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Inhibitory effect of phloretin and biochanin A on IgE-mediated allergic responses in rat basophilic leukemia RBL-2H3 cells

Mi Ja Chung [a], Jae Kyung Sohng [b], Doo Jin Choi [c], Yong Il Park [c,*]

[a] Department of Food Science and Nutrition, College of Health, Welfare and Education, Gwangju University, Gwangju 503-703, Republic of Korea
[b] Department of Pharmaceutical Engineering, Institute of Biomolecule Reconstruction, Sun Moon University, Asansi, Chungnam 336-708, Republic of Korea
[c] Department of Biotechnology, The Catholic University of Korea, Bucheon, Gyeonggi-do 420-743, Republic of Korea

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ABSTRACT

Aims: Anti-allergic effects and action mechanism of phloretin (Phl) and biochanin A (BioA) on the IgE–antigen complex-mediated allergic responses in rat basophilic leukemia RBL-2H3 cells were investigated.
Main methods: Cell viability, formation of reactive oxygen species (ROS), DPPH radical-scavenging activity, β-hexosaminidase release, production of interleukin (IL)-4, IL-13, and tumor necrosis factor-alpha (TNF-α) and phosphorylation of Akt and mitogen-activated protein kinase (MAPK) were determined by MTT assay, 2,7-dichlorofluorescein diacetate (DCF-DA) assay, DPPH radical-scavenging assay, reverse transcriptase polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and western blot analysis, respectively.
Key findings: Ph1 and BioA dose-dependently inhibited the formation of ROS and the release of β-hexosaminidase from the RBL-2H3 cells and also showed DPPH radical-scavenging activity. Ph1 and BioA suppressed the antigen-induced phosphorylation of the downstream signaling intermediates, including MAPK and Akt, which are critical for the production of pro-inflammatory cytokines, and also significantly attenuated the production of IgE-mediated pro-inflammatory cytokines, such as IL-4, IL-13, and TNF-α.
Significance: Phloretin and biochanin A attenuate the degranulation and allergic cytokine production through inhibition of intracellular ROS production and the phosphorylation of Akt and the MAPKs, such as ERK1/2, p38, and JNK. The results of this study suggested that these two plant flavonoids may have potent anti-allergic activity in vitro.

Introduction

Allergic reactions are known to be triggered by the binding of antigens to IgE molecules and are bound to a high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. The reactions subsequently lead to the synthesis and secretion of allergy related cytokines, intracellular reactive oxygen species (ROS) production and antigen-stimulated degranulation (Itoh et al., 2011). β-Hexosaminidase is considered a degranulation marker and has been widely used for allergy studies (Chung et al., 2012a,b). The phosphorylation of Akt, a marker of phosphatidylinositol 3-kinase (PI3-kinase) activation, and the downstream signaling molecules of allergic activity play a critical role in mast cell activation, and the pro-inflammatory cytokines and mitogen-activated protein kinase (MAPK) signaling cascades are important in the degranulation of mast cells (Han et al., 2011). Activation of mast cells can increase the phosphorylation of the MAPKs, including extracellular signal-regulated kinase (MEK/ERK), p38 kinase, and c-jun N-terminal kinase (JNK) (Han et al., 2011). The phosphorylation of MAPK has been implicated in the production of interleukin-4 (IL-4), IL-3 and tumor necrosis factor (TNF)-α (Han et al., 2011), and the secretion of large amounts of these cytokines plays a pivotal role in initiating and perpetuating inflammatory responses in allergic reactions (Chung et al., 2012a,b). Each step of these events can be a target for the development of effective therapeutics for the treatment of allergic asthma.

Flavonoids are a group of polyphenolic compounds that occur ubiquitously in foods of plant origin. The six major subclasses of flavonoids are flavones, flavonols, flavanones, flavanols, chalcones, anthocyanins, and isoflavones, based on variation in the heterocyclic C-ring (Ross and Kasum, 2002). Although phloretin (Phl), which is a flavonoid found exclusively in apples (Shao et al., 2008), inhibits the degranulation of mast cells (Grosman, 1988), the mechanism of its anti-allergic action is largely unknown. Biochanin A (BioA) is an isoflavone that has been identified in red clover, cabbage and alfalfa (Cassady et al., 1988). BioA was observed to have inhibitory effects in vivo on mouse lung tumor induced by benzo(a)pyrene (Lee et al., 1991) and showed anti-proliferative and anti-inflammatory activities in vitro through the inhibition of iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NFκB nuclear translocation in LPS-stimulated RAW 264.7 cells (Kole et al., 2011). However, the effects of BioA for anti-allergic activity and its mechanism of action have not been investigated. Naringenin (Nar), which is an aglycone of naringin found in grapefruit and the main metabolite of

* Corresponding author. Tel.: +82 2 2164 4512; fax: +82 2 2164 4846.
E-mail address: yongil382@catholic.ac.kr (Y.I. Park).

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naringin (Jayachitra and Nalini, 2012), has been reported for its anticancer, antimutagenic, anti-inflammatory, anti-atherogenic, and free radical scavenging activities (Jayachitra and Nalini, 2012). Quercetin (Que), a plant-derived flavonoid, has been reported for its inhibitory effects on antigen-stimulated degranulation in RBL-2H3 cells (Itoh et al., 2011), and fisetin (Fis) was reported for its anti-allergic inflammatory actions in human mast cells (Park et al., 2007). In this study, we evaluated a possible anti-allergic activity of Phl and BioA and their mechanism of action in vitro using rat basophilic leukemia RBL-2H3 cells. Que, Fis, and Nar were used as reference compounds in this study.

Materials and methods

Cell culture and cytotoxicity assay

Rat basophilic leukemia RBL-2H3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and were grown in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PEST) at 37 °C in a humidified incubator (5% CO_2, 95% air). The flavonoids used in this study, Nar, Que, Ph1, Fis, and BioA, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nar, Que, Ph1, Fis, and BioA were separately dissolved in dimethyl sulfoxide (DMSO). The dilutions were made in MEM, and the final concentration of DMSO never exceeded 0.05% (vol/vol), which showed no influence on cell growth. The viability of the RBL-2H3 cells was determined by the method reported by Chung et al. (2012a) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co., St. Louis, MO, USA) assay.

DPPH radical scavenging activity

To direct the antioxidant activity, the DPPH (1,1-Diphenyl-2-picrylhydrazyl) (Sigma Chemical Co., St. Louis, MO, USA) radical-scavenging assay was performed as described previously with slight modifications (Choi et al., 2010). Various concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μM) of each flavonoid (Nar, Que, Phl, Fis, and BioA) or α-tocopherol (Sigma, USA) were mixed with 120 μl of DPPH radical solution (1.5×10^{-4} M), and the decrease in absorbance at 517 nm was monitored. The DPPH solution plus methanol was used as a control and α-tocopherol was used as a standard antioxidant and positive control. The inhibition percentage was calculated using the following equation:

$$\text{Scavenging activity } (\%) = \left(1 - A_{samples}/A_{control}\right) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the flavonoids or standard.

Intracellular ROS measurement and β-hexosaminidase release assay

RBL-2H3 cells (2×10^5 cells) in 24-well plates were stimulated with 0.5 μg/ml mouse anti-dinitrophenol (DNP) monoclonal IgE (Sigma Chemical Co., St. Louis, MO, USA) for 24 h. The cells were washed with Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, and 25 mM PIPES) and incubated in a buffer containing 5.6 mM $CaCl_2$ and 0.1% BSA for an additional 10 min. The cells were incubated with 1 ml MEM containing 0, 12.5, 25 and 50 μM of flavonoids (Nar, Que, Phl, Fis, and BioA) for 2 h and washed with Siraganian buffer. The cells were stimulated for 1 h with DNP-conjugated human serum albumin (DNP-HSA) (Sigma Chemical Co., MO, USA) (10 μg/ml), which activates RBL-2H3 cells to trigger allergic reactions, and the cells and the culture supernatants were used for analysis by RT-PCR, western blotting, intracellular ROS measurement, β-hexosaminidase release assay or ELISA. The intracellular ROS was measured by 2,7-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, OR, USA) methods (Kim et al., 2012). After incubation of the cells with 50 μM DCF-DA for 45 min and removal of the DCF-DA medium, the cells were washed twice with 1× PBS (pH 7.4), and the DCF fluorescence was measured using a fluorometric microplate reader (BioTek, Winooski, VT, USA) (excitation wavelength, 485 nm; emission wavelength, 538 nm).

To determine the degranulation of the mast cells, the β-hexosaminidase activity was measured. The supernatant (50 μl) was mixed with an equal volume of the substrate solution (p-nitrophenyl-*N*-acetyl-β-D-glucosaminide 2 mM in 0.1 M sodium citrate buffer, pH 1.5), and the mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 500 μl of stopping buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10). The absorbance at 405 nm was measured with a microplate reader (Molecular Devices, Menlo Park CA, USA). As a control, the release of β-hexosaminidase induced with IgE and DNP-HSA was used (100%). The inhibition of degranulation was calculated as follows:

$$\text{Inhibition } (\%) = [1 - T(405 \text{ nm})/C(405 \text{ nm})] \times 100$$

where C is the absorbance from the reaction mixture of IgE (+) + DNP-HSA (+) + test sample (−), and T is IgE (+) + DNP-HSA (+) + test sample (+).

RNA extraction and RT-PCR analysis of cytokine mRNA production

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. The RNA was reverse-transcribed using a Power cDNA synthesis kit (iNtRON Biotech, Seongnam, Korea) with an oligo $(dT)_{15}$ primer according to the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed with a Maxime PCR PreMix kit (iNtRON Biotech, Seongnam, Korea) in 20 μl of the total reaction mixture containing 1 μl of the RT-reaction mixture and 2 μl of each primer (forward and reverse, 10 pmol/μl). The following primers were used: TNF-α, F, 5′-CGGAAT TCGGCTCCCTCTCATCAGTTC and R, 5′-GCTCTAGACCCTT GAAGAGAAC CTGGG; IL-4, F, 5′-ACCTTGCTGTCACCCTGTTC and R, 5′-TTGTGAGCGTGG ACTCATTC; IL-13, F, 5′-GCTCTCGCTTGCCTTGGTGGTC and R, 5′-CATCCG AGGCCTTTTGGTTAGAG. The GAPDH (F, 5′-ACCACAGTC CATGCCATCAC and R, 5′-TCCACCACCCTGTTGCTGTA) transcripts were used as internal references. The PCR using the TNF-α, IL-4 and IL-13 primers was performed with an initial cycle of 2 min at 94 °C, followed by 35 cycles for 20 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C, and a final extension for 5 min at 72 °C. The PCR using the GAPDH primers was performed using the same settings, with the exception of the annealing temperature (GAPDH, 55 °C).

Enzyme-linked immunosorbent assay (ELISA) and western blot analysis

For the ELISAs and western blot analysis, the protein concentration of the lysate was determined using a Bio-Rad protein kit (Hercules, CA, USA) with bovine serum albumin (BSA, Sigma) as the standard. The concentrations of IL-13 in the cell lysates and IL-4 and TNF-α in the cell culture supernatants were measured using ELISA kits according to the manufacturer's instructions (IL-4 and TNF-α, Abcam, Cambridge, UK; IL-13, RayBiotech, Norcross, GA, USA).

The aliquots of the lysates were separated on a 10% polyacrylamide gel, then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK) using a glycine transfer buffer (1.2 l dH_2O, 0.3 l methanol, 16.875 g glycine and 3.63 g Tris base). After blocking with 5% non-fat dried milk and 0.1% (v/v) Tween 20 in Tris-buffered saline (TBS-T), the membranes were incubated with the diluted primary antibodies for 1 h at room temperature on an orbital shaker, followed by an additional 30 min of incubation with the secondary antibodies in TBS-T. The primary antibodies such as Akt, phospho-Akt (p-Akt), phospho-p44/42 MAPK (p-Erk1/2), p44/42 MAPK (Erk1/2), phospho-p38 MAPK (p-p38), p38 MAPK (p-38), phospho-

SAPK/JNK (Thr183/Tyr185) (p-JNK), SAPK/JNK (JNK), and β-actin (Cell Signaling Technology, Beverly, MA) were used at 1:1000 dilution, and horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG antibodies (Cell Signaling Technology, Beverly, MA) at 1:2000 were used as the secondary antibody. After washing, the immune reactivity was detected with an enhanced chemiluminescence (ECL) system using an ECL Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ). The membrane was exposed to X-ray film. Equal protein loading was confirmed by the β-actin antibody (Cell Signaling Technology, Beverly, MA).

Statistical analysis

The data from three independent experiments were expressed as the mean ± S.D. One-way analysis of variance (ANOVA) followed by the Tukey's test was used to compare the results from different treatments. The data were considered to have statistical significances at $P < 0.05$.

Results

Cytotoxicity of flavonoids

The cytotoxicity of Nar, Que, Phl, Fis, and BioA on the RBL-2H3 cells was evaluated quantitatively by the MTT assay 24 h after the treatment of cells with different concentrations of flavonoids. As shown in Fig. 1, all the flavonoids up to 50 μM had no detectable effect on the cell viability of the RBL-2H3 cells, compared with the control. These concentrations were used for subsequent experiments.

Effects of flavonoids on IgE–antigen-stimulated intracellular ROS production and DPPH radical scavenging activity

Accumulation of intracellular ROS has been reported in RBL-2H3 mast cells upon stimulation with IgE + DNP-HSA (Han et al., 2011). In Fig. 2A, it was shown that Nar, Que, Ph1, Fis, and BioA inhibit the generation of intracellular ROS in the IgE + DNP-HSA-stimulated RBL-2H3 cells. Although somewhat less effective than Que and Fis, both Ph1 and BioA inhibited IgE + antigen-stimulated ROS production in a dose-dependent manner (Fig. 2A). Phl was more potent than BioA in inhibiting intracellular ROS formation. Nar had no effect on the intracellular ROS levels in the IgE + DNP-HSA-stimulated RBL-2H3 cells. The inhibitory percentages of the intracellular ROS production at 50 μM concentration of each flavonoid were in the following order: Fis (55.6%) > Que (39.4%) > Phl (28.2%) > BioA (17.4%).

The DPPH radical scavenging activities of these flavonoids were presented in Fig. 2B. Among the flavonoids tested, Que and Fis were more effective DPPH radical scavengers than Nar, Phl and BioA. While Que and Fis showed DPPH scavenging activities similar to that of the positive control (α-tocopherol) at a concentration of 12.5–200 μM, lower activity was detected for Nar and BioA. Ph1 showed significantly higher antioxidant activity than BioA. The DPPH radical scavenging activity of Ph1 gradually increased in a dose-dependent manner, with the 57% DPPH radical scavenging activity at a concentration 50 μM, compared to the untreated control (0%). The DPPH radical scavenging activities of the samples at 100 μM were shown to be in the following order: α-tocopherol (99.1%) = Que (99.0%) = Fis (98.3%) > Phl (70.1%) > BioA (29.0%) > Nar (24.1%). It was found that there was a positive correlation between the reduced intracellular ROS levels and the DPPH radical scavenging activity ($r = 0.913$; $P < 0.0001$).

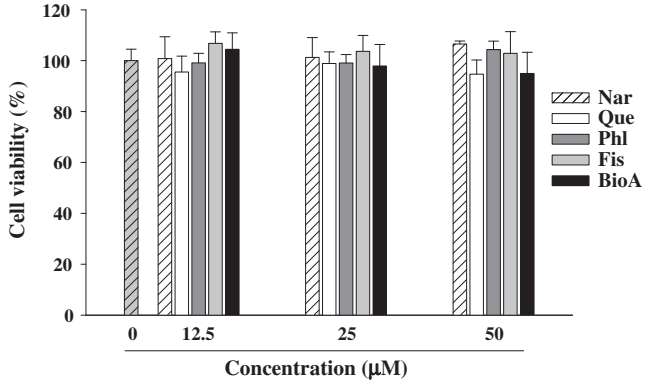

Fig. 1. Cytotoxicity of naringenin (Nar), quercetin (Que), phloretin (Phl), fisetin (Fis), and biochanin A (BioA) on RBL-2H3 cells. The cells were incubated with different concentrations (0–50 μM) of flavonoids for 24 h. The cytotoxicity of the samples was determined by MTT assay. The results are expressed as the mean ± SD (n = 5–8). The cell viability results were not significantly different ($P > 0.05$). The control was untreated cells.

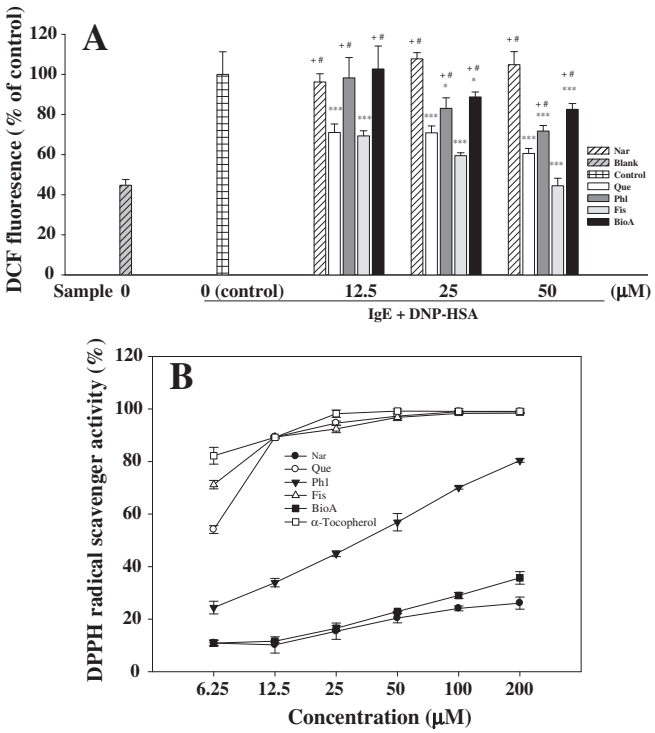

Fig. 2. Effects of Nar, Que, Phl, Fis, and BioA on intracellular ROS production in IgE–antigen complex-stimulated RBL-2H3 cells and DPPH radical scavenger activity. (A) IgE-sensitized RBL-2H3 cells were pretreated with Nar, Que, Ph1, Fis, and BioA, respectively, for 2 h prior to exposure to DNP-HAS (10 μg/ml) for 1 h. The ROS-mediated DCF oxidation was measured for intracellular ROS level detection. Control, IgE + DNP-HSA; blank, without IgE + DNP-HSA and samples. (B) The DPPH radical-scavenging activities of Nar, Que, Ph1, Fis, and BioA were measured at the indicated concentrations. The values are means ± SD (n = 8). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the control (IgE–antigen complex-treated group). $^{+}P < 0.05$ compared to Que. $^{\#}P < 0.05$ compared to Fis.

Effects of flavonoids on degranulation

β-Hexosaminidase has been generally accepted as a marker of degranulation (Itoh et al., 2011). We examined the effects of flavonoids on β-hexosaminidase release in the IgE–antigen complex stimulated RBL-2H3 cells. The release of β-hexosaminidase by DNP-HSA stimulation was inhibited by Que, Phl, Fis, and BioA in a dose-dependent manner (Fig. 3). Ph1 and BioA, at a concentration of 50 μM each, inhibited the β-hexosaminidase release by 66.2% and 28.4%, respectively, compared to the untreated control. The inhibitory effects of Phl and BioA were lower than that of Que (82.2%) and Fis (88.6%), which are known for their inhibitory effects on IgE-mediated allergic responses including degranulation (Itoh et al., 2011; Park et al., 2007). The strength of the inhibitory effects on degranulation was in the following order: Fis > Que > Phl > BioA (Fig. 3). Nar treatment did not inhibit DNP-HSA-induced degranulation.

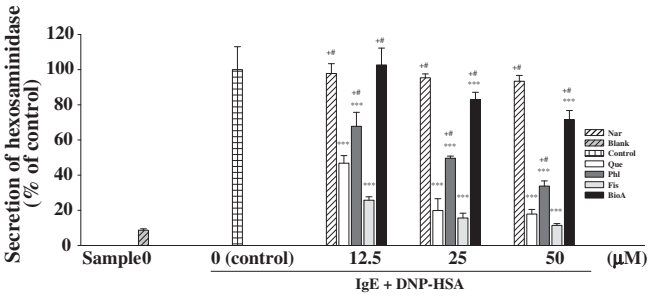

Fig. 3. Effects of Nar, Que, Phl, Fis, and BioA on β-hexosaminidase release in IgE–antigen complex-stimulated RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were pretreated with the test compounds for 2 h, followed by treatment with the antigen (DNP-HSA, 10 μg/ml) for 1 h. The absorbance was measured on a microplate reader at 450 nm. Control, IgE + DNP-HSA; blank, without IgE + DNP-HSA and samples. The values are means ± SD (n = 8). $^{***}P < 0.001$ compared to the control (IgE–antigen complex-treated group). $^{+}P < 0.05$ compared to Que. $^{\#}P < 0.05$ compared to Fis.

According to the results of their inhibitory effects on degranulation, intracellular ROS and DPPH radical scavenging activity, Que, Ph1, Fis, and BioA were selected for subsequent signaling pathway experiments. Nar showed negligible antioxidant and anti-allergy effects.

Flavonoids inhibit antigen-induced phosphorylation of Akt and MAPK pathways in IgE-sensitized mast cells

To determine the effects of Phl, Que, BioA, and Fis on the MAPK signaling pathways, we examined the effects of these four flavonoids on the phosphorylation of Erk 1/2, p38, JNK, and Akt (a marker of PI3-kinase activation) in the antigen-stimulated RBL-2H3 cells.

The IgE–antigen complex induced the phosphorylation of Akt and MAPK pathways including Erk 1/2, p38, and JNK in the RBL-2H3 cells (Fig. 4A, B). Treatment with Phl, Que, BioA, and Fis attenuated the DNP-HSA-induced phosphorylation of the Akt (p-Akt) and MAPK pathways (Fig. 4A, B). The antigen-induced p-Akt was inhibited by Phl (12.5,

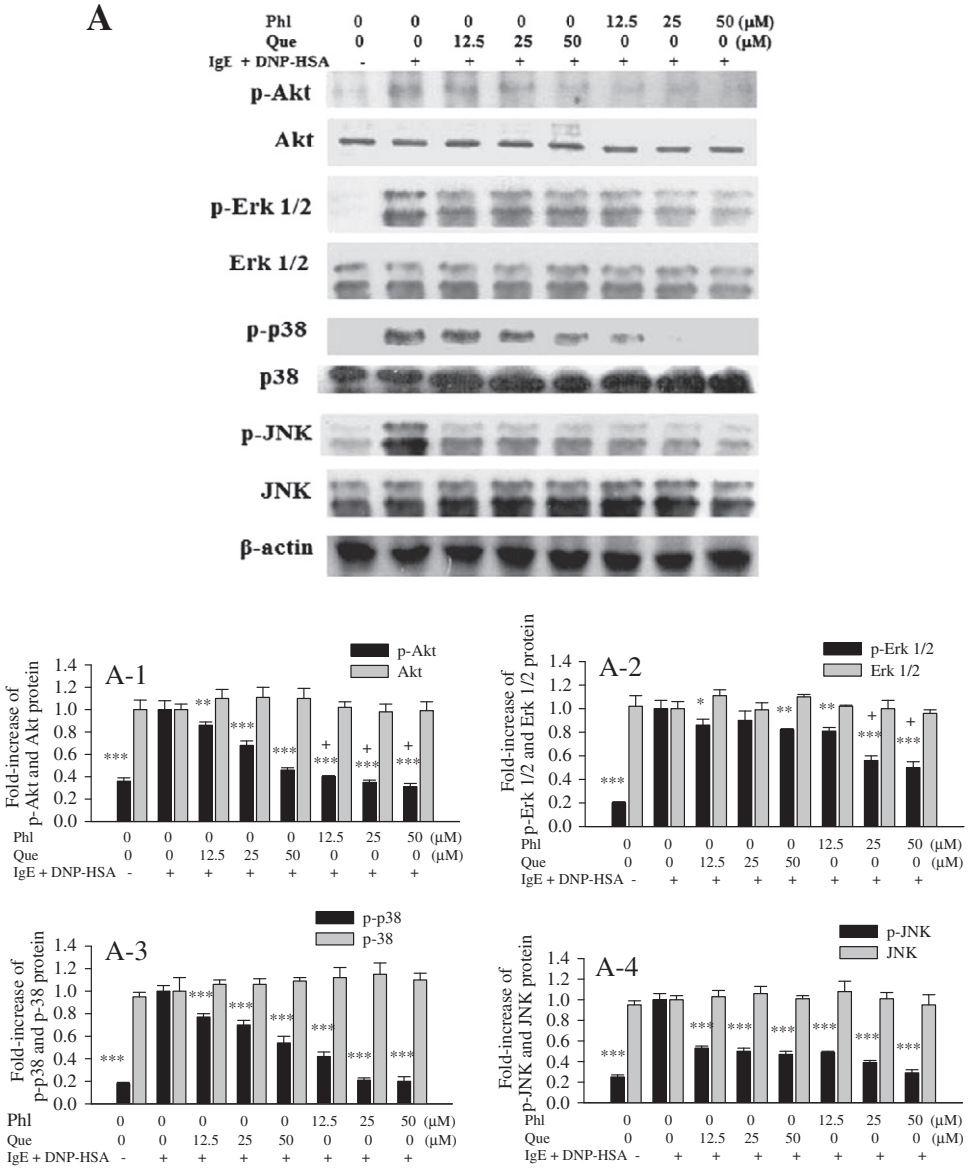

Fig. 4. Effects of Phl, Que, BioA, and Fis on phosphorylation of Akt and MAPKs (Erk 1/2, p38 and JNK) in IgE–antigen complex-stimulated RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were pre-incubated with the test compounds (A, Phl and Que; B, BioA and Fis) for 2 h, followed by treatment with the antigen (DNP-HSA, 10 μg/ml) for 1 h. The protein samples were analyzed using western blots with specific antibodies or phospho-specific antibodies. The Akt, p-Akt, p-Erk 1/2, Erk 1/2, p-p38, p38, p-JNK and JNK levels in each sample were normalized to the β-actin levels. The density of each protein band was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA). The phosphorylation of Akt, Erk 1/2, p38 and JNK was abbreviated by p-Akt, p-Erk 1/2, p-p38, and p-JNK, respectively. A representative blot from three independent experiments is shown. The results are expressed as the mean ± SD (n = 3). $P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control (IgE–antigen complex-treated group). $^{+}P < 0.05$ is compared to Que in the same concentration. $^{\#}P < 0.05$ compared to Fis in same concentration.

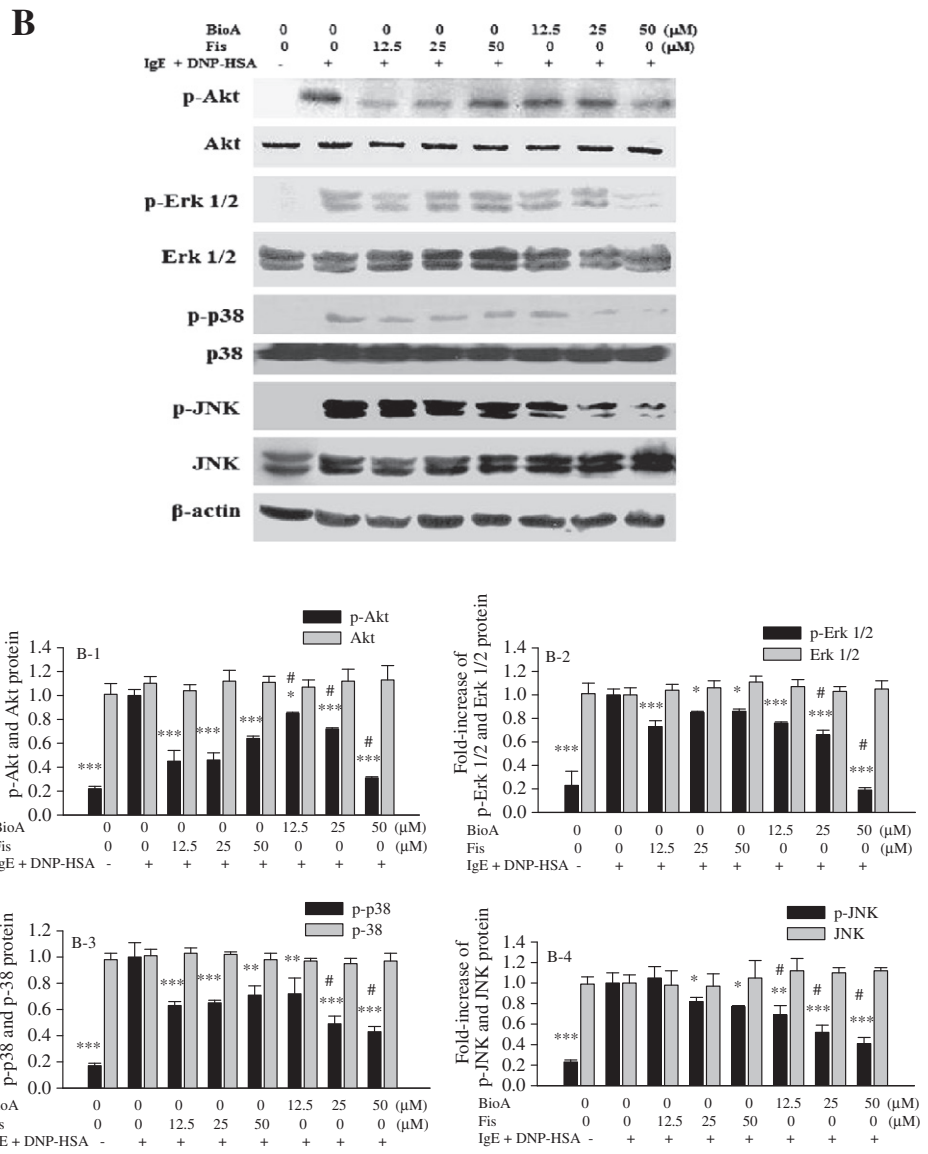

Fig. 4 (*continued*).

25, and 50 μM) in the IgE-sensitized RBL-2H3 cells. In addition, Phl significantly and dose-dependently inhibited the phosphorylation of Erk 1/2, p38 and JNK (p-Erk 1/2, p-p38, and p-JNK). The inhibitory effects on the phosphorylation of the Akt and MAPK pathways by Phl were considerably higher compared to that of Que. Que markedly inhibited the IgE–antigen complex stimulated phosphorylation of the Akt, Erk 1/2, p-38 and JNK protein but not that of the Erk 1/2 protein at 25 μM concentration (Fig. 4A). As shown in Fig. 4B, BioA also effectively inhibited the DNP-HSA-induced phosphorylation of Akt, Erk 1/2, p38, and JNK (p-Akt, p-Erk 1/2, p-p38, and p-JNK) in a dose-dependent manner. Fis attenuated the phosphorylation of Akt, Erk 1/2, p38, and JNK protein. Both Phl and BioA were shown to possess much higher inhibitory effects on the phosphorylation of the Akt and MAPK pathways than those of Que and Fis, which were used as reference compounds. These results suggest that Phl and BioA may have anti-inflammatory effects that can alleviate allergic symptoms.

Effects of flavonoids on cytokine expression in IgE–antigen-stimulated RBL-2H3 cells

Activation of MAPKs and Akt has been known to affect the release of pro-inflammatory cytokines such as IL-4, IL-13 and TNF-α in antigen-stimulated RBL-2H3 cells (Itoh et al., 2011). We examined whether these four flavonoids could modulate the expression of pro-inflammatory cytokines such as TNF-α, IL-4, and IL-13 mRNA induced by DNP-HSA in IgE-sensitized RBL-2H3 cells. As shown in Fig. 5, pretreatment of Que, Phl, Fis, or BioA for 2 h prior to stimulation with DNP-HSA dose-dependently inhibited the DNP-HSA-induced gene expression of TNF-α, IL-4, and IL-13. At a concentration of 50 μM, Que, Phl, Fis and BioA inhibited the IL-4 mRNA expression by 0.21-, 0.35-, 0.41- and 0.31-fold, respectively, which are lower than the control (1-fold) (Fig. 5A). Similarly, the antigen-stimulated IL-13 mRNA expression was also significantly and dose-dependently inhibited by Que, Ph1 and Fis at a concentration of 12.5–50 μM (Fig. 5B). Among the four flavonoids the highest inhibitory activity on IL-13 mRNA expression was observed by Que (reference compound). Phl and Fis exhibited more significant inhibition of the IL-13 mRNA expression than BioA or the control. BioA was less effective than the three other flavonoids. In the cells treated with 50 μM Que, Phl, Fis, or BioA, the IL-13 mRNA levels were determined to be 0.59-, 0.64-, 0.62- and 0.8-fold, respectively, which are lower than that of the control (1-fold). In addition, pretreatment with 25 or 50 μM Que, Ph1, Fis, or BioA, except with 25 μM Ph1, significantly suppressed the TNF-α mRNA expression compared with that of control group (IgE–antigen-stimulated RBL-2H3 cells) (Fig. 5C). Que, Ph1 and

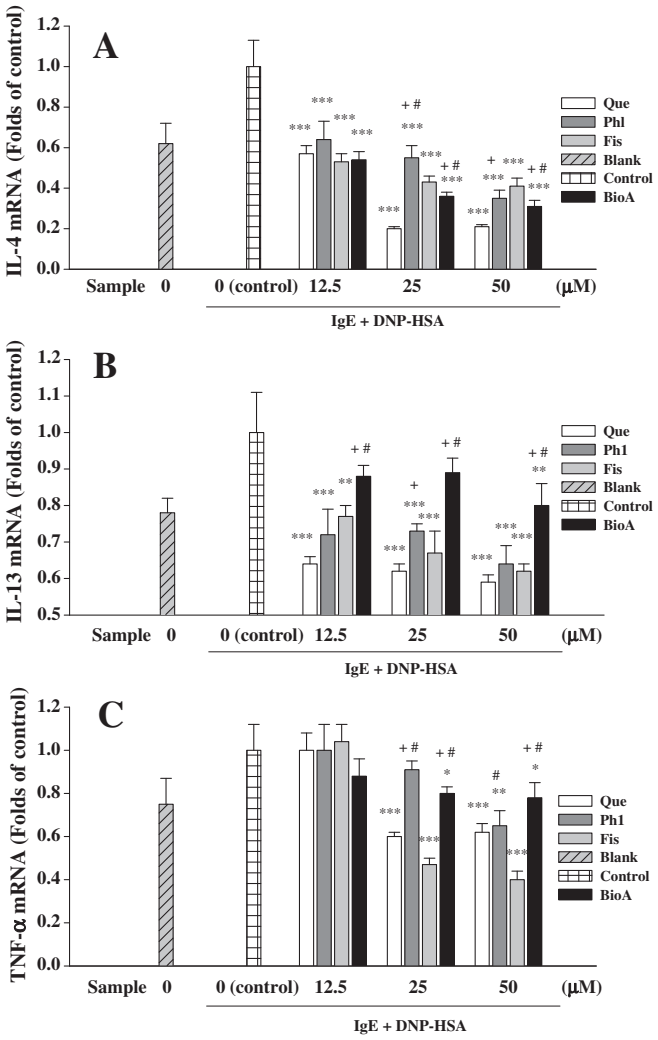

Fig. 5. Effects of Que, Phl, Fis, and BioA on the IL-13, IL-4, and TNF-α mRNA levels IgE–antigen complex-stimulated RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were pre-incubated with the test compounds for 2 h, followed by treatment with the antigen (DNP-HSA, 10 μg/ml) for 1 h. The cytokine expressions (A, IL-4; B, IL-B; C, TNF-α) were measured by RT-PCR. The cytokine levels in each sample were normalized to the GAPDH levels. The density of each mRNA was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA). Results are from three experiments and expressed as the mean ± SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control (IgE–antigen complex-treated group). $^{+}P < 0.05$ compared to Que. $^{\#}P < 0.05$ compared to Fis.

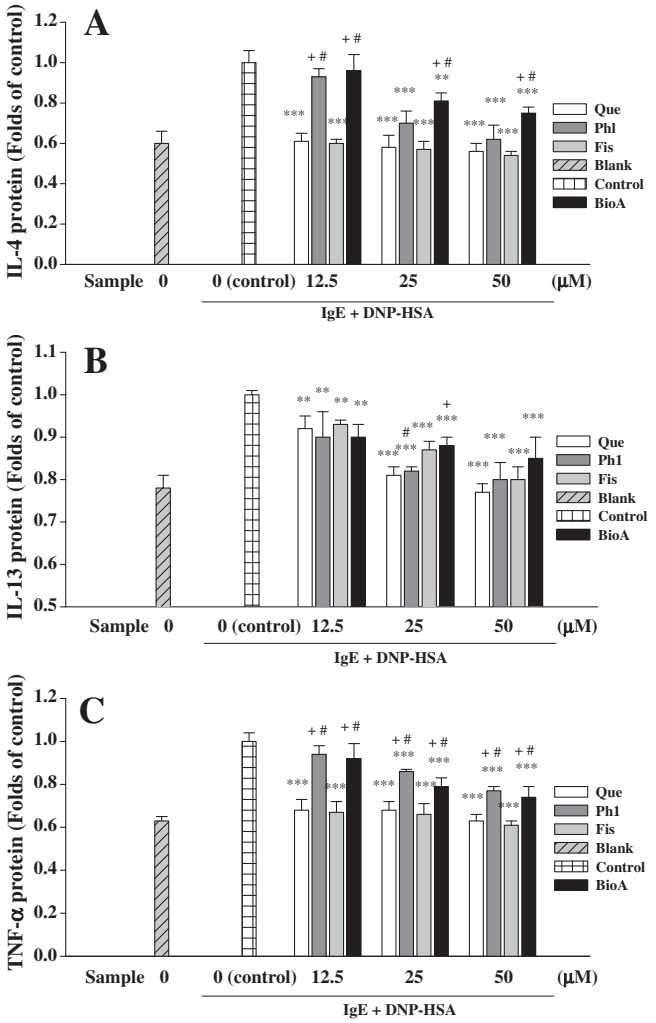

Fig. 6. Effects of Que, Phl, Fis, and BioA on the IL-4, IL-13, and TNF-α protein levels in IgE + DNP-HSA-stimulated RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were pre-incubated with the test compounds for 2 h, followed by treatment with antigen (DNP-HSA, 10 μg/ml) for 1 h. The production of A) IL-4, B) IL-13, and C) TNF-α protein was determined in cultured media (IL-4 and TNF-α) and cell lysates (IL-13) using a commercial ELISA kit. The results are from three experiments and expressed as the mean ± SD (n = 4). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control (IgE–antigen complex-treated group). $^{+}P < 0.05$ compared to Que. $^{\#}P < 0.05$ compared to Fis.

Fis were shown to be better suppressors of IL-13 and TNF-α mRNA expression in the IgE–antigen-stimulated RBL-2H3 cells than BioA.

To confirm the effects of the four flavonoids on the gene expression of the pro-inflammatory cytokines, the culture supernatants and cell lysates were assayed for cytokine levels using ELISA (Fig. 6). Pretreatment with Que and Fis as the reference compounds significantly reduced the IL-4, IL-13, and TNF-α protein levels at all concentrations tested (12.5, 25 and 50 μM) compared with the control group (IgE–antigen-stimulated cells). Pretreatment with Phl and BioA also inhibited IL-4, IL-13 and TNF-α protein production in a concentration-dependent manner, although the effect on IL-4 and TNF-α protein levels was not significant at a lower concentration (12.5 μM). Que and Fis were shown to be better suppressors of IL-4 and TNF-α levels than Ph1 and BioA. IL-4, IL-13, and TNF-α protein levels at a concentration of 50 μM were 0.62-, 0.80-, and 0.77-fold, respectively, which were lower than that of the control (1.0-fold). A similar effect was observed with mRNA expression and protein production of IL-4, IL-13 and TNF-α. Taken collectively, these results showed that, although less effective in anti-inflammatory action than Que and Fis, both Ph1 and BioA are markedly effective in reducing allergic responses through anti-inflammatory action.

Discussion

Mast cells secrete chemical mediators such as histamine, β-hexosaminidase, ROS, Ca^{2+}, MAPKs, Atk, and cytokines during specific immune responses (Barnes, 2008; Kips, 2001). RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells, display characteristics of mucosal-type mast cells and express several hundred thousand FcεRI on the membrane surface. After stimulation with antigen, RBL-2H3 cells release β-hexosaminidase, a marker of mast cell degranulation through the antigen-induced aggregation of FcεRI and cytokines. Kim et al. (2009), Itoh et al. (2011) and Murata et al. (2013) reported inhibitory effects of flavonoids on antigen-stimulated granulation and the mechanism of anti-allergic action of flavonoids in rat basophilic leukemia RBL-2H3 cells. The RBL-2H3 cells are widely used to study anti-allergic activity of flavonoids and their mechanism of action in vitro. They are therefore considered a good model for studying the anti-allergic effects of the compounds tested in this study in the responses

of mast cells to multivalent allergens (Chung et al., 2012a,b; Han et al., 2011; Marchand et al., 2003).

In this study, Que and Fis were used as reference compounds due to their anti-allergic effects and/or antioxidant activities (Itoh et al., 2011; Park et al., 2007). ROS are involved in allergic inflammation (Springer et al., 2007). We found that Phl and BioA significantly suppressed intracellular ROS production in IgE–antigen complex stimulated RBL-2H3 cells. Previous reports demonstrated that intracellular ROS act as a Ca^{2+} regulator and the IgE–antigen complex induces mast cell degranulation by increasing the intracellular Ca^{2+} (Itoh et al., 2011). Allergic disease due to degranulation is closely associated with oxidative stresses caused by an allergic reaction. We hypothesized that Phl and BioA may influence the antigen-stimulated degranulation by their inhibitory effects on intracellular ROS. To test this hypothesis, we measured β-hexosaminidase activity, a marker of mast cell degranulation, in IgE–antigen complex stimulated RBL-2H3 cells (Giudice et al., 2007). The binding of the IgE–antigen (DNP-HSA) to FcεRI on the RBL-2H3 cell surface increased the degranulation, and treatment with Phl or BioA markedly inhibited the antigen-stimulated degranulation in this study. Suzuki et al. (2003) showed that inhibition of antigen-stimulated degranulation by diphenyleneiodonium, a broad-spectrum inhibitor of flavoprotein-containing oxidoreductases, is primarily due to the suppression of intracellular Ca^{2+} by the inhibition of intracellular ROS production. Therefore, the inhibition of intracellular ROS production by Phl and BioA may be a major influence in the inhibition of antigen-stimulated degranulation in this study. Although the inhibitory effects were lower than those of the reference compounds such as Que and Fis, the observed capacity of Phl and BioA on intracellular ROS and degranulation inhibition in the IgE–antigen complex stimulated RBL-2H3 cells suggests that these two flavonoids may be effective agents in the treatment of allergic symptoms.

The results of this study also showed that Ph1 and BioA possess DPPH radical scavenging activities, although to a lesser extent than that of Fis and Que, which have been reported to effectively suppress intracellular ROS and the DPPH radical (Itoh et al., 2011). The DPPH radical scavenger activity of Phl and BioA was positively correlated with the inhibitory effects of intracellular ROS formation ($r = 0.913$; $p < 0.0001$). In Que-treated RBL-2H3 cells, the inhibition of antigen-stimulated degranulation was primarily due to the suppression of intracellular Ca^{2+} elevation, which is caused by the inhibition of intracellular ROS by DPPH radical scavenger activity (Itoh et al., 2011). Therefore, the DPPH radical scavenger activity of Phl and BioA may provide an additional benefit in the inhibition of degranulation in mast cells. Although Nar was reported for its free radical scavenging activities (Kumar et al., 2003), it showed little or no effect on the IgE–antigen complex stimulated degranulation, intracellular ROS and DPPH radical scavenging activity in this study. Leung et al. (1984) showed that Phl was able to inhibit ConA-induced histamine release from peritoneal mast cells of the rat, mouse and hamster. This study is a very basic research in that the results of this study only showed inhibitory effect of Phl on degranulation. However, our result describes the anti-allergic effects and action mechanism of Phl on the IgE–antigen complex-mediated allergic responses in rat basophilic leukemia RBL-2H3 cells. The allergic cell model in Leung's study was also different from our allergic cell model. Nar inhibits allergen-induced airway inflammation and airway responsiveness and inhibits NF-*k*B activity in a murine model of asthma (Shi et al., 2009). Nar has anti-degranulating activities and its target molecules in the degranulating signaling were Lyn, Syk and Akt. Nar suppressed the phosphorylation of Syk and Akt (Murata et al., 2013). In our study, Nar showed only modest effects at low concentration but, at higher concentration (100 μg/ml), it showed significant anti-degranulating activities (data not shown). However, the key compounds in our study were Phl and BioA. Since Nar (including Que and Fis) was used only as the reference compound, we did not further test more detailed anti-allergic action mechanism of Nar.

The activation of the downstream signaling molecules, PI3-kinase and Akt, is important in the antigen-stimulated degranulation because antigen-stimulated degranulation is involved in activation of the PI3-kinase pathway (Balatt et al., 2012; Huang et al., 2008). The phosphorylation of Akt was used as a marker of PI3-kinase activation (Balatt et al., 2012; Huang et al., 2008). In our study, Phl was identified as an inhibitor of Akt, and its inhibitory effect was higher than that of Que (a reference compound). In contrast, the inhibitory effect of BioA (12.5 and 25 μM) on the phosphorylation of Akt was lower than that of Fis (a reference compound). The phosphorylation of Akt and degranulation by these flavonoids showed a similar pattern with regard to the inhibitory effects. The inhibition of Akt phosphorylation by Phl and BioA may result in the inhibition of the antigen-stimulated degranulation pathway.

The MAPK cascade is one of the important signaling pathways of immune response because the MAPKs play important roles in regulating the transcription activity of various pro-inflammatory cytokine genes such as IL-4, L-13 and TNF-α in mast cells (Vo et al., 2011). MAPKs including ERK 1/2, JNK 1/2, and p38 have been used as good targets for the pharmacological treatment of allergic inflammation (Huang et al., 2008; Vo et al., 2011). Akt, ERK1/2, JNK1/2, and p38 are the downstream signaling intermediates in the antigen-stimulated activation of the intracellular signaling pathways in the RBL-2H3 cells (Balatt et al., 2012; Huang et al., 2008). In this study, it was shown that the antigen-induced phosphorylation of ERK 1/2, JNK 1/2, and p38 was inhibited by Phl and BioA. Phl inhibited the phosphorylation of ERK 1/2, JNK 1/2, and p38 more potently than did the reference compounds (Que and Fis). Treating cells with BioA showed lower p-ERK 1/2, p-JNK 1/2, and p-p38 protein levels than Fis. That the inhibitory effects on the antigen-induced phosphorylation of the MAPK pathway by Fis and BioA were higher than those by Que and Fis (the reference compounds) may suggest their strong anti-allergic effects.

Subsequently, we investigated whether inhibition of the phosphorylation of the major MAPKs influences the production of pro-inflammatory cytokines in the IgE–antigen complex stimulated RBL-2H3 cells. IL-4, IL-13, and TNF-α are important cytokines in allergic reactions (Kips, 2001; Springer et al., 2007). It was shown that Phl and BioA significantly inhibited IL-4, IL-13, and TNF-α mRNA expression and protein production, although their effect was somewhat lower compared to that by Que and Fis. Production of IL-13 protein in response to 50 μM of Phl was similar to that produced by 50 μM of Fis. The inhibitory effects of BioA on IL-4, IL-13 and TNF-α production were lower than those of Phl, suggesting that Phl may be more effective in anti-allergic activity than BioA. Phl was shown to be approximately equal to Que and Fis in anti-allergic activity effectiveness. Taken together, these results suggest that the anti-allergy effects of Phl and BioA may be mediated by their anti-inflammatory action through the inhibition of IL-4, IL-13 and TNF-α production. Interestingly, however, the results showed that, although Ph1 and BioA were better than the reference compounds (Que and Fis) in the inhibition of Akt and MAPK phosphorylation, they were shown to be less effective suppressors in IL-4, IL-13 and TNF-α production than Que and Fis. Activation of the MAPKs and Akt has been known to affect the release of these pro-inflammatory cytokines in antigen-stimulated RBL-2H3 cells (Itoh et al., 2011). Therefore, our results may suggest that, although these four flavonoids, Ph1, BioA, Que, and Fis, behave in a similar way in activating mast cells, Ph1 and BioA, or, Que and Fis, may adopt additional signaling pathways as well as Akt and MAPK signaling cascades. We observed weaker inhibitory effects of Phl and BioA on allergic cytokine release, ROS production and degranulation compared to Que and Fis. However, their effects on Akt and MAPK activation were stronger than Que and Fis. Thus, these four flavonoids, Ph1, BioA, Que, and Fis, may differently adopt additional signaling pathways. The possible additional signaling pathways on allergic cytokine release and degranulation are intracellular calcium (Ca^{2+})-related pathway and/or PLCγ 1/2 pathways. It is known that an increase in the concentration of free intracellular calcium is a necessary and sufficient stimulus for degranulation in mast cells (Neher, 1988). PLCγ 1/2 is a downstream target of syk and plays a critical role in degranulation and cytokine gene transcription of RBL-2H3 cells. PLCγ 1/2 is

inactivated by Que (Itoh et al., 2011). Therefore, it seems that Que and Fis may be related with PLCγ 1/2 signaling, coupled with an influx of Ca^{2+} ions, and this may play an important role in degranulation and cytokine expression. The weaker inhibitory effects of Phl and BioA than Que and Fis on allergic cytokine release and degranulation, which was observed in our study, may explain that Que and Fis are more strongly, or at least differently, connected to PLCγ 1/2 pathway signaling, coupled with Ca^{2+} influx, compared to Ph1 and BioA, although this speculation needs to be proved in the future. In the possible additional signaling pathways for intracellular ROS production, NOX is an important multi-subunit enzyme complex and it is composed of membrane-bound subunits (gp91phox and p22phox), cytosolic subunits (p40phox, p47phox and p67phox) and a monomeric GTP-binding protein of the Rho family, Rac2 (Sarfstein et al., 2004). The inhibition of NOX-related pathway by Que and Fis may inhibit intracellular ROS and their inhibitory effect on NOX-related pathway may be stronger than Ph1 and BioA. Further studies are needed to address this speculation in the future.

Conclusions

Our results suggest that Phl and BioA have an anti-allergic effect that reduces levels of degranulation and pro-inflammatory cytokines by the inhibition of intracellular ROS production and the phosphorylation of Akt and major MAPKs, such as p38, Erk1/2, and JNK 1/2.

Conflict of interest statement

The authors declare no conflict of interest.

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