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Inhibitory effects of phloroglucinol derivatives isolated from *Ecklonia stolonifera* on FcεRI expression

Sun-Yup Shim^a, Jae-Sue Choi^b, Dae-Seok Byun^{b,*}^a Institute of Marine Life Science, Pukyong National University, Daeyeon 3-dong, Busan, 608-737, Republic of Korea^b Division of Food Science and Biotechnology, Pukyong National University, Daeyeon 3-dong, Nam-Gu, Busan, 608-737, Republic of Korea

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ABSTRACT

Two bioactive phloroglucinol derivatives, dioxinodehydroeckol (DHE) and phlorofucofuroeckol A (PFF-A) were isolated from edible marine brown alga, *Ecklonia stolonifera*, and evaluated for effects on cell surface FcεRI expression in KU812F cells. DHE and PFF-A were found to reduce the cell surface expression, and total cellular protein and mRNA levels for the FcεRI α chain. Moreover, both compounds exerted inhibitory effects against the elevation of intracellular calcium concentration [Ca²⁺]_i, and histamine release from anti-FcεRI α chain antibody (CRA-1)-stimulated cells. These inhibitory effects were stronger for PFF-A than for DHE. These results show that two phloroglucinol derivatives, DHE and PFF-A, may exert anti-allergic effects via the inhibition of FcεRI expression, calcium influx, and degranulation in basophils, and contributes to the pharmacological activities of marine brown alga, including *E. stolonifera*.

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1. Introduction

The high-affinity IgE receptor, FcεRI, which is expressed on the surface of mast cells and basophils, is known to be involved in the regulation of IgE-mediated allergic reactions.^{1,2} The aggregation of FcεRI by multivalent allergen–IgE complexes or by anti-FcεRI antibody initiates a cascade of biochemical events which results ultimately in degranulation, and the secretion of inflammatory mediators such as histamine, prostaglandins, and leukotrienes from activated mast cells and basophils, which results in allergic diseases-including asthma, atopic dermatitis, and allergic rhinitis.^{3–6} Thus, the down-regulation of FcεRI expression may result in an attenuation of the IgE-mediated allergic reaction. The FcεRI molecule is a tetrameric receptor composed of one-α, one-β, and two disulfide-linked γ-chains. Among the three subunits of FcεRI, the γ-chain is expressed in FcεRI-positive cells and mostly extends out into the extracellular region where it binds directly to the Fc portion of the IgE antibody with high affinity.

Ecklonia stolonifera Okamura is a perennial edible marine brown alga which belongs to the Laminariaceae, is distributed extensively throughout countries such as Korea and Japan, and contains

phlorotannins with various pharmaceutical properties including anti-diabetic,⁷ anti-oxidative,^{8–10} anti-cancer,¹¹ anti-HIV,¹² and anti-allergic activities.^{13–16} Studies have reported that phlorotannins inhibited histamine release in response to A23187 or IgE, and reduced binding activity between IgE and FcεRI.^{13,14}

However, the regulation of FcεRI expression by phlorotannins has yet to be clearly characterized. Recently, many studies on anti-allergic activities of bioactive components have been reported, and (–)-epigallocatechin-3-O-gallate (EGCG) of a green tea catechin and kaempferol of *Nelumbo nucifera* stamens, have been demonstrated to have anti-allergic activities through the suppression of FcεRI expression.^{17–19}

In this report, we isolated phlorotannins, DHE and PFF-A from marine brown alga, *E. stolonifera*, and investigated the suppressive effects of DHE and PFF-A on FcεRI expression in human basophilic KU812F cells.

2. Results and discussion

2.1. Structure elucidation of DHE and PFF-A

DHE: Off-white powder, C₁₈H₁₀O₉, ¹H NMR (400 MHz, DMSO-d₆) δ: 9.77 (1-OH), 9.64 (9-OH), 9.60 (6-OH), 9.27 (3-OH), 9.26 (11-OH), 6.10 (1H, s, H-7), 6.04 (1H, d, J = 2.7 Hz, H-2), 6.01 (1H, d, J = 2.7 Hz, H-10), 5.84 (1H, d, J = 2.7 Hz, H-4), 5.82 (1H, d, J = 2.7 Hz, H-12). ¹³C NMR (100 MHz, DMSO-d₆) δ: 153.3 (C-3),

Abbreviations: [Ca²⁺]_i, intracellular calcium concentration; CRA-1, anti-human FcεRI antibody; DHE, dioxinodehydroeckol; FcεRI, the high affinity IgE receptor; PFF-A, phlorofucofuroeckol A.

* Corresponding author. Tel.: +82 51 629 5844; fax: +82 51 629 5842.

E-mail address: dsbyun@pknu.ac.kr (D.-S. Byun).

153.0 (C-11), 146.1 (C-1), 146.0 (C-9), 142.1 (C-4a), 141.7 (C-12a), 140.1 (C-6), 137.2 (C-7a), 131.6 (C-13b), 125.9 (C-5a), 122.7 (C-8a), 122.5 (C-13a), 122.3 (C-14a), 98.8 (C-2, 10), 97.6 (C-7), 93.9 (C-4, 12) (Fig. 1).

PFF-A: Amorphous powder, $C_{30}H_{18}O_{14}$, ^1H NMR (400 MHz, CD_3OD) δ : 6.63 (1H, s, H-7), 6.40 (1H, s, H-11), 6.26 (1H, s, H-2), 5.97 (2H, d, $J = 2.1$ Hz, H-2'', 6''), 5.94 (1H, t, $J = 1.9$ Hz, H-4'), 5.92 (1H, t, $J = 2.0$ Hz, H-4''), 5.88 (2H, d, $J = 2.1$ Hz, H-2', 6'), ^{13}C NMR (100 MHz, CD_3OD) δ : 162.7 (C-1''), 162.6 (C-1'), 161.0 (C-3', 5'), 161.0 (C-3'', 5''), 154.0 (C-7a), 152.5 (C-10), 152.0 (C-8a), 149.1 (C-3), 149.0 (C-12), 146.7 (C-6), 144.7 (C-1), 139.2 (C-4a), 136.2 (C-12c), 128.9 (C-5a), 125.9 (C-13a), 125.6 (C-4), 123.2 (C-9), 106.2 (C-12a), 106.1 (C-12b), 100.8 (C-11), 100.2 (C-2), 98.6 (C-4'), 98.5 (C-4''), 97.0 (C-7), 96.2 (C-2'', 6''), 96.2 (C-2', 6') (Fig. 1).

2.2. Cytotoxic effects of KU812F cells

To assess the cytotoxic effects, the viabilities of cells cultured in serum-free medium with various concentrations of DHE and PFF-A are provided in Figure 2. These compounds were evidenced no cytotoxic effects in a concentration range of 1–50 μM , and no reduction of cell viability was observed even at higher concentrations (data not shown).

2.3. DHE and PFF-A inhibit the cell surface Fc ϵ RI expression

To assess the inhibitory effects on cell surface Fc ϵ RI expression, KU812F cells were treated with different concentrations of these

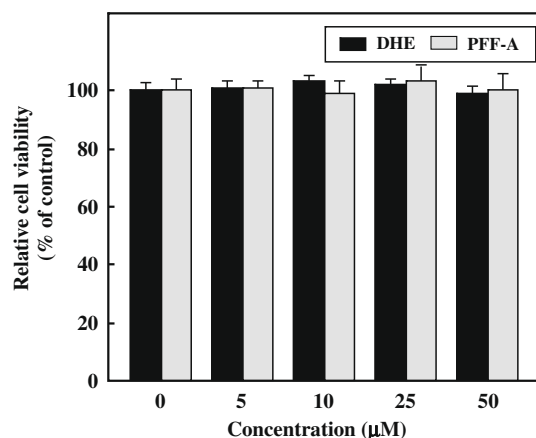


Figure 2. Effects of DHE and PFF-A on cell viability. KU812F cells were cultured in the presence of DHE and PFF-A (0, 5, 10, 25, and 50 μM) for 24 h under serum-free conditions. The cell viabilities were determined via MTS assay. Each determination was made in triplicate and the data are expressed as means $\pm$ S.D.

compounds for 24 h, and the cell surface Fc ϵ RI was evaluated via indirect immunofluorescence and flow cytometry using CRA-1, and found to have been reduced by these compounds in a dose-dependent manner.

The Fc ϵ RI expression on the cell surface of DHE-treated cells was observed to be reduced from 28.1% to 25.2%, 23.1%, 19.5% and 16.9% by the treatment of DHE at a level of 5, 10, 25, and 50 μM , respectively (Fig. 3). And Fc ϵ RI expression in PFF-A-treated cells was observed to be reduced to 27.6%, 23.8%, 22.1%, 17.2% and 15.4% by the treatment of PFF-A at a level of 5, 10, 25, and 50 μM , respectively (Fig. 3).

2.4. Effects on total cellular Fc ϵ RI α chain protein expression

To determine whether the suppression of cell surface Fc ϵ RI α chain expression by the compounds was attributable to the reduction in the levels of total cellular Fc ϵ RI α chain protein, we applied Western blot analysis. As shown in Figure 4, we determined that treatment with these compounds also reduced total cellular Fc ϵ RI α chain protein expression in a dose-dependent manner. DHE and PFF-A induced decrease in the level of the cellular Fc ϵ RI α chain may be associated with the suppression of the cell surface Fc ϵ RI expression.

2.5. Effects on Fc ϵ RI α chain mRNA level

To further study the inhibitory effects of these compounds on Fc ϵ RI α chain gene expression, the mRNA levels were measured via RT-PCR. The mRNA of the Fc ϵ RI α chain of non-treated cells was clearly detected; however, the corresponding mRNA of these compound-treated cells was shown to be reduced in a dose-dependent manner (Fig. 5). These results show that the DHE and PFF-A-induced reduction in total cellular protein and mRNA levels for the Fc ϵ RI α chain may be associated with the suppression of cell surface expression.

Fc ϵ RI α -chain is expressed in Fc ϵ RI-positive cells and is crucial for the proper functioning of the cell surface receptor for IgE.⁶ Gene expression of the Fc ϵ RI α chain, as determined by the mRNA level, was found to be downregulated by these compounds. The gene expression of the Fc ϵ RI α -chain is known to be regulated by at least two transcription factors, GATA-1 and Elf-1.^{20–23} Further studies into the regulation of the signal transduction of the gene-encoding Fc ϵ RI α -chain will be necessary for a

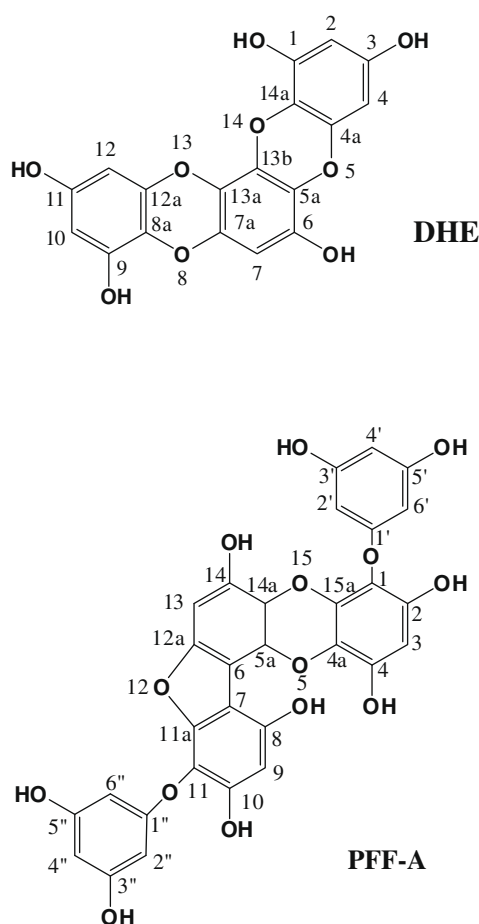


Figure 1. Chemical structures of DHE and PFF-A isolated from *Ecklonia stolonifera*.

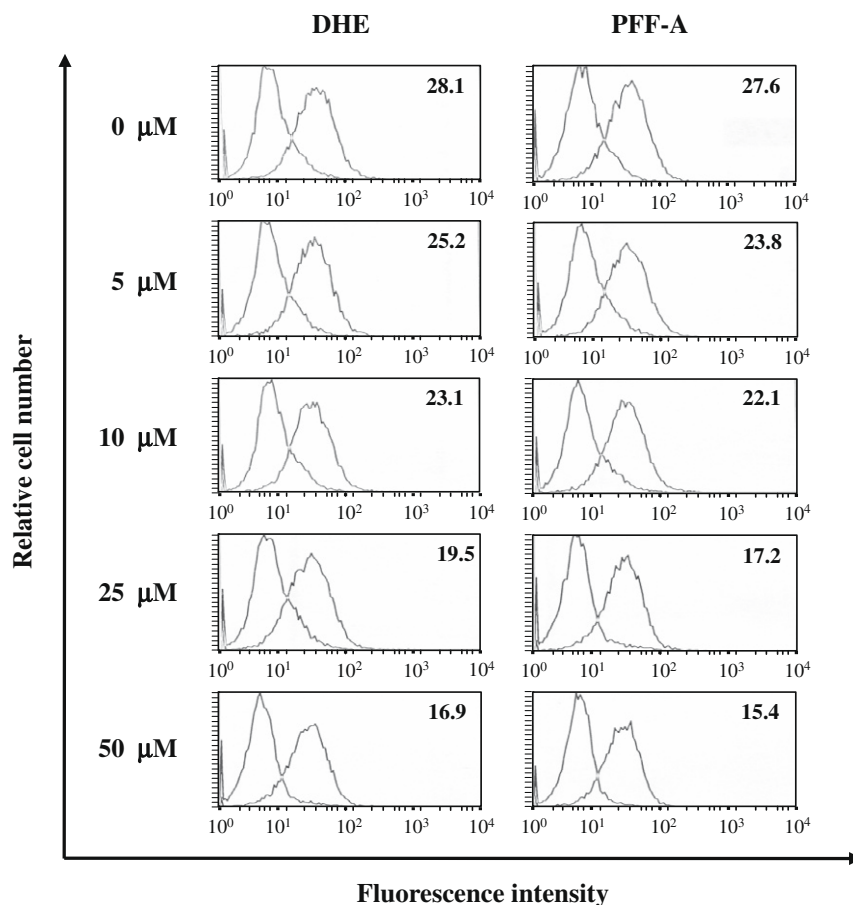


Figure 3. Effects on the cell surface FcεRI expression. The pretreated cells were then incubated with CRA-1 followed by staining with the FITC-conjugated F(ab')₂ goat anti-mouse immunoglobulins. The stained cells were then analyzed via flow cytometry. The number in the figures indicates the percentage of CRA-1-positive cells.

clearer understanding of the suppressive mechanism of FcεRI expression by DHE and PFF-A compounds.

2.6. Effects on FcεRI-mediated [Ca²⁺]_i elevation

The FcεRI-cross linking leads to the phosphorylation of several protein tyrosine kinases, and activates downstream signaling cascades including calcium influx that are required for the degranulation of mast cells and basophils.^{24,25} In order to determine the effects of these compounds on calcium influx, KU812F cells were labeled with a calcium-specific fluorescence probe, Fura 2-AM, and stimulated with CRA-1. Both compounds inhibited the elevation of [Ca²⁺]_i in CRA-1-stimulated cells in a dose-dependent manner, and PFF-A treatment caused stronger inhibition than DHE treatment (Fig. 6).

2.7. DHE and PFF-A inhibit FcεRI-mediated degranulation

The aggregation of FcεRI with anti-FcεRI antibody or multivalent allergen and IgE complexes initiates a cascade of biochemical events that result in degranulation which induces the secretion of inflammatory mediators and contributes to allergic responses.^{3–6} Histamine is a inflammatory mediator stored in the secretory granules, which is released in immunologically activated mast cells and basophils. Thus, the histamine in the medium is utilized as a marker of the degranulation of mast cells and basophils.²⁶

In order to assess the inhibitory effects on degranulation, KU812F cells were treated with DHE and PFF-A, and stimulated

with CRA-1, and the amount of histamine released from the cells was determined spectrofluorometrically using OPA. The treatment of these compounds inhibited the histamine released from CRA-1-stimulated KU812F cells in a dose-dependent manner, and PFF-A treatment evidenced stronger inhibitory effects than were seen with DHE treatment (Fig. 7).

The FcεRI-mediated degranulation of mast cells and basophils is closely related to FcεRI activation. Both the DHE and PFF-A-induced modulation of FcεRI expression may be of particular importance in IgE-mediated allergic reactions, because such effects elucidated in the present study can theoretically influence all of FcεRI-mediated down-stream signaling events after FcεRI activation. Here we demonstrated that down-regulation of FcεRI expression by DHE and PFF-A leads to inhibition of FcεRI-stimulated calcium influx and histamine release, and these inhibitory effects may be exerted by suppression of downstream signaling events occurring after FcεRI activation.

3. Conclusion

In conclusion, our present study show that DHE and PFF-A, phenolic compounds isolated from *E. stolonifera*, may exert anti-allergic effects via the down-regulation of FcεRI expression and a subsequent reduction in FcεRI-mediated calcium influx and degranulation of basophils. These results suggest that DHE and PFF-A contribute to attenuation of allergic reactions and might be a promising candidate for the design of novel inhibitor of FcεRI-mediated allergic reaction.

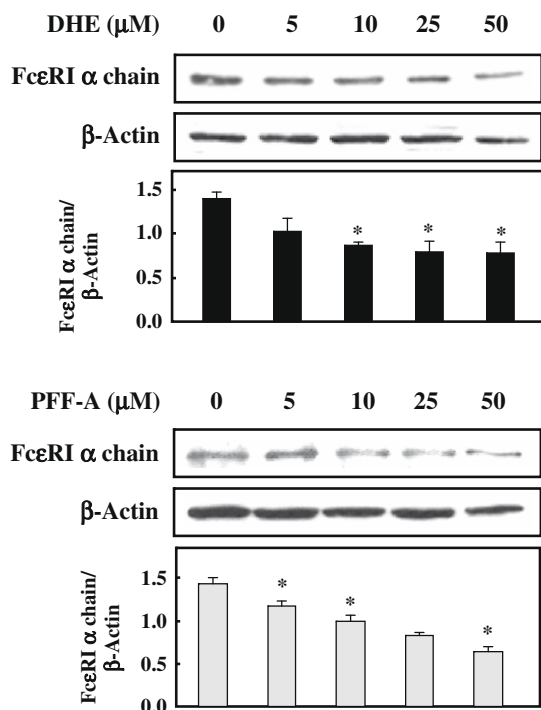


Figure 4. Effects on the cellular protein expression for the FcεRI α chain. KU812F cells were cultured in the presence of DHE or PFF-A (0, 5, 10, 25, and 50 μM) for 24 h under serum-free conditions. Whole cell lysates were isolated from the cells, and were analyzed via Western blot analysis using CRA-1 and β-actin antibodies, and the amount of protein in each band was quantified via densitometry. The upper panel shows a representative Western blot, and the lower panel presents the relative levels of the FcεRI α chain corrected to β-actin. Each value is expressed as the mean ± S.D. of two different experiments. *Values are significantly different from control (**p* < 0.05).

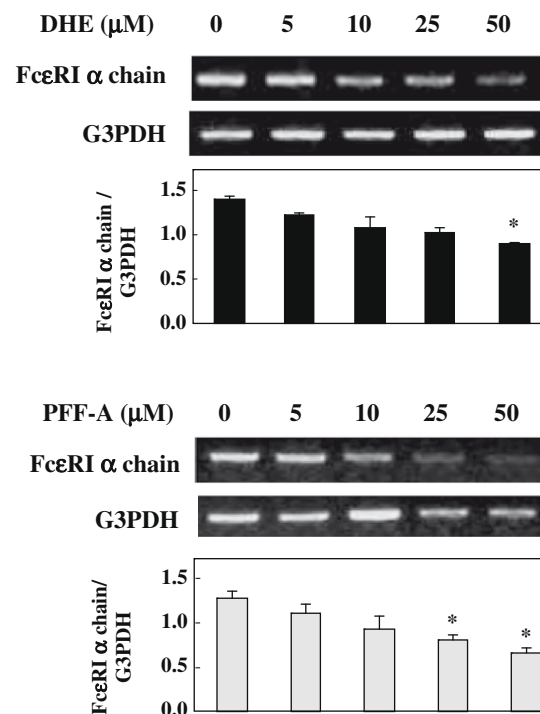


Figure 5. Effects on the mRNA level for the FcεRI α chain. Total cellular RNA was extracted from the pretreated cells, and the FcεRI α chain mRNA level was quantified by RT-PCR. The mRNA expression in each band was quantified via densitometry. The upper panel shows a representative RT-PCR, and the lower panel presents the relative levels of the amount of total cellular protein and gene expression for the FcεRI α chain. Each value is expressed as the mean ± S.D. of two different experiments. *Values are significantly different from control (**p* < 0.05).

4. Materials and methods

4.1. Materials

The leafy thalli samples of *E. stolonifera* were collected from Gijang-gun, Korea, and the collected samples were authenticated by Professor H. G. Kim (Kangnung National University, Korea).

4.2. Isolation of DHE and PFF-A

The lyophilized powder (3 kg) of *E. stolonifera* was percolated in hot EtOH (3 × 9 L) for 3 days, and the resultant extract (700 g) was then partitioned using organic solvents to yield *n*-hexane (27.9 g), CH₂Cl₂ (25.6 g), EtOAc (25.0 g), and *n*-BuOH (99.6 g) fractions. The EtOAc fraction (25.0 g) was subjected to column chromatography over a silica gel with EtOAc–MeOH (50:1 to 5:1) under gradient conditions (50:1–5:1), which yielded 10 fractions (F1–F10). The first fraction, F1 (3.44 g) was then subjected to column chromatography over the same matrix using a solvent consisting of *n*-hexane and EtOAc, which yielded 11 fractions (F1-1–F1-11). DHE (60 mg) was yielded by RP-18 column chromatography (20% MeOH–100% MeOH, gradient) of F1-5 (1.01 g). PFF-A (57 mg) was isolated RP-18 column chromatography (20% MeOH–100% MeOH, gradient) followed by purification with Sephadex LH-20 (MeOH).

4.3. Cell culture

KU812F cells were maintained in an RPMI-1640 medium supplemented with 10% heat-inactivated FBS, antibiotics and antimy-

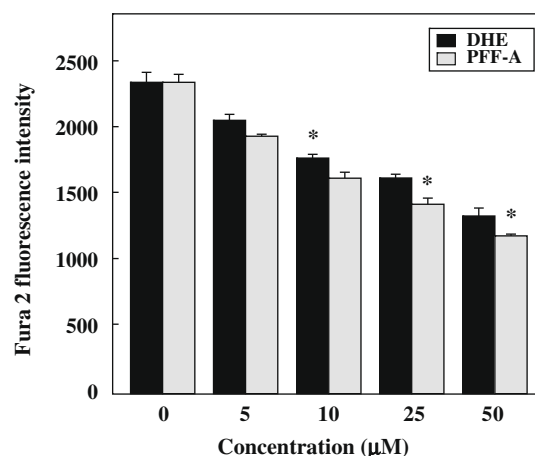


Figure 6. Effects on FcεRI-mediated [Ca²⁺]_i elevation. KU812F cells were cultured in the presence of DHE or PFF-A for 24 h and the cells were incubated with Fura 2-AM and stimulated for 30 min with CRA-1. [Ca²⁺]_i was determined spectrofluorometrically. Each value is expressed as the mean ± S.D. of three different experiments. *Values are significantly different from control (**p* < 0.05).

cotics, and cultured at 37 °C in a humidified atmosphere with 5% CO₂, and passaged every 3–4 days.

4.4. Cell viability

Cell viability was assayed via Celltiter 96[®] AQueous One Solution Cell Proliferation Assay in accordance with the manufacturer's instructions. KU812F cells were seeded at a density of 2.5 × 10⁴

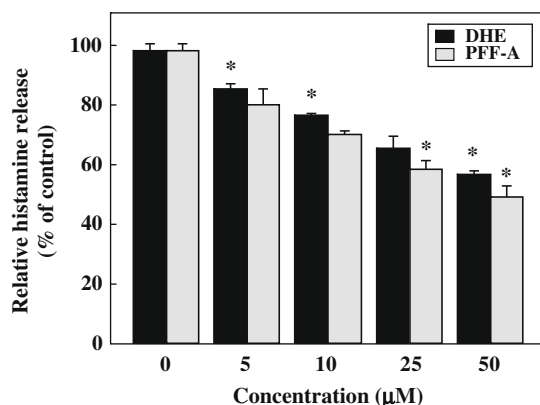


Figure 7. Effects on FcεRI-mediated histamine release. The pretreated-cells with DHE or PFF-A were stimulated for 30 min with CRA-1. Histamine content was determined via a spectrofluorometric method. Each value represents the mean $\pm$ S.D of three different experiments. *Values are significantly different from control ($p < 0.05$).

cells/well and incubated with serum-free media in the presence of DHE and PFF-A. After 24 h of treatment, reagents were added and incubated for 4 h. The colored formazan products were then measured at 490 nm and relative cell viability was calculated and compared with absorbance of untreated cells.

4.5. Flow cytometric analysis

FcεRI expression on the cell surface was measured via flow cytometry. In brief, KU812F cells were incubated with 10 μg/ml of CRA-1 on ice for 60 min. The cells were then stained with anti-mouse IgG FITC antibody (20 μg/ml) on ice for 60 min, followed by washing with ice-cold PBS for flow cytometry. As a negative control, a mouse IgG antibody (10 μg/ml) was used instead of CRA-1. The number in the figures indicates the percentage of CRA-1-positive cells.

4.6. Western blot analysis

For the western blot analysis of total cellular FcεRI α chain expression, the cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Sodium Dodecyl Sulfate, 1% Nonidet P-40, 1 mM EDTA, protease inhibitor cocktail, and 2 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min, and used for immunoblot analysis. Protein samples were subjected to 10% SDS-PAGE, transferred to the nitrocellulose membrane, and blocked with plain buffer (20 mM Tris (pH 7.6), and 136 mM NaCl) containing 10% skim milk. The membrane was incubated with CRA-1 (1:500 dilution) and anti-mouse immunoglobulin HRP antibody (1:2000 dilution). The immunoreactive proteins were visualized using the enhanced chemiluminescent ECL assay kit according to the manufacturer's instructions.

4.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

FcεRI α chain mRNA level was determined by RT-PCR. Total cellular RNA was extracted from the treated cells using TRIzol reagent in accordance with the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using a oligo (dT)₁₅ primer and MMLV reverse transcriptase. The resultant cDNA samples were subjected to PCR amplification in the presence of specific sense and antisense primers. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as internal control, and the primer sequences are as follows: for the FcεRI α -chain,

sense 5'-CTTAGGATGTGGGTTTCAGAAAGT-3' and antisense 5'-GACAG TGGAGAATACAAATGTCA-3'; for the G3PDH, sense 5'-GCTCAGACA CCATGGGGAAGGT-3' and antisense 5'-GTGGTGCAGGAGGCATTGCT GA-3'. The PCR was conducted as follows; 94 °C, 30 s denaturing; 55 °C, 30 s annealing; and 72 °C, 1 min extension, and subjected to 18 cycles for FcεRI α chain and G3PDH. The amplified PCR products were visualized via 1% agarose gel electrophoresis, ethidium bromide staining, then analyzed.

4.8. [Ca²⁺]_i measurement

[Ca²⁺]_i was measured using the calcium reactive fluorescence probe, Fura 2-AM. KU812F cells were suspended in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 12 mM NaHCO₃, 1.8 mM CaCl₂) and incubated with 2.0 μM Fura 2-AM at 37 °C for 30 min. The cells were then washed three times, resuspended in fresh buffer, and stimulated with 10 μg/ml of CRA-1. The Fura 2 fluorescence was monitored at an excitation wavelength of 360 nm and an emission wavelength of 528 nm.

4.9. Histamine assay

Histamine released from the stimulated cells was assessed via a spectrofluorometric assay using OPA.²⁷ The treated cells were stimulated with 10 μg/ml of CRA-1 for 30 min. After centrifugation, 1 N NaOH and 0.2% OPA were added to the supernatant and the mixtures were incubated on ice for 40 min. The reaction was terminated via the addition of 3 N HCl, and the fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

4.10. Statistical analysis

The statistical differences between the control and test groups were determined via Student's *t*-test. Differences were considered significant at $p < 0.05$.

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