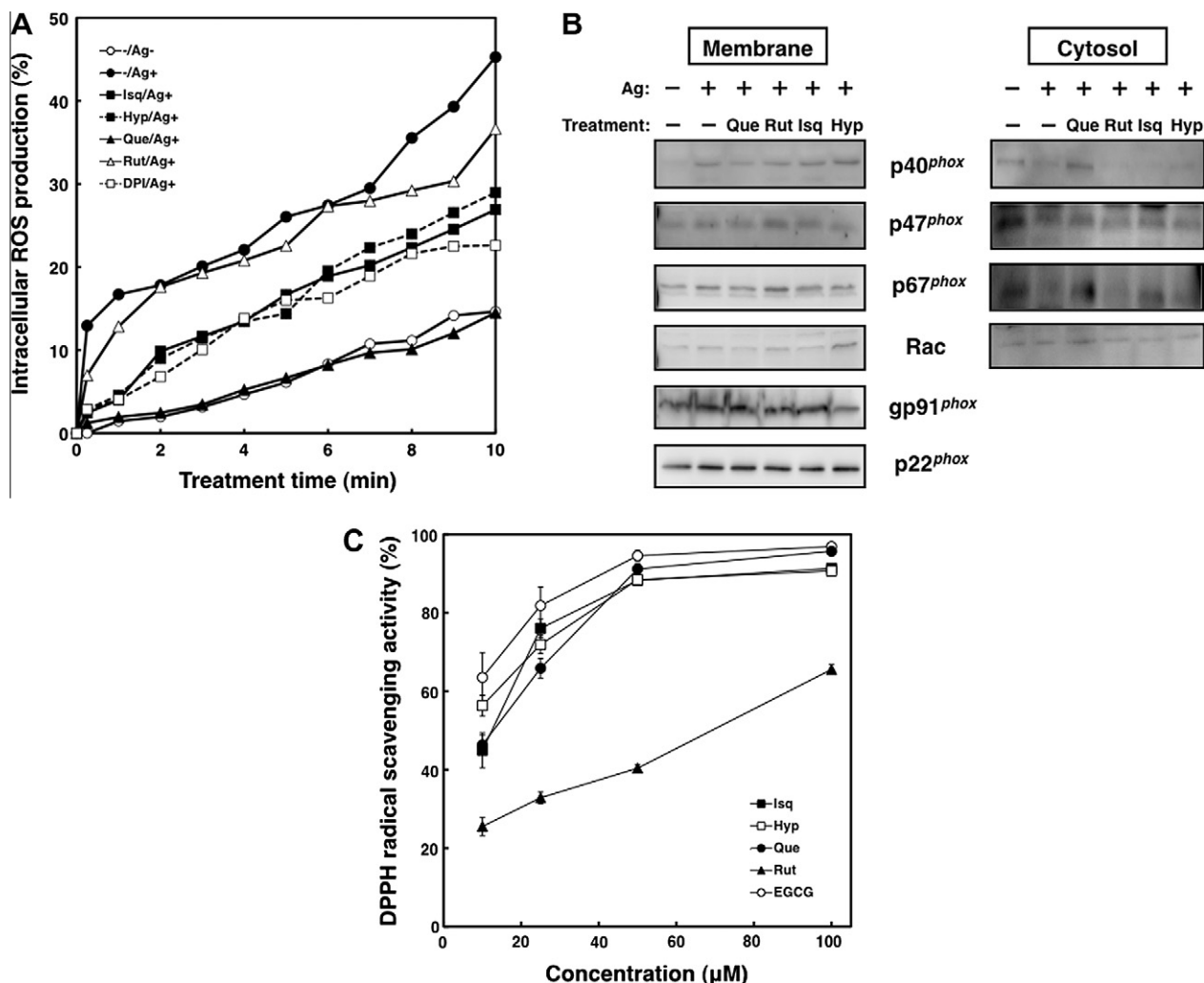


Fig. 4. Effects of Isq and Hyp on Ag-stimulated intracellular ROS production in RBL-2H3 cells. (A) IgE-sensitized RBL-2H3 cells were treated with CM-H₂DCF-DA and incubated without or with 50 μM Isq, Hyp, Que or Rut, or 200 nM diphenyleneiodonium chloride (DPI) for 30 min. Then, cells were stimulated without or with DNP-BSA and ROS-mediated DCF oxidation was measured. Values are means ± SEM (*n* = 10). (B) IgE-sensitized RBL-2H3 cells were treated without or with 50 μM of Isq, Hyp, Que or Rut for 30 min. Then, cells were stimulated without or with DNP-BSA. The cytosolic and membrane fractions were subjected to Western blot analysis for subunits of NOX (p40^{phox}, p47^{phox}, p67^{phox}, Rac, gp91^{phox} and p22^{phox}). A representative blot from three independent experiments is shown. (C) The DPPH radical-scavenging activities of epigallocatechin gallate (EGCG), and Isq, Hyp, Que and Rut were measured at indicated concentrations. Values are means ± SEM (*n* = 12).

2.9. Statistical analysis

All data were analysed, first by one-way ANOVA, and subsequently by Fisher's-multiple range test. The differences were considered significant at *p* < 0.05.

3. Results and discussion

We have recently isolated two major flavonoid glycosides, Isq (15 mg) and Hyp (15 mg), from the peel of Japanese persimmon (520 g; dried peel) (Ohguchi et al., 2010). Isq and Hyp are glycosylated forms of quercetin (Que), quercetin 3-O-glucoside and quercetin 3-O-galactoside, respectively. In the present study, we studied effects of Isq and Hyp on Ag-stimulated degranulation in RBL-2H3 cells. Both Isq and Hyp inhibited Ag-stimulated degranulation in a dose-dependent manner (Fig. 2). However, the inhibitory effects of Isq and Hyp were lower than that of Que, which has been reported to effectively suppress degranulation (Kempuraj et al., 2006; Matsushima et al., 2009). By contrast, rutin (Rut; quercetin 3-O-rutinoside) showed little or no effect on Ag-stimulated

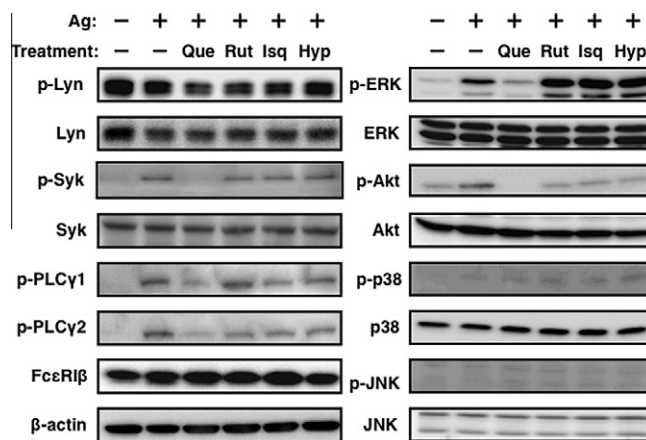


Fig. 5. Effects of Isq and Hyp on Ag-stimulated activation of intracellular signalling pathways in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated without or with 50 μM Isq, Hyp, Que or Rut for 30 min. Then, cells were stimulated without or with DNP-BSA. Cell lysates were subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

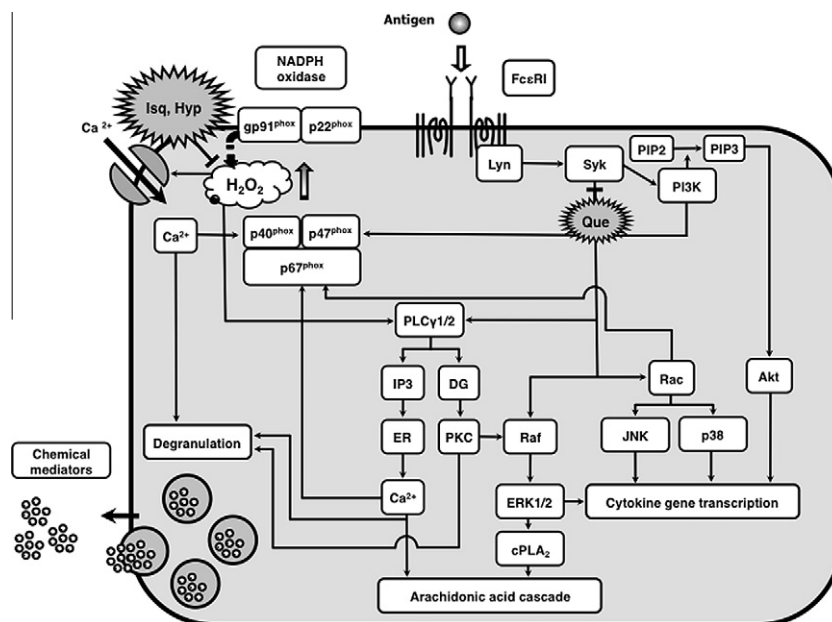


Fig. 6. A scheme showing the inhibitory effects of Isq and Hyp on Ag-stimulated degranulation in RBL-2H3 cells. Isq and Hyp inhibit Ag-stimulated intracellular Ca^{2+} elevation through direct scavenging of ROS that are generated by NOX. Que suppresses Syk activation following Ag-stimulation.

degranulation. These results suggest that there is an inverse correlation between the activity of inhibiting degranulation and the number of glycochains, which might be explained, in part, by differences in the cell permeability of the compounds, depending on their number.

In order to clarify the mechanisms underlying the inhibitory effects of Isq and Hyp on Ag-stimulated degranulation, we measured the intracellular levels of Ca^{2+} and ROS levels following Ag stimulation. Both Isq and Hyp significantly suppressed Ag-stimulated intracellular Ca^{2+} elevation (Fig. 3) and ROS production (Fig. 4A). Que completely abolished Ag-stimulated elevation of intracellular Ca^{2+} and ROS, whereas Rut did not affect them at all. Since previous reports demonstrated that intracellular ROS acts as a Ca^{2+} regulator (Suzuki, Yoshimaru, Inoue, Niide, & Ra, 2005; Yoshimaru et al., 2002), it is likely that Isq and Hyp attenuate Ag-stimulated ROS production and thereby inhibit Ca^{2+} elevation. ROS production, after Ag stimulation, is supposed to be due to activation of NADPH oxidase, because it is abolished by treatment with DPI, a potent inhibitor of the enzyme (Suzuki et al., 2003; Swindle & Metcalfe, 2007). NOX 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 the Rho family, Rac2 (Sarfstien et al., 2004). Upon activation, the cytosolic subunits of NOX are translocated to the membrane, initiating the production of superoxide, which is readily converted to hydrogen peroxide (Bedard & Krause, 2007). However, Isq and Hyp did not inhibit the translocation of all cytosolic subunits (Fig. 4B), suggesting that suppression of ROS production is not mediated by NOX inhibition. Instead, DPPH radical-scavenging assay revealed that Isq and Hyp possessed *in vitro* anti-oxidant activity which is similar to that of Que and EGCG, a potent anti-oxidant and higher than that of Rut (Fig. 4C). As shown in Fig. 6, Ag binding to FcεRI activates multiple signal transduction pathways, which plays a crucial role in degranulation (Costello et al., 1996; Xiao et al., 2005). Treatment with Isq and Hyp showed little or no effects on Ag-stimulated Syk activation (Fig. 5). However, PLCγ1/2, which is a downstream target of Syk, was slightly inactivated by Isq and Hyp treatments. By contrast, Que markedly inhibited Ag-stimulated phosphorylation of Syk and PLCγ1/2 and also their downstream signalling mol-

ecules, ERK and JNK. Suzuki et al. (2003) reported that ROS generated by Ag stimulation regulate PLCγ1/2 activations. Thus, weak suppression of PLCγ1/2 by Isq or Hyp may be ascribed to inhibition of ROS production. On the other hand, Que, Rut, Isq and Hyp did not show the suppression of Lyn and Syk. It is also well known that antigen-stimulated degranulation is involved in activation of T cells (LAT)-independent pathway (Fyn/Gab2/PI3k pathway) (Rivera, Fierro, Olivera, & Suzuki, 2008; Rivera & Olivera, 2006; Rivera & Olivera, 2007). The inactivation of Akt by Rut, Isq and Hyp may be due to the suppression of the Fyn/Gab2/PI3k pathway. Further study is needed to clarify the Akt inactivation mechanisms of Rut, Isq and Hyp treatments.

Taken together, our findings suggest that the inhibitory effect of Isq and Hyp on Ag-stimulated degranulation is mainly due to suppression of intracellular Ca^{2+} elevation, which is caused by direct scavenging of ROS that are generated by NOX.

4. Conclusions

We demonstrated that flavonoid glycosides isolated from *D. kaki* Fuyu, Isq and Hyp, were capable of inhibiting Ag-stimulated degranulation in RBL-2H3 cells, and elucidated the mechanisms underlying the inhibitory effect. These flavonoid glycosides would be beneficial for alleviating symptoms of type I allergy.

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