



Inhibitory effects of an ellagic acid glucoside, okicamelliaside, on antigen-mediated degranulation in rat basophilic leukemia RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice

Megumi Kuba-Miyara^{a,*}, Kengo Agarie^a, Rina Sakima^b, Shihoko Imamura^b, Kazuyo Tsuha^b, Takeshi Yasumoto^b, Shinichi Gima^c, Goro Matsuzaki^d, Tsuyoshi Ikehara^{b,**}

^a Department of Health Sciences, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan

^b Tropical Technology Center Ltd, 5-1 Suzuki, Uruma, Okinawa 904-2234, Japan

^c Instrumental Research Center, University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan

^d Molecular Microbiology Group, Center of Molecular Biosciences, Tropical Biosphere Research Center, University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan

ARTICLE INFO

Article history:

Received 16 September 2011

Received in revised form 25 January 2012

Accepted 29 January 2012

Available online 11 February 2012

Keywords:

Allergy

Degranulation

Ellagic acid glucoside

Okicamelliaside

Passive cutaneous anaphylaxis

RBL-2H3

ABSTRACT

Degranulation inhibitors in plants are widely used for prevention and treatment of immediate-type allergy. We previously isolated a new ellagic acid glucoside, okicamelliaside (OCS), from *Camellia japonica* leaves for use as a potent degranulation inhibitor. Crude extracts from leaves also suppressed allergic conjunctivitis in rats. In this study, we evaluated the *in vivo* effect of OCS using a pure sample and performed *in vitro* experiments to elucidate the mechanism underlying the extraordinary high potency of OCS and its aglycon. The IC₅₀ values for degranulation of rat basophilic leukemia cells (RBL-2H3) were 14 nM for OCS and 3 μM for aglycon, indicating that the two compounds were approximately 2 to 3 orders of magnitude more potent than the anti-allergic drugs ketotifen fumarate, DSCG, and tranilast (0.17, 3, and >0.3 mM, respectively). Antigen-induced calcium ion (Ca²⁺) elevation was significantly inhibited by OCS and aglycon at all concentrations tested (*p*<0.05). Upstream of the Ca²⁺ elevation in the principle signaling pathway, phosphorylation of Syk (Tyr525/526) and PLCγ-1 (Tyr783 and Ser1248) were inhibited by OCS and aglycon. In DNA microarray-screening test, OCS inhibited expression of proinflammatory cytokines [interleukin (IL)-4 and IL-13], cytokine-producing signaling factors, and prostaglandin-endoperoxidase 2, indicating that OCS broadly inhibits allergic inflammation. During passive cutaneous anaphylaxis in mice, OCS significantly inhibited vascular hyperpermeability by two administration routes: a single intraperitoneal injection at 10 mg/kg and per os at 5 mg/kg for 7 days (*p*<0.05). These results suggest the potential for OCS to alleviate symptoms of immediate-type allergy.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Allergy is a hypersensitivity disorder of the immune system. According to Coombs and Gell, allergic reactions can be classified into four types [1]. Type I allergy, also called immediate-type allergy, is characterised by inappropriate immunoglobulin E (IgE) production by plasma cells and excessive activation of mast cells by IgE. Increases in allergic diseases become social problems in developed countries, including Japan, where about 30% of the population reportedly suffers from allergies. Thus, it is highly desirable to identify appropriate measures to mitigate allergies. Although there have been enormous improvements in the medical practices for allergy treatment, difficulty still lies in the regulation of IgE production. Therefore, suppressive

treatment for mast cell activation is mainly used in therapy. The mast cell-activating mechanism has been recently clarified in detail [2]. Specifically, signal transduction is triggered by the cross-linking of high-affinity receptors for IgE (FcεRI) following IgE and antigen binding. The beginning stage of the signaling factor phosphorylation cascade is composed of two main pathways: the principle pathway (Lyn-Syk-LAT-PLCγ-1), including Src-family kinases, and complementary pathways (Fyn-GAB2-PI3K). After the phosphorylation cascade, calcium ion (Ca²⁺) mobilization, known as the second messenger system, occurs. Finally, conformational changes in the cytoskeleton induce 'degranulation', which is a release of chemical mediators such as histamine, serotonin, and leukotriene, followed by allergic reactions. These signaling events at the molecular level, including phosphorylation and calcium mobilization, have been analysed by Western blotting [3,4] and fluorescence imaging [5–8], respectively. Several drugs and natural products have been analyzed and demonstrated to inhibit degranulation via suppression of signaling pathways, suggesting that they could be valuable compounds for allergy therapy [3–8].

* Corresponding author. Tel.: +81 98 895 3331x2652; fax: +81 98 895 1433.

** Corresponding author. Tel.: +81 98 982 1100; fax: +81 98 982 1101.

E-mail addresses: kubame93@med.u-ryukyu.ac.jp (M. Kuba-Miyara), tikehara@ttc.co.jp (T. Ikehara).

Recently, we isolated an ellagic acid glucoside named okicamelliaside (OCS) from *Camellia japonica* leaves, guided by anti-degranulation assays in a rat basophilic leukemia cell line (RBL-2H3) [9]. For comparison, the aglycon, 3,4-dioxoloellagic acid, was also prepared by hydrolyzing OCS (3,4-dioxoloellagic acid 4'-O- β -D-glucopyranoside) with β -glucosidase (Fig. 1). OCS and aglycon prominently exhibited anti-degranulation activities that were 12,000- and 50-fold higher, respectively, than that of the antihistaminic drug ketotifen fumarate [9]. A crude extract of *C. japonica* leaves containing OCS also suppressed allergic conjunctivitis in rats by oral administration [10]. The revelation of these potent activities encouraged us to continue efforts to accumulate pure material in high enough quantities for further *in vivo* and *in vitro* experiments.

In this study, the anti-allergic activities of OCS were evaluated for the prospect of various uses by *in vivo* experiments. OCS was found to suppress immediate-type allergy in mice. In the *in vitro* experiments, we aimed to shed light on the mechanisms underlying degranulation-inhibitory activity, focusing especially on signaling pathways. OCS and aglycon were found to inhibit degranulation through suppression of signal transduction.

2. Materials and methods

2.1. Materials

OCS was purified from *C. japonica* leaves as previously described [9]. In brief, dried leaves of *C. japonica* were extracted with hot water (80°C) for 30 min. The extract was treated twice in a Diaion HP-20 resin column (100×300 mm, Mitsubishi Chemical Industries, Tokyo, Japan) with methanol (MeOH):H₂O:acetic acid (AcOH) (4:6:0.02 and 8:2:0.02, 30 L each). The residue from the second fraction was chromatographed in a Wakogel 50C₁₈ column (80×240 mm, Wako Pure Chemical Industries, Osaka, Japan) with MeOH:H₂O:AcOH (4:6:0.05, 3.6 L) and then in a Toyopearl HW40F gel filtration column (30×180 mm, Tosoh, Tokyo, Japan) with MeOH:H₂O:AcOH (8:2:0.05). The fractions containing OCS were evaporated and suspended in ethanol. After centrifugation, OCS was collected as a pellet. Further HPLC purification was first carried out in a Cosmosil 5C₁₈-ARII column (20×250 mm) with MeOH:H₂O:AcOH (7:3:0.05) and second in the same column by linear gradient elution of 35–80% acetonitrile in 0.05% AcOH. The elution of OCS was monitored by UV absorption at 255 nm. OCS was finally purified to greater than 95% by liquid chromatography-mass spectrometry analysis. OCS aglycon was synthesized from ellagic acid with 95% purity and gifted from Dr. Eiji Asato (University of the Ryukyus, Okinawa, Japan) [11]. Anti-dinitrophenyl (DNP)-IgE, anti-rabbit IgG (goat) conjugated with peroxidase, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, and Evans blue were purchased from Sigma–Aldrich (St. Louis, MO, USA). DNP-bovine serum albumin (BSA) was obtained from Cosmo Bio (Tokyo, Japan). Anti-actin polyclonal antibody, anti-PLC γ -1 polyclonal antibody, and anti-Syk polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-PLC γ -1 (Tyr783 and Ser1248) polyclonal antibody, anti-Lyn polyclonal antibody, anti-phospho-Lyn (Tyr507) polyclonal antibody, and anti-phospho-Syk (Tyr525/526)

monoclonal antibody were from Cell Signaling Technology (Beverly, MA, USA). Fluo 3-AM and Cell Counting Kit-8 were from Dojindo Laboratories (Kumamoto, Japan).

2.2. Cell culture and cytotoxicity assay

The RBL-2H3 cell line is of basophilic tumor origin, but is similar to mast cells with respect to Fc ϵ RI-mediated signal transduction and chemical mediators [12,13], making it a suitable alternative for use in *in vitro* analyses. RBL-2H3 cells were purchased from the Health Science Research Resources Bank (Osaka, Japan) and grown in Eagle's minimum essential medium (MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel), 2 mM L-glutamine, 100 units/ml penicillin (Sigma–Aldrich), 100 μ g/ml streptomycin (Sigma–Aldrich), and non-essential amino acids for Eagle's MEM (Dainippon Sumitomo Pharma, Osaka, Japan) in a humidified atmosphere of 5% CO₂ at 37°C.

RBL-2H3 cells (5×10^4 cells/0.1 ml/well) were cultured in a 96-well plate overnight and incubated with OCS and OCS aglycon for 1 h. The cells were further incubated with 10 μ l of a Cell Counting Kit-8 solution containing water-soluble tetrazolium salt for 3 h. The absorbance at 450 nm was then measured to calculate cell viability according to the kit instructions.

2.3. β -Hexosaminidase release assay

A β -hexosaminidase release assay was used to evaluate antigen-induced degranulation [14,15]. β -hexosaminidase is stored in granules and released in parallel with histamine after antigen stimulation of mast cells [16]. RBL-2H3 cells (5×10^4 cells/0.1 ml/well) in a 96-well plate were cultured with anti-DNP IgE (0.2 μ g/ml) overnight. The supernatants were discarded, and cells were washed twice with 200 μ l of phosphate buffered physiological saline (PBS) and 130 μ l of releasing mixture (25 mM HEPES-KOH, pH 7.6; 116.9 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 2 mM CaCl₂, 5.6 mM glucose, 0.1% BSA) was added. Cells were incubated with 10 μ l of sample solution for 10 min at 37°C and stimulated with 10 μ l of DNP-BSA (2 μ g/ml) for 1 h at 37°C. To determine the released β -hexosaminidase activity, 45 μ l of supernatant and 15 μ l of 5 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 50 mM citrate buffer (pH 4.5) was incubated for 3 h at 37°C. The reaction was stopped by adding 180 μ l of carbonate buffer (0.1 M NaHCO₃/Na₂CO₃, pH 10.0). The absorbance due to the formation of *p*-nitrophenol was measured at 415 nm by a microplate reader. Three anti-allergic drugs, ketotifen fumarate, disodium cromoglycate (DSCG), and tranilast, all obtained from Sigma–Aldrich, were measured for comparison. Tranilast and OCS aglycon were dissolved in 1% dimethylsulfoxide (DMSO). The final concentration of DMSO was 0.067%, and there were no effects of DMSO on this assay. Anti-degranulation activity (%) was calculated by the following equation: anti-degranulation activity (%) = $[1 - (S - B)/(C - B)] \times 100$. *C* represents the absorbance of the control and *B* represents the

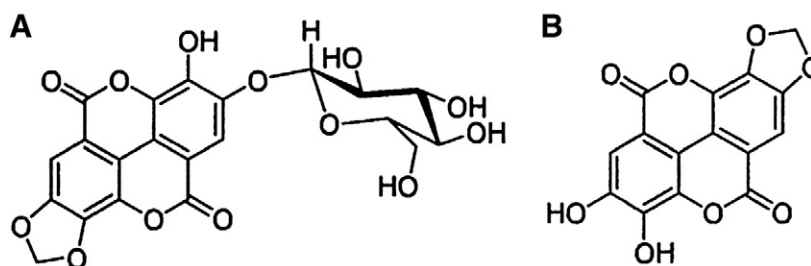


Fig. 1. Chemical structures of okicamelliaside (OCS) isolated from *Camellia japonica* leaves and chemically synthesized OCS aglycon. (A) 3,4-dioxoloellagic acid 4'-O- β -D-glucopyranoside: okicamelliaside (OCS). (B) 3,4-dioxoloellagic acid (OCS aglycon).

Table 1
IC₅₀ values of anti-degranulation activity on RBL-2H3 cells.

	IC ₅₀ (M)
OCS	1.4×10^{-8} ^a
OCS aglycon	3.0×10^{-6} ^a
Ketotifen fumarate	1.7×10^{-4} ^a
DSCG	3.0×10^{-3}
Tranilast	$> 3.0 \times 10^{-4}$

^a IC₅₀ values of OCS, OCS aglycon, and ketotifen fumarate were cited from reference [9] with permission from the Japan Society for Bioscience, Biotechnology, and Agrochemistry.

absorbance of the blank (without cells) for the control. *S* represents the absorbance of the samples.

2.4. Measurement of intracellular Ca²⁺ levels

Intracellular Ca²⁺ levels were determined using the calcium-reactive fluorescence probe Fluo 3-AM. RBL-2H3 cells (1×10^5 cells/ml) in 10 mM PIPES buffer (pH 7.4; 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, and 1% BSA) were incubated with anti-DNP IgE (0.2 µg/ml) and Fluo 3-AM (4.4 µM) for 30 min at 37°C. After washing and resuspension, 10 µl of each sample was added to 0.5 ml of cell suspension and incubated for 10 min at 37°C. The cells were stimulated with DNP-BSA (2 µg/ml) and monitored for fluorescence (excitation and emission at 488 and 525 nm, respectively) by a MACSQuant flow cytometer (Miltenyi Biotech, North Rhine-Westphalia, Germany). Mean fluorescence intensity (MFI) was calculated using associated equipment software. Intracellular concentration of Ca²⁺ ([Ca²⁺]) was thought to be almost 0% at the stationary phase before antigen stimulation, and alterations in intracellular [Ca²⁺] were calculated using the following equation: increase of [Ca²⁺] (%) = $(S - B)/B \times 100$. *B* represents the MFI of the blank (adding vehicle without antigen stimulation), and *S* represents the MFI of the samples (adding samples with antigen stimulation).

2.5. Western blot analysis

IgE-sensitized RBL-2H3 cells in a 35-mm dish were incubated with 15 µM OCS aglycon or 100 nM OCS for 10 min and then treated with DNP-BSA (2 µg/ml) or PBS for 10 min. The cells were lysed in 0.3 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1 mM EDTA-2Na, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na₃VO₄, and 1 mM NaF) using a sonicator (Ultrasonic Processor XL, Astron Electronic, Feistritz, Austria). The

cell lysates were resolved by 7.5% sodium dodecylsulfate electrophoresis (SDS-PAGE) and transferred onto nitrocellulose acetate membranes (Hybond-ECL, GE Healthcare Japan, Tokyo, Japan). After blocking, the membranes were incubated with the appropriate primary antibody overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Immunoreactive proteins were detected with an ECL Advance Western Blotting Detection Kit (GE Healthcare Japan) by a chemiluminescence detector (LAS 4000mini, GE Healthcare Japan).

2.6. DNA microarray analysis

IgE-sensitized RBL-2H3 cells were incubated with 2.1 µM OCS or vehicle for 10 min. As described in β-hexosaminidase release assay, cells were stimulated by DNP-BSA for 1 h at 37°C and collected by trypsin-EDTA treatment. Total RNAs were extracted with an RNeasy Plus Mini Kit (QIAGEN K.K., Tokyo, Japan). DNA microarray analysis using rat oligo DNA microarray (22,575 spots; Agilent Technologies, Santa Clara, CA, USA) was consigned to Hokkaido System Science (Hokkaido, Japan).

2.7. Animals

Male ICR mice were purchased from CLEA Japan (Shiga, Japan). They were housed in air-conditioned rooms maintained at 23°C ± 3°C with relative humidity of 55% ± 15% under a 12-h light-dark cycle. The animals had *ad libitum* access to laboratory rodent chow (Oriental Yeast, Tsukuba, Japan) and tap water. The experimental protocols of this study were approved by the Animal Experiment Ethics Committee of the University of the Ryukyus. All animals were maintained according to the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan.

2.8. Passive cutaneous anaphylaxis reactions

The ICR mice (10–12 weeks of age) were sensitized with 50 µl of anti-DNP-IgE/PBS by intradermal injection in the ears using a 0.5-ml insulin syringe. After 24 h, DNP-BSA (0.2 mg/head) in PBS containing 1% Evans blue was intravenously injected to induce passive cutaneous anaphylaxis (PCA) reactions. Samples were administered per os (p.o.) or intraperitoneally (i.p.) 1 h before the antigen challenge. Tranilast (RIZABEN Fine Granules 10%, Kissei Pharmaceutical, Nagano, Japan) suspended in 0.5% methyl cellulose solution (Wako Pure Chemical Industries, Osaka, Japan) was used as a comparison sample. Thirty minutes after the antigen challenge, mice were sacrificed under

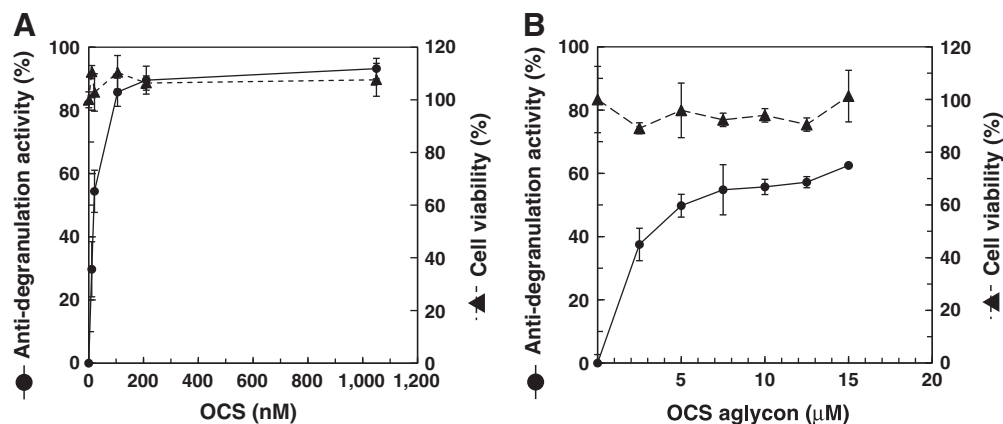


Fig. 2. Effects of OCS and OCS aglycon on antigen-induced degranulation and RBL-2H3 cell viability. Cells were sensitized with anti-DNP IgE (0.2 µg/ml) and stimulated with DNP-BSA (2 µg/ml) for 1 h. Before stimulation, cells were incubated with OCS (A) or OCS aglycon (B) for 10 min. OCS was tested at 11, 21, 105, 210, and 1050 nM. Aglycon was assayed at 2.5, 5, 7.5, 10, 12.5, and 15 mM. Anti-degranulation activities (closed circle) are expressed as the percentage of inhibition of β-hexosaminidase release as compared with that of DNP-BSA alone. Cell viability was evaluated by the Cell Counting Kit-8 (closed triangle). Each point represents the mean ($n = 4$) ± SD.

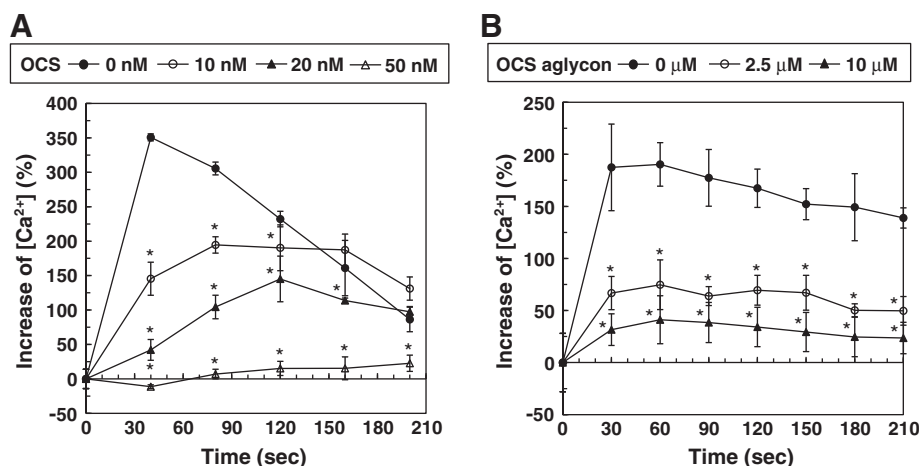


Fig. 3. Effects of OCS and OCS aglycon on antigen-induced intracellular $[Ca^{2+}]$ elevation in RBL-2H3 cells. Cells were incubated with anti-DNP IgE (0.2 μg/ml) and Fluo 3-AM (4.4 μM) for 30 min. After washing, cells were incubated with OCS (A) or OCS aglycon (B) for 10 min and then stimulated with DNP-BSA (2 μg/ml). Increases in $[Ca^{2+}]$ were evaluated by mean fluorescence intensity. Each point represents the mean ($n=3$) $\pm$ SD. *OCS and aglycon significantly inhibited antigen-induced increases in $[Ca^{2+}]$ as compared with DNP-BSA alone ($p<0.05$).

anesthesia, and their ears were removed and immersed in 1 ml of formamide. The Evans blue was extracted at 80°C for 2 h followed by extraction at 40°C for 14 h, and the absorbance at 620 nm was measured. The first experiment was performed by single sample administration with the following 5 experimental groups: normal group (PBS was injected into the ears instead of anti-DNP IgE, $n=6$), allergy group ($n=23$), tranilast 200 mg/kg p.o. group ($n=5$), and OCS 10 mg/kg p.o. and i.p. groups ($n=9$ and 6, respectively). OCS has been evaluated for safety by single administration at 10 mg/kg p.o. and i.p. in mice (unpublished data). Next, repetitive administration (once a day, from 7 days before antigen challenge) was performed to investigate the preventive effects of OCS using the following two experimental groups: allergy group ($n=15$) and OCS 5 mg/kg p.o. group ($n=8$). Because of the limited availability of

OCS, the repetitive administration dose was set at 5 mg/kg body weight.

2.9. Statistical analysis

In vitro assay data were expressed as the mean $\pm$ standard deviation (SD) and analyzed by a Student's *t*-test, which compared the control and each sample. *In vivo* assay data were expressed as the mean $\pm$ standard error (SE) and analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test with Statistical Analysis System software (ver. 9.2, SAS Institute Japan Ltd., Tokyo, Japan). In all cases, probability (*p*) values of <0.05 were considered statistically significant.

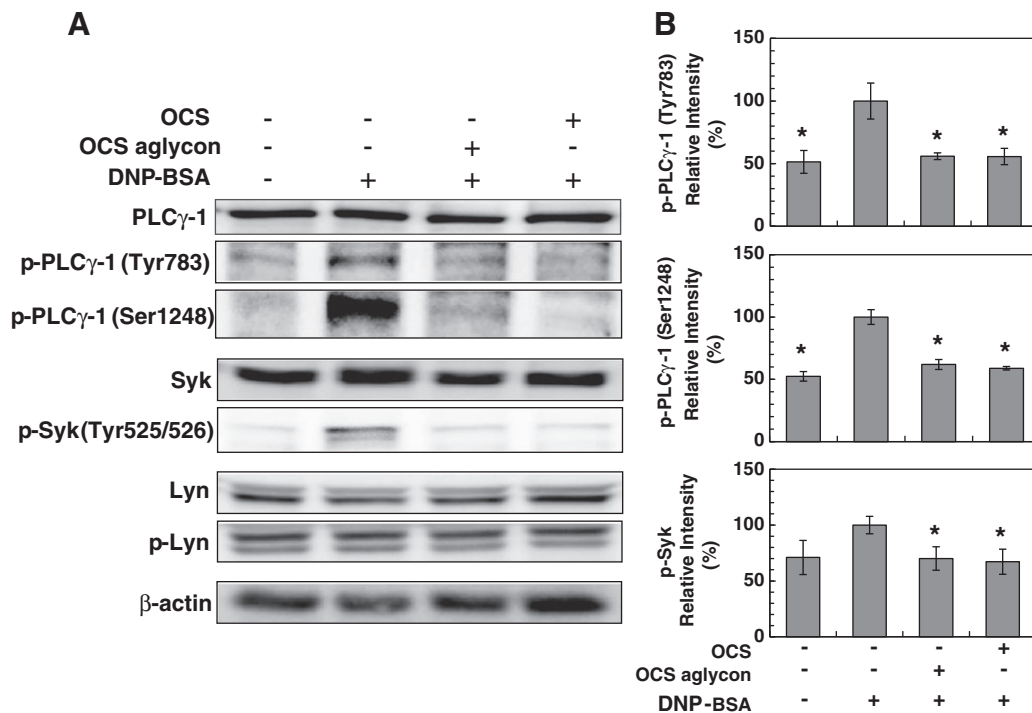


Fig. 4. Effects of OCS and OCS aglycon on antigen-stimulated signal transduction in RBL-2H3 cells. Cells were sensitized with anti-DNP IgE and stimulated with DNP-BSA (2 μg/ml) for 10 min. Before stimulation, cells were incubated with 15 μM OCS aglycon or 100 nM OCS. Whole cell lysates were analyzed by Western blotting as described in the [Materials and methods](#). (A) The blots are representative of three independent experiments. (B) The data are presented as means ($n=3$) $\pm$ SD. * $p<0.05$ compared with antigen stimulated cell.

Table 2

DNA microarray analysis of antigen-stimulated RBL-2H3 cells. (A) Genes downregulated by more than 2-fold as a result of OCS treatment.

No.	Gene name	Fold decrease
1	Interleukin 3 (Il3)	35.71
2	Early growth response 1 (Egr1)	30.42
3	Interleukin 4 (Il4)	16.48
4	Chemokine (C-C motif) ligand 2 (Ccl2)	9.58
5	Zinc finger protein 36 (Zfp36)	8.13
6	Sphingosine kinase 1 (Sphk1)	7.85
7	Immediate early response 3 (Ler3)	5.68
8	Early growth response 3 (Egr3)	4.64
9	Protein phosphatase 1, regulatory (inhibitor) subunit 14c (Ppp1r14c)	3.83
10	Interleukin 13 (Il13)	3.55
11	Colony stimulating factor 1 (macrophage) (Csf1)	3.26
12	Immediate early response 2, predicted	3.21
13	G protein-coupled receptor 37 (Gpr37)	2.93
14	G-protein coupled receptor 65 (Gpr65), predicted	2.69
15	SH2 domain protein 2A (Sh2d2a)	2.67
16	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	2.64
17	Regulator of G-protein signaling 2 (Rgs2)	2.36
18	Nuclear factor, interleukin 3 regulated (Nfil3)	2.32
19	MAP kinase-activated protein kinase 2 (Mapkapk2)	2.18
20	Mitogen-activated protein kinase kinase kinase 8 (Map3k8)	2.16
21	Nuclear receptor subfamily 2, group F, member 1 (Nr2f1)	2.10
22	Protein tyrosine phosphatase, non-receptor type 12 (Ptpn12)	2.09
23	Cytokine inducible SH2-containing protein (Cish), predicted	2.09
24	Regulator of G-protein signaling 1 (RGS1)	2.04

(B) Genes upregulated by more than 2-fold as a result of OCS treatment.

No.	Gene name	Fold increase
1	Hematopoietically expressed homeobox (Hhex)	3.11
2	Phosphoinositide-3-kinase, regulatory subunit, polypeptide p101, predicted	2.95
3	Stress-induced protein SIP18, partial (77%)	2.68
4	ATPase, Ca ²⁺ transporting, plasma membrane 3 (Atp2b3), predicted	2.65
5	Nuclear receptor coactivator 7, predicted	2.57
6	Senataxin; DEAXQ-box helicase; tRNA splicing endonuclease regulator 1, predicted	2.46
7	Actin related protein M1, predicted	2.37
8	ADP-ribosylation factor-like 11 (Arl11), predicted	2.16
9	Calcium/calmodulin-dependent protein kinase kinase 1, alpha (Camkk1)	2.10
10	GDP-L-fucose:beta-D-galactoside 2-alpha-1-fucosyltransferase, predicted	2.04
11	Fat tumor suppressor homolog 2 (Fath2)	2.01

3. Results

3.1. Inhibitory effects of OCS and OCS aglycon on antigen-induced degranulation of RBL-2H3 cells

OCS and OCS aglycon showed more potent anti-degranulation activities than the antihistaminic drug ketotifen fumarate, as shown in a previous study [9]. However, an additional experiment was necessary to compare their activities to major mast cell stabilizers such as DSCG and tranilast. In addition, the cytotoxicity of both compounds was unclear. To address these issues, a β -hexosaminidase release assay and cytotoxicity assay were performed. Table 1 summarizes the IC₅₀ values of degranulation for OCS, aglycon, and typical anti-allergic drugs. Tranilast showed <50% inhibition at 0.3 mM, which was the maximum concentration in this assay. IC₅₀ values of OCS and aglycon (14 nM and 3 μ M, respectively) were approximately 2 to 3 orders of magnitude lower than those of ketotifen fumarate, DSCG, and tranilast (0.17, 3.0, and >0.3 mM, respectively). OCS, which has a hydrophilic glucose residue, was about 200-fold more potent than aglycon in terms of inhibitory activity. There were no observed effects of OCS or aglycon on cell viability at concentrations capable of

exhibiting anti-degranulation activities (Fig. 2). Hence, the inhibitory effects of both compounds are suggested not to result from their cytotoxicity.

3.2. Effects of OCS and OCS aglycon on antigen-induced intracellular calcium mobilization and phosphorylation of signaling factors in RBL-2H3 cells

To investigate the mechanisms of degranulation inhibition by OCS and OCS aglycon, we measured antigen-induced intracellular Ca²⁺ mobilization after sample treatments. As shown in Fig. 3, intracellular [Ca²⁺] maximally increased at approximately 30 s after antigen stimulation. The antigen-induced [Ca²⁺] elevation was significantly inhibited by OCS (30 to 90 s) and aglycon (30 to 210 s) at all concentrations tested when compared with DNP-BSA alone ($p < 0.05$). These results indicate that OCS and aglycon inhibit antigen-induced degranulation in RBL-2H3 cells via inhibition of intracellular Ca²⁺ mobilization.

To further elucidate the inhibitory mechanisms of degranulation, we examined the effects of OCS and OCS aglycon on the phosphorylation of protein tyrosine kinases (Lyn and Syk) and PLC γ -1 located upstream of the [Ca²⁺] elevation in the principle signaling pathway. As shown in Fig. 4, phosphorylation of Syk (Tyr525/526) and PLC γ -1 (Tyr783 and Ser1248) by DNP-BSA stimulation for 10 min was inhibited by OCS and OCS aglycon, whereas OCS and OCS aglycon did not affect Lyn. These results indicate that OCS and aglycon inhibit degranulation via the suppression of signaling molecules for phosphorylation in the principle pathway.

3.3. DNA microarray analysis of RBL-2H3 cells treated with OCS

Because OCS showed strong anti-degranulation activity, a DNA microarray analysis was performed for a more comprehensive understanding of the anti-allergic effect of OCS. Compared with the control, 113 genes were downregulated by >2-fold as a result of OCS treatment (annotated genes totalled 53). On the other hand, 41 genes were upregulated by >2-fold as a result of OCS treatment (annotated genes totalled 11). Typical genes related to allergy and inflammation are shown in Table 2. Proinflammatory cytokines such as interleukin (IL)-3, IL-4, and IL-13, and cytokine-producing signaling molecules such as MAP kinase-activated kinase 2 and mitogen-activated protein kinase-kinase-kinase were markedly downregulated. The expression of prostaglandin-endoperoxidase (cyclooxygenase; COX) 2, which produces prostaglandins that are important chemical mediators, was also suppressed by OCS treatment. These results indicate that OCS suppresses allergy not only by inhibition of degranulation, but also by downregulation of cytokines and chemical mediator production.

3.4. Effects of OCS and OCS aglycon on PCA reaction in mice

PCA reaction tests in mice were designed to clarify the anti-allergic effects of OCS *in vivo*, as a model for immediate-type allergy. As shown in Fig. 5A, Evans blue leakage was significantly increased from 1.98 ± 0.06 μ g/ml in the normal group to 12.14 ± 0.97 μ g/ml in the allergy group by PCA reaction ($p < 0.05$). Tranilast (200 mg/kg p.o.), an anti-allergic drug, significantly suppressed Evans blue leakage (6.21 ± 0.14 μ g/ml, 58.4% inhibition, $p < 0.05$). Oral administration of OCS at 10 mg/kg had no effect; however, intraperitoneal administration significantly inhibited Evans blue leakage to 6.33 ± 0.85 μ g/ml (57.2% inhibition, $p < 0.05$). Furthermore, repetitive sample administration from 7 days prior to antigen challenge was examined to reveal the preventive effects of OCS (Fig. 5B). Repetitive administration of OCS (5 mg/kg p.o.) had no effect on body weights of mice (data not shown). Evans blue leakage was significantly decreased from 12.20 ± 0.81 μ g/ml in the allergy group to 8.74 ± 0.92 μ g/ml by OCS (33.9% inhibition, $p < 0.05$).

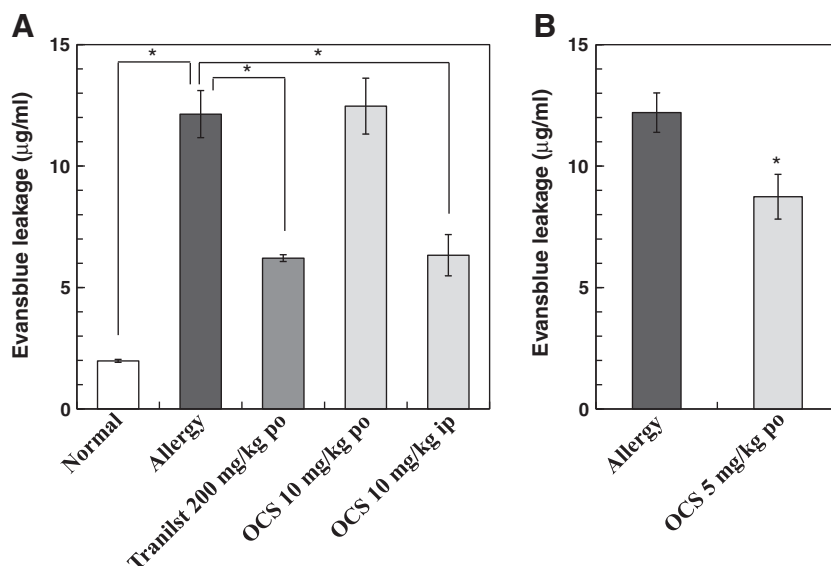


Fig. 5. Effects of OCS on the passive cutaneous anaphylaxis reaction in mice. ICR mice were sensitized with anti-DNP IgE (0.5 μg/50 μl/head) by intradermal injection in the ears. After 24 h, passive cutaneous anaphylaxis (PCA) was induced by intravenous injection of DNP-BSA/PBS containing 1% Evans blue (0.2 mg/0.2 ml/head). Single samples were administered at 1 h before the antigen challenge (A) and repeated for 7 days (B). Thirty minutes after the antigen challenge, Evans blue leakage was measured. Values represent the mean ± SE. **p* < 0.05 compared with allergy group.

4. Discussion

Natural organic compounds are widely used for allergy therapy. DSCG and tranilast are well-known mast cell stabilizers whose lead compounds were originally derived from plants. The structures of these lead compounds were then modified to enhance anti-allergic activities. Compared with these medicines, OCS purified from *C. japonica* leaves and chemically synthesized OCS aglycon had much higher anti-degranulation activities *in vitro* (Table 1). OCS and aglycon are ellagic acid analogues with a cyclic structure at the 3,4-OH position (Fig. 1) and low molecular weights (476 and 314, respectively)

[9]. These structural features seem to contribute to their remarkable activities. Although the role of the glucose residue is unclear, the amphipathic properties caused by the cyclic structure and glucose residue are considered to be necessary for the high activity, as OCS was more potent than aglycon.

A schematic diagram of the anti-allergic effects of OCS and OCS aglycon on RBL-2H3 cells based on our results is shown in Fig. 6. OCS and aglycon inhibit degranulation via the suppression of Syk-phosphorylation followed by inhibition of PLCγ-1-phosphorylation and Ca²⁺-mobilization in the principle pathway. At the start of signal transduction, phosphorylation of Lyn might immediately occur after

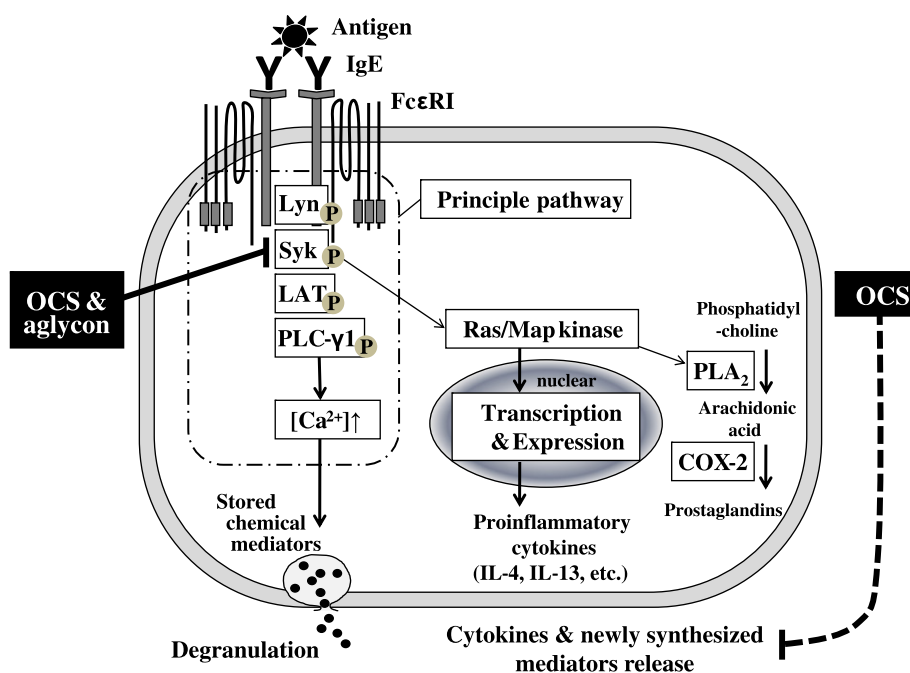


Fig. 6. Schematic diagram of the anti-allergic effects of OCS and OCS aglycon on RBL-2H3 cells. Bold lines drawn from 'OCS & aglycon' on the left side express the inhibitory effects observed by intracellular Ca²⁺ measurement and Western blot analysis. Bold dashed lines drawn from 'OCS' on the right side express inhibitory effects predicted by DNA microarray analysis.

antigen stimulation; however, it was not detected at 10 min. Therefore, the effects of OCS and aglycon on Lyn are unknown. Both compounds may act at an earlier stage (e.g., cross-linking of FcεRI on cell surfaces or Lyn-phosphorylation). Additional studies are necessary to reveal the precise mechanisms of action. With respect to PLCγ-1, phosphorylation at Ser1248 was reportedly involved in a signaling pathway related to the epidermal growth factor receptor [17]. It was also found in the FcεRI induced-signaling pathway.

OCS should inhibit the production of proinflammatory cytokines and chemical mediators. It is well known that IL-4 and IL-13 promote the proliferation and activation of helper T2 cells and accelerate humoral immunity [18,19]. Prostaglandins are also known to induce vascular hyperpermeability, smooth muscle contraction, and eosinophil migration. In addition, they lead to deterioration in allergic inflammation [20]. OCS interferes with these critical factors, indicating that it broadly suppresses allergic inflammation. Further effort is being made to confirm the suppression of proinflammatory cytokines and chemical mediators with decreased amounts of OCS and with quantitative methods such as real time PCR and enzyme linked immunoassay.

In the *in vivo* analysis, OCS significantly inhibited the PCA reaction in mice via two routes of administration: (i) single i.p. at 10 mg/kg and (ii) repetitive p.o. at 5 mg/kg for 7 days (Fig. 5). We previously reported the anti-allergic activity of the crude extract of *C. japonica* leaves [10]. In addition, the present study demonstrates the anti-allergic activity of purified OCS. Because oral administration is available, OCS may be useful in health foods or as a lead compound of oral drugs. The remarkable anti-degranulation activity of OCS *in vitro* led us to expect powerful anti-allergic activities *in vivo*. However, judging from the 33.9% inhibition of vascular permeability by OCS at 5 mg/kg p.o., the potency can be regarded as moderate. Plausible explanations include a low rate of absorption through the digestive membrane and conversion to metabolites of lower potency, as exemplified by the deglycosylation of OCS to aglycon.

In conclusion, OCS and its aglycon exert prominent anti-degranulation activities by suppressing Syk-phosphorylation and inhibiting intracellular calcium mobilization in the principle signaling pathway. OCS is also likely to prevent deterioration of allergic reactions by inhibiting the expression of proinflammatory cytokines and COX-2. Moreover, preventive oral intake of OCS could exert anti-allergic activity *in vivo*. Thus, OCS may be a valuable source for functional foods and lead compounds in medicine. For further utilization, optimization of effective doses or chemical modifications to enhance absorption and metabolic stability would be required.

Acknowledgements

We express our thanks to Dr. Eiji Asato, a Professor from the University of the Ryukyus, for supplying OCS aglycon. We are also grateful to Dr. Kouya Yamaki, an Associate Professor from Kobe Pharmaceutical University, for the helpful technical support. We thank Okinawa

Prefecture for providing purified OCS. This work was supported by a JST Collaboration of Regional Entities for the Advancement of Technological Excellence in Okinawa and the JST Adaptable and Seamless Technology Transfer Program through target-driven R&D.

References

- [1] Coombs RRA, Gell PGH. Classification of allergic reactions responsible for clinical hypersensitivity and disease. In: Coombs RRA, Gell PGH, editors. Clinical aspects of immunology. Oxford: Blackwell Science Publication; 1968. p. 575–96.
- [2] Gilfillan AM, Tkaczyk C. Integrated signaling pathways for mast-cell activation. *Nat Rev Immunol* 2006;6:218–30.
- [3] Hanashiro K, Sunagawa M, Nakasone T, Nakamura M, Kosugi T. Inhibition of IgE-mediated phosphorylation of FcεRIγ protein by antiallergic drugs in rat basophilic leukemia (RBL-2H3) cells: a novel action of antiallergic drugs. *Int Immunopharm* 2007;7:994–1002.
- [4] Han EH, Hwang YP, Kim HG, Park JH, Choi JH, Im JH, et al. Ethyl acetate extract of *Psidium guajava* inhibits IgE-mediated allergic responses by blocking FcεRI signaling. *Food Chem Toxicol* 2011;49:100–8.
- [5] Huang F, Yamaki K, Tong X, Fu L, Zhang R, Cai Y, et al. Inhibition of the antigen-induced activation of RBL-2H3 cells by sinomenine. *Int Immunopharm* 2008;8:502–7.
- [6] Itoh T, Ohguchi K, Iinuma M, Nozawa Y, Akao Y. Inhibitory effects of xanthenes isolated from the pericarp of *Garcinia mangostana* L. on rat basophilic leukemia RBL-2H3 cell degranulation. *Bioorg Med Chem* 2008;16:4500–8.
- [7] Tanifuji S, Aisu-Yokota E, Funakoshi-Tago M, Sonoda Y, Inoue H, Kasahara T. Licochalcones suppress degranulation by decreasing the intracellular Ca²⁺ level and tyrosine phosphorylation of ERK in RBL-2H3 cells. *Int Immunopharm* 2010;10:769–76.
- [8] Miyata N, Gon Y, Nunomura S, Endo D, Yamashita K, Matsumoto K, et al. Inhibitory effects of parthenolide on antigen-induced microtubule formation and degranulation in mast cells. *Int Immunopharm* 2008;8:874–80.
- [9] Onodera K, Tsuha K, Yasumoto-Hirose M, Tsuha K, Hanashiro K, Naoki H, et al. Okicamelliaside, an extraordinarily potent anti-degranulation glucoside isolated from leaves of *Camellia japonica*. *Biosci Biotechnol Biochem* 2010;74:2532–4.
- [10] Kuba M, Tsuha K, Tsuha K, Matsuzaki G, Yasumoto T. *In vivo* analysis of the anti-allergic activities of *Camellia japonica* extract and okicamelliaside, a degranulation inhibitor. *J Health Sci* 2008;54:584–8.
- [11] Asato E, Nakazawa K. Method for producing ellagic acid derivative and new ellagic acid derivative produced thereby. Japan Patent 2009–221189 (Oct. 1, 2009).
- [12] Maeyama K, Hohman RJ, Metzger H, Beaven MA. Quantitative intracellular signals, and histamine secretion in rat basophilic leukemia (2H3) cells. Enhanced responses with heavy water. *J Biol Chem* 1986;261:2583–92.
- [13] Passante E, Frankish N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflamm Res* 2009;58:737–45.
- [14] Matsuda H, Morikawa T, Tao J, Ueda K, Yoshikawa M. Bioactive constituents of Chinese natural medicines. VII. Inhibitors of degranulation in RBL-2H3 cells and absolute stereostructures of three new diarylheptanoid glycosides from the bark of *Myrica rubra*. *Chem Pharm Bull* 2002;50:208–15.
- [15] Kataoka M, Takagaki Y. Screening of the crude drugs having the antiallergic effect using rat basophilic leukemia cells (RBL-2H3). *Shoyakugaku Zasshi* 1992;46:25–9 (in Japanese).
- [16] Schwartz LB, Austen KF, Wasserman SI. Immunologic release of β-hexosaminidase and β-glucuronidase from purified rat serosal mast cells. *J Immunol* 1979;123:1445–50.
- [17] Wang Y, Wu J, Wang Z. Akt binds to and phosphorylates phospholipase C-γ1 in response to epidermal growth factor. *Mol Biol Cell* 2006;17:2267–77.
- [18] Swain S, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990;145:3796–806.
- [19] Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just Th2 cells. *Nature Rev Immunol* 2010;10:838–48.
- [20] Ricciotti E, FitzGerald G. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 2011;31:986–1000.