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Urolithin A represses IgE-induced degranulation in RBL-2H3 cells

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ABSTRACT

Background: Urolithins, intestinal metabolites of ellagic acid, have various physiological properties including anti-inflammatory effects. In addition, the anti-allergic effect of urolithin ingestion in a mouse model of pollinosis has been reported. However, the effect of urolithins on mast cell degranulation has not been reported. To investigate the anti-allergic effect of urolithin A (UA), we examined whether UA suppressed the degranulation reaction in a rat mast cell/basophil cell line, RBL-2H3. Furthermore, we examined the effect of UA on the activation of the signal pathway involved in the degranulation reaction.

Methods: We measured antigen-induced β-hexosaminidase and histamine release from RBL-2H3 cells to study the ability of UA to inhibit degranulation activity. Furthermore, we performed western blotting to determine whether Akt phosphorylation, which is involved in the signal transduction pathway leading to antigen-antibody-induced degranulation, was inhibited.

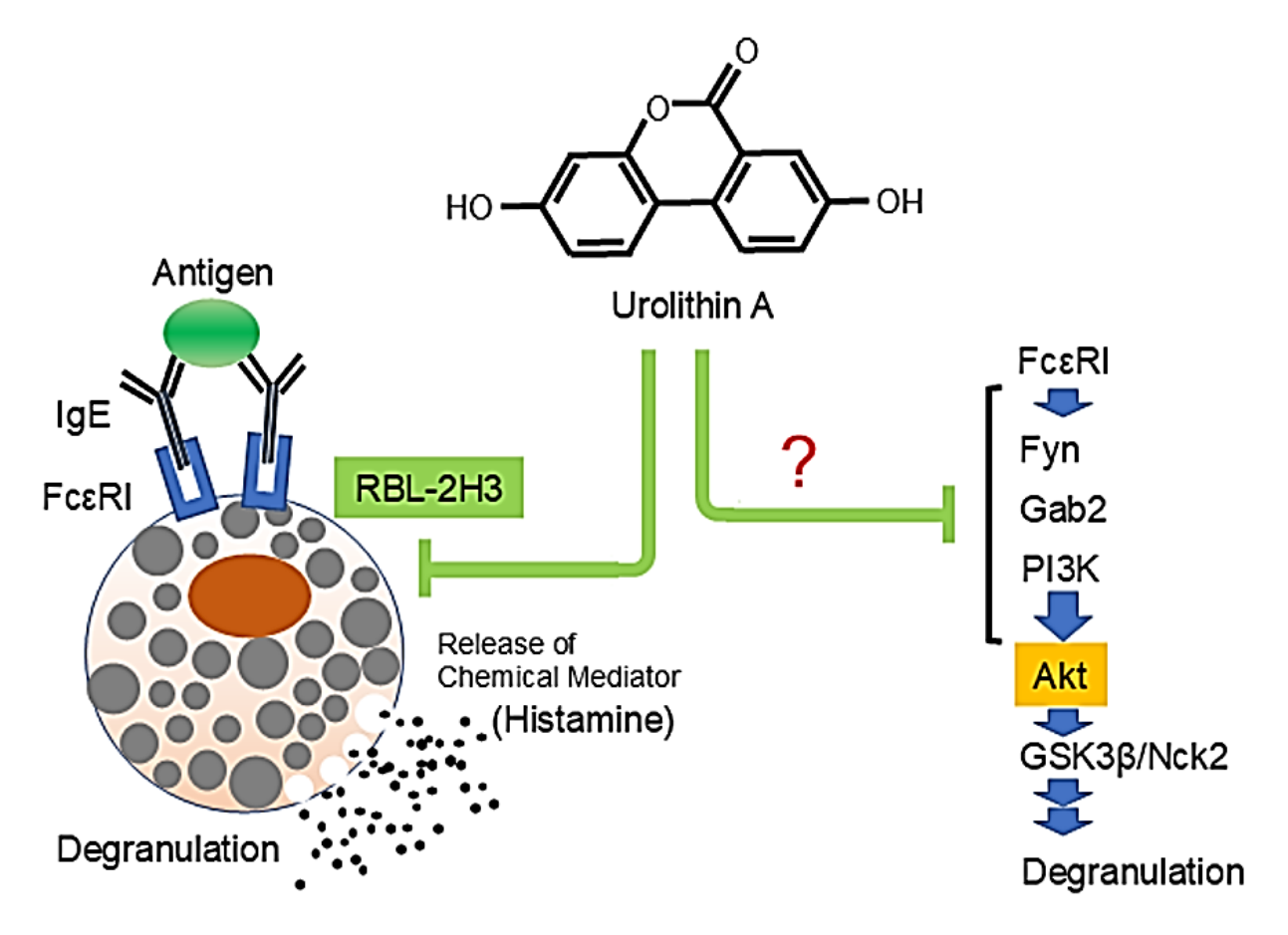
Results: UA suppressed β-hexosaminidase activity up to 10 μM in a concentration-dependent manner—2 and 5 μM UA suppressed activity by 46% and 85%, respectively, and 10 μM UA suppressed activity by 96%. The increase in the amount of histamine was also suppressed depending on the concentration of UA—5 and 10 μM of UA suppressed the increase by 60% and 86%, respectively. UA suppressed IgE-mediated Akt phosphorylation in a concentration-dependent manner.

Conclusions: UA (10 μM) effectively suppressed the degranulation reaction in RBL-2H3 cells induced by an antigen-antibody reaction. This inhibitory effect was stronger than that of tranilast (50 μM), ketotifen (50 μM), and sodium cromoglicate (50 μM) tested in parallel. Accompanying the antigen-antibody reaction, peak Akt phosphorylation was

observed at 20 minutes. UA suppressed Akt phosphorylation in a concentration-dependent manner. This suggests that UA might suppress the degranulation reaction in RBL-2H3 cells by suppressing the signal transduction system associated with the antigen-antibody reaction.

Novelty of the Study: This study is the first to report the effect of UA on mast cell degranulation. Additionally, it reveals that UA suppresses IgE-mediated Akt phosphorylation, suggesting a novel mechanism for its anti-allergic properties. These findings provide new insights into UA as a potential therapeutic compound for allergic reactions.

Keywords: urolithin A; degranulation; mast cells; anti-allergy; Akt



Graphical abstract: Urolithin A represses IgE-induced degranulation in RBL-2H3 cells

INTRODUCTION

Type I allergy is a pathological condition that causes a rapid change in the general condition, mainly by an IgE antibody-mediated mechanism. Within minutes of contact with a causative antigen, measles, facial flushing, mucosal edema, and decreased blood pressure occur. In some cases, it causes cardiovascular insufficiency and status epilepticus and may be accompanied by serious

life-threatening symptoms. When IgE antibody is re-exposed to the antigen while adhering to mast cells or the surface of basophils, cross-linking between the IgE antibodies occurs and intracellular granules are released. Because intracellular granules contain a large number of inflammatory mediators such as histamine, serotonin, and heparin, degranulation causes rapid vasodilation and increased vascular permeability. Thus, mast cells have an important role in type I allergic reactions, and it is thought that suppressing the degranulation reaction directly leads to the alleviation of symptoms. Therefore, control of the degranulation reaction of mast cells is important and the search for natural ingredients and functional food ingredients that have this effect is being vigorously carried out.

Ketotifen, sodium cromoglicate, and tranilast, which have been developed as anti-allergic agents, all suppressed degranulation in mast cells [1-4]. It has been reported that polyphenols such as nobiletin [5], curcumin [6], hesperidin [5], and resveratrol [7] reduced Tumor Necrosis Factor (TNF) and Lipopolysaccharide (LPS)-induced vascular endothelial cell damage in pollinosis animal models and relieved allergic symptoms [8-9].

Recently, it has been reported that urolithins, an intestinal metabolite of ellagic acid [10-12], have anti-inflammatory effects [13-15], inhibit cancer metastasis [16, 17], and activate autophagy [17] and sirtuin genes [18]. In addition, the anti-allergic effect of urolithin ingestion in a mouse pollinosis model was reported [19]. The effect of urolithin on the degranulation reaction in mast cells that occurs during allergic reactions has not been reported. However, it was reported that the metabolism of ellagic acid to urolithin is difficult unless the conditions of the intestinal flora are met, even if foods containing ellagic acid are ingested [20-21]. Recently, urolithin A (UA; 3,8-hydroxydibenzo-α-pyrone, Figure 1) has been produced from pomegranate peel in Japan using a fermentation method, and its application as a functional food material has begun [22].

To investigate the anti-allergic effect of UA, a rat mast cell/basophil cell line, RBL-2H3, was used to investigate whether UA suppresses the degranulation reaction in mast cells. Furthermore, we examined the effect of UA on the activation of the signal pathway involved in the degranulation reaction [23].

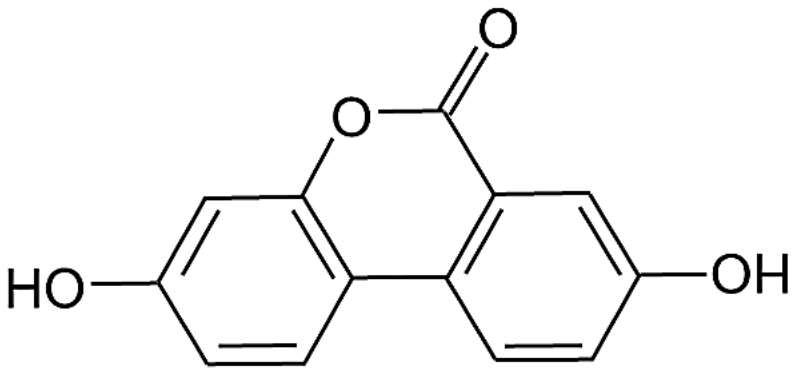


Figure 1. The structure of urolithin A.

METHODS

Chemicals and reagents: UA was purchased from AdooQ BioScience (Irvine, CA, USA). A Histamine ELISA kit was purchased from ImmuSmol (Talence, France). Other reagents were purchased from FUJIFILM Wako Chemicals Corporation (Osaka, Japan) unless otherwise indicated.

Cell culture: The rat mast cell/basophil cell line RBL-2H3 (JCRB0023) was obtained from the JCRB Cell Bank

(Ibaraki-Osaka, Japan) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO_2 atmosphere [24].

Cells were seeded into plates and allowed to settle for 24 h. After incubation, anti-DNP IgE (0.2 µg/mL) was added and incubated for another 2 h. Then, wells were washed with modified Tyrode (MT) buffer twice. Cells were treated with or without the test compound and incubated for 10 min. After incubation, human serum albumin conjugated with DNP (DNP-HSA) (0.1 µg/mL) was added and incubated for another 30 min.

Cell viability assay: Cells were treated with various concentrations of UA for 40 min, followed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution. After incubation for 20 min, the medium was removed, dimethyl sulfoxide was added, and the absorbance at 620 nm was measured by a Multiscan FC microplate reader (Thermo Fisher Scientific Inc., MA).

Effects of UA on the degranulation of RBL-2H3 cells: RBL-2H3 is a widely used mast cell line that shares some characteristics with basophils [24-26]. β-hexosaminidase is stored in the secretory granules of mast cells, and it is commonly used as a marker of degranulation [26]. We measured antigen (Ag)-induced β-hexosaminidase release from RBL-2H3 cells to study the ability of UA to inhibit degranulation activity. The assay of β-hexosaminidase release was performed as described by Murata et al. [27] with slight modifications.

Briefly, the cells were seeded in a 24-well plate (2.5 × 10^5/well) and cultured overnight. After incubation, medium in each well was exchanged with DMEM (FBS-) twice. The cells were sensitized with DNP-specific IgE at 50 ng/mL for 2 h. After the cells were washed with MT buffer containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, and 0.1% BSA at pH 7.4, polyphenol samples diluted in MT buffer were added. After 10 min of incubation, DNP-HSA (final concentration 50 ng/mL) was added, and the culture was incubated for 30 min. The supernatant was collected, and the cells were lysed with MT buffer containing 0.1% Triton X-100. The cell lysate was purchased by centrifugation at 15,000 rpm for 15 min. Aliquots of each supernatant and cell lysate were examined for β-hexosaminidase or histamine release. β-hexosaminidase release was performed as described by Watanabe et al. [24]. Aliquots of each supernatant and cell lysate were incubated with 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamide solubilized in 0.05 M citrate buffer (pH 4.5) for 30 min at 37°C. The enzyme reaction was terminated by the addition of 100 µl of 2 M glycine buffer (pH 10), and the absorbance (at 405 nm) was measured. The results are expressed as a percentage of the total content of β-hexosaminidase in the cells. Histamine release was measured by a Histamine ELISA kit (BAE-1000, ImmuSmol SAS) using the same supernatant used in the β-hexosaminidase assay.

Western blot analysis: To investigate the effect of UA on the activation of the signal pathway involved in the degranulation reaction, we detected the phosphorylation of signal proteins by western blot analysis. RBL-2H3 cells were treated using the same method as described above. Cells were rinsed with PBS, scraped into cell lysis buffer M (FUJIFILM Wako Pure Chemical Corporation), and dissolved in complete protease inhibitor cocktail (Roche) and PhosSTOP® phosphatase inhibitor cocktail (Roche). After incubation on ice for 20 min, cell lysates were obtained by centrifugation at 15,000 ×g for 15 min at 4°C. Protein concentrations were determined by Protein Assay (Bio-Rad, CA) and equal amounts (15 µg) of total protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels at a constant current of 20 mA. Separated proteins were then transferred to Immobilon

polyvinylidene difluoride membranes (Millipore, Burlington, MA) at 4 mA/cm^2 membrane for 1 h using a semi-dry blotting system. The membrane was blocked with 5% (w/v) Difco Skim Milk (BD Biosciences, NJ) for 1 h at room temperature. The blocked membrane was subsequently probed overnight at 4°C with a 1:2,500 dilution of the primary antibody in a 1:10 dilution of blocking buffer. After the membrane had been washed three times with Tris Buffered Saline with 0.05%Tween 20 (TBST), it was incubated for 2 h at room temperature with horseradish peroxidase-conjugated antibodies against IgG of the animal species of the primary antibody and hybridized with the primary antibody. After washing the membrane with TBST, a chemiluminescent substrate (Super Signal West Pico chemiluminescent substrate, Thermo Fisher Sciences, Waltham, MA) was added. The band intensities of p-Akt, Akt, and *β*-actin were analyzed using a LAS4000 mini® Image analyzer (FUJIFILM, Tokyo).

Statistical analysis: Data are expressed as the mean ± standard deviation (SD). Results were analyzed using one-way ANOVA test, and statistical significance for all comparisons was assigned when $p < 0.05$ or $p < 0.01$.

RESULTS

Effect of UA on cell viability: The effect of UA on RBL-2H3 cell activity was examined using the MTT assay. The incubation time with UA was set to 40 min considering the reaction time with DNP-HSA. UA at 50 μM caused a 20% decrease in viability, but at 20 μM there was little effect. Therefore, in this study, we used UA at a concentration of 20 μM or less (Figure 2).

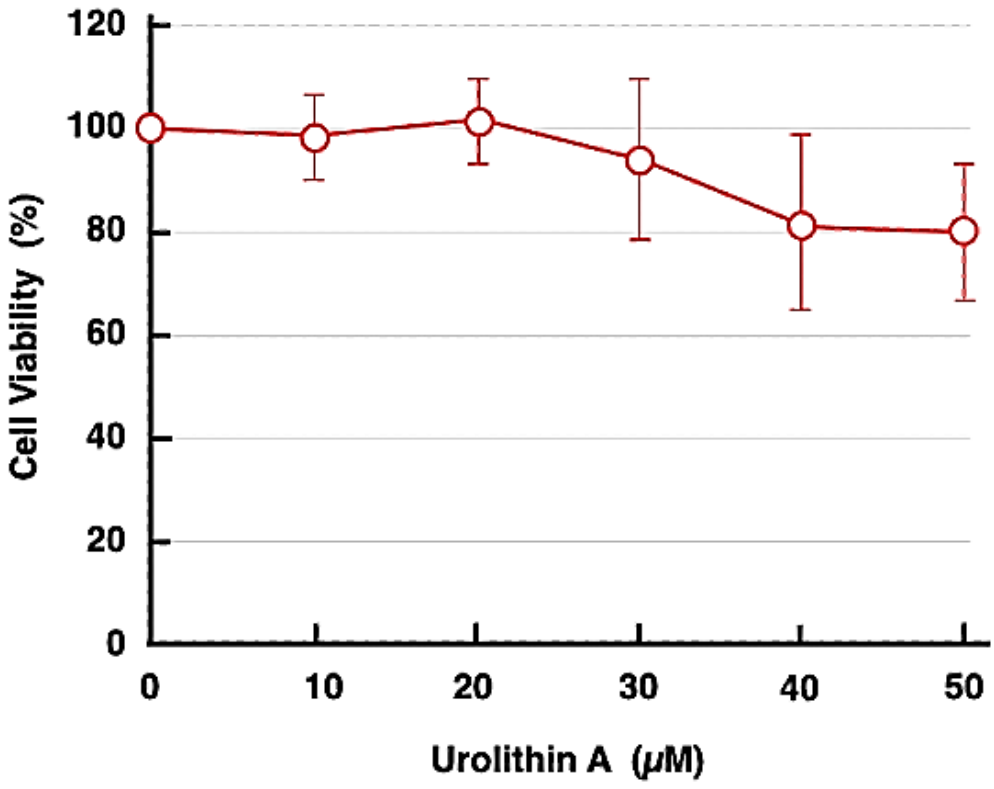


Figure 2. Effect of UA on the cell viability of RBL-2H3. RBL-2H3 cells were treated with UA at various concentrations for 40 min, and cell viability was assayed by the MTT method, as described in the Methods. All results shown are means ± SD from four independent experiments.

Inhibition of β-hexosaminidase release by UA: Degranulation caused by the reaction between IgE and DNP-HSA was investigated by measuring the β-hexosaminidase activity in the culture supernatant and RBL-2H3 cells. Under the conditions of this experiment, approximately 43% of the β-hexosaminidase activity originally present in the cells related to the IgE-mediated reaction was released into the supernatant. This value was then set to 100% and the amount of suppression by UA was calculated and graphed. The degree of inhibition is shown with the values obtained from cells treated with the positive control 0.1 μM wortmannin [28] (Figure 3). We found 50 μM ellagic acid had little effect on β-hexosaminidase activity in the supernatant, which was increased by the IgE-mediated reaction. However, UA suppressed β-hexosaminidase activity up to 10 μM in a concentration-dependent manner—1 μM and 5 μM UA suppressed the activity by 46% and 85%, respectively, and 10 μM UA suppressed the activity by 94% (Figure 3). We also investigated the inhibitory effects of tranilast (50 μM), ketotifen (50 μM), and sodium cromoglicate (50 μM), which are currently used as allergic agents, but these only suppressed activity by 29%, 22%, and 12%, respectively.

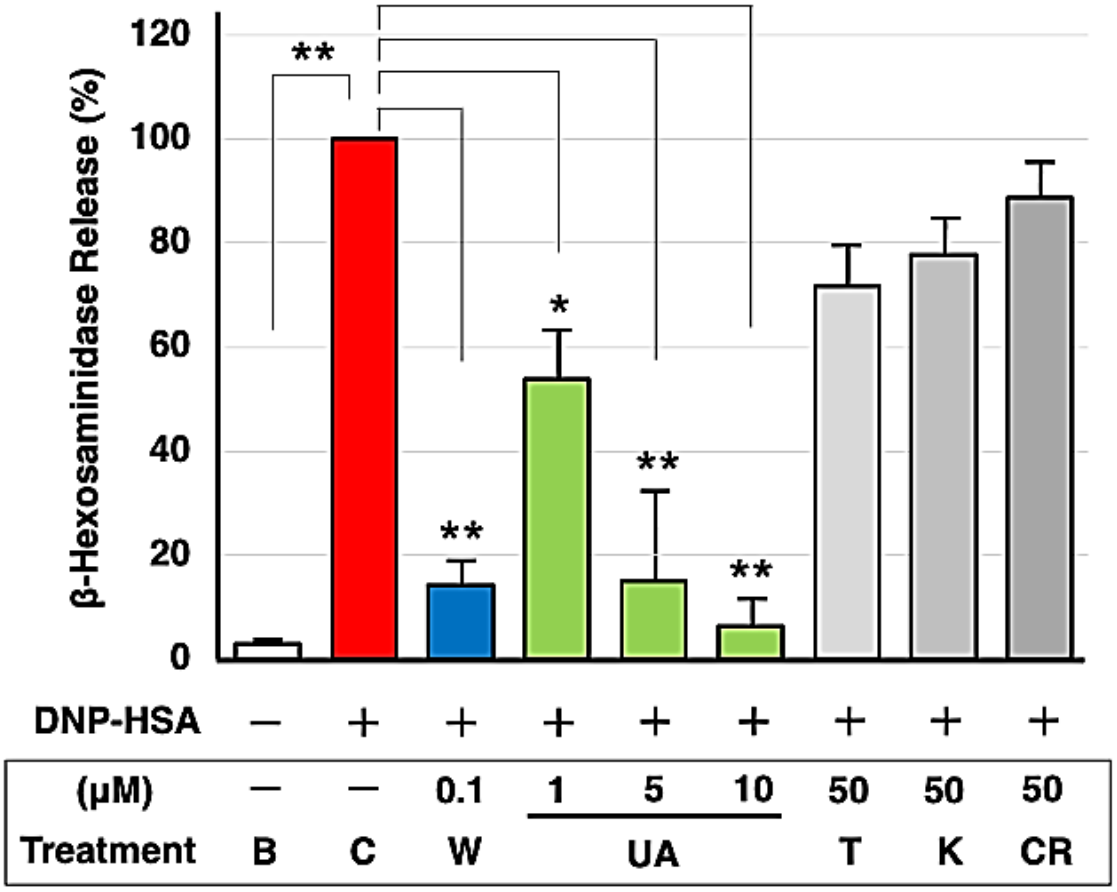


Figure 3. UA represses the release of β-hexosaminidase from antigen-treated RBL-2H3 cells**.**

RBL-2H3 cells were treated with anti-DNP IgE. After 2 h of incubation, test compounds were added to each well and incubated for 10 min. DNP-HSA was added to wells and incubated for 30 min to induce degranulation. β-hexosaminidase activity in the incubated supernatant and cell lysates were assayed as described in the Methods section. The compounds tested were blank (B), control (C), wortmannin (W), urolithin A (UA), tranilast (T), ketotifen (K), and cromoglicate (CR). All results shown are means ± SD of four independent experiments. Results are expressed as percentages of the control value and represent the mean of four independent experiments ($n = 4$). Significant differences between groups are indicated by $*p < 0.05$ or ** $p < 0.01$.

Inhibition of histamine release by UA: Because UA might selectively suppress β-hexosaminidase enzyme activity, the amount of histamine released during the degranulation reaction was measured by ELISA using the cell supernatant in which the β-hexosaminidase activity was measured. The reaction between IgE and DNP-HSA showed an increase in the amount of histamine in the culture supernatant, but in cells treated with UA, the increase in the amount of histamine was suppressed depending on the concentration of UA—5 μM and 10 μM UA suppressed histamine release by 60% and 86%, respectively (Figure 4). This demonstrated UA did not directly inhibit β-hexosaminidase activity but suppressed IgE and DNP-HSA-induced degranulation. However, the inhibitory effects of tranilast (50 μM), ketotifen (50 μM), and sodium cromoglicate (50 μM) were 17.5%, 3.1%, and 1.0%, respectively (Figure 4).

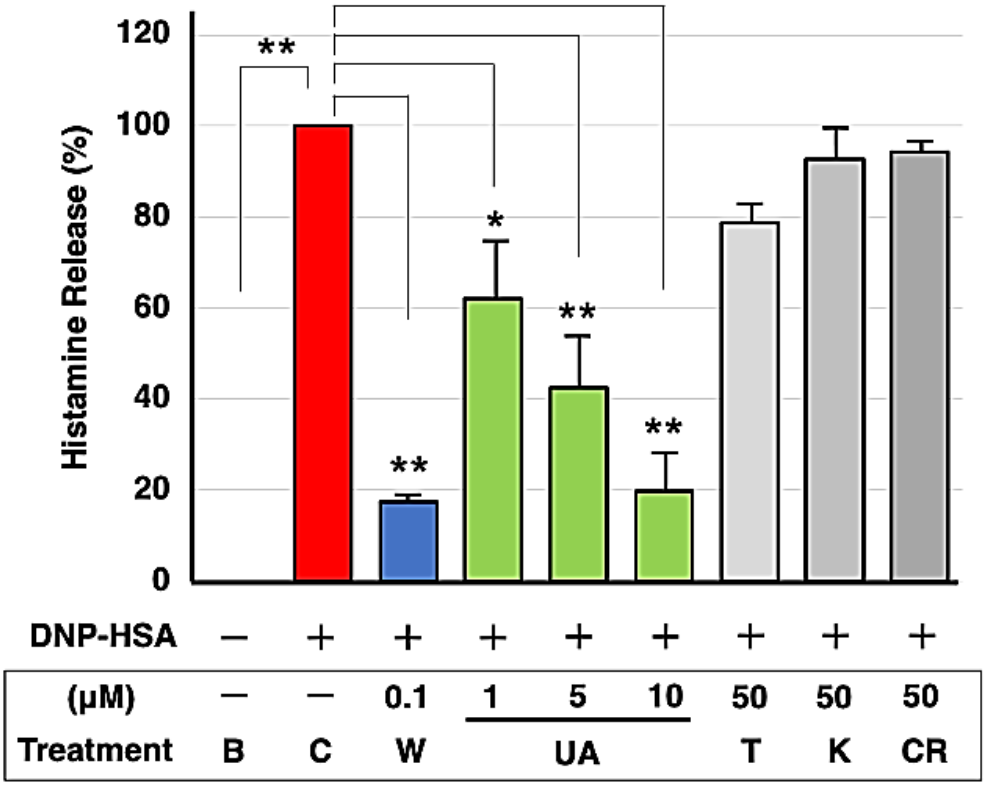


Figure 4. UA represses the release of histamine from antigen-treated RBL-2H3 cells. RBL-2H3 cells were treated with anti-DNP IgE. After 2 h of incubation, test compounds were added to each well and incubated for 10 min. DNP-HSA was added to wells and incubated for 30 min to induce degranulation. The histamine concentration in incubated supernatants was assayed by ELISA. The compounds tested were blank (B), control (C), wortmannin (W), urolithin A (UA), tranilast (T), ketotifen (K) and cromoglicate (CR). All results shown are means ± SD of four independent experiments. Results are expressed as percentages of the control value and represent the mean of four independent experiments (n = 4). Significant differences between groups are indicated by $*p < 0.05$ or ** $p < 0.01$.

Western blot analysis:

It has been reported that phosphoinositide 3-kinase (PI3K)/Akt is involved in the signaling pathway leading to IgE-mediated degranulation [29, 30]. To investigate whether UA suppresses the phosphorylation of Akt, western blot analysis of the phosphorylation signal induced by antigen was performed. After sensitization by the antigen, the phosphorylation of Akt induced by IgE and DNP-HSA peaked at 20 or 30 minutes and then gradually disappeared (Figure 5A, B).

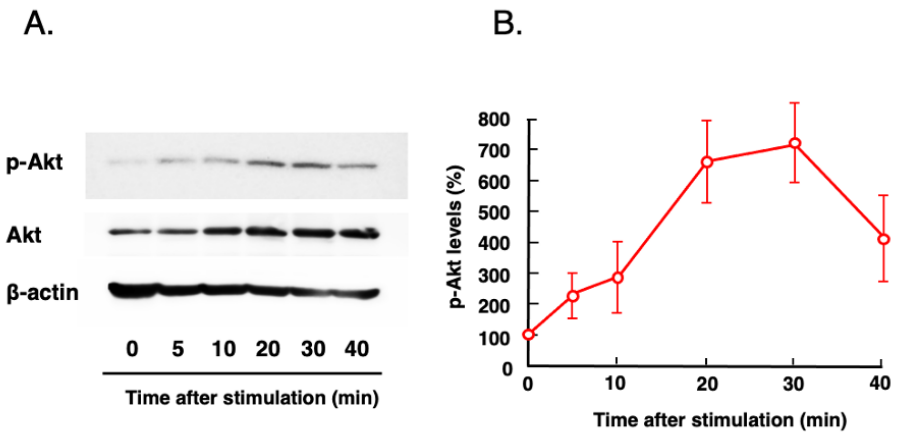


Figure 5. Changes in the phosphorylation of Akt after the antigen-stimulation of RBL-2H3 cells. RBL-2H3 cells were incubated with IgE in DMEM (without FBS) for 2 h and then washed with MT buffer. Cells were treated with DNP-HSA for various times. Cell lysates were prepared and used for western blot analysis. Experiments were performed three times. (A) shows a representative pattern of detected bands. Each band was analyzed by a densitometrical analyzer and calculated the ratio of p-Akt to the p-Akt level in cells without stimulation by DNP-HSA. All results shown are means ± S.E. from three independent experiments. (B).

The level of phosphorylated Akt at 20 or 30 min was 6.4-fold and 7.0-fold, respectively. Therefore, the effect of UA on the phosphorylation of Akt at a reaction time of 20 min was investigated. UA suppressed IgE-mediated Akt phosphorylation in a concentration-dependent manner at 20 min after sensitization by antigen (Figure 6A, B). The p-Akt/Akt ratio of 10 μM UA-treated cells was reduced by 97% relative to the p-Akt/Akt ratio of DNP-HSA-stimulated cells alone (Figure 6A, B).

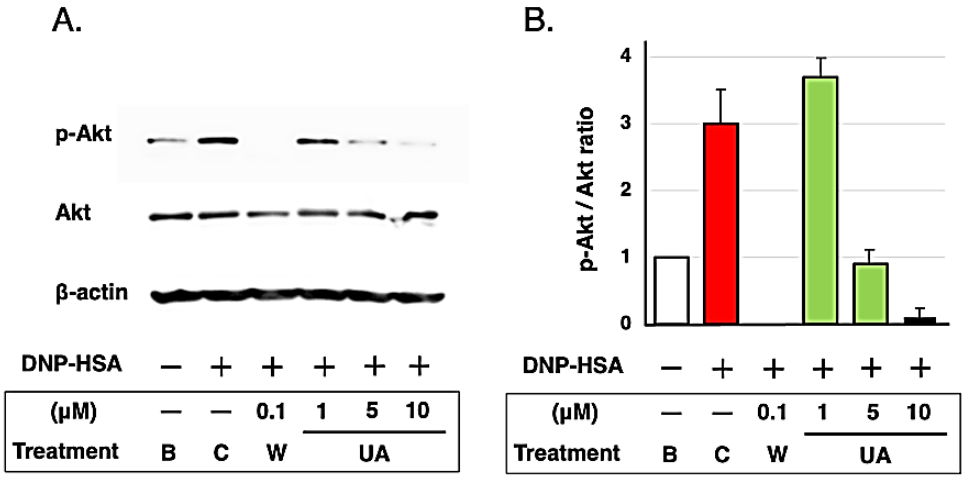


Figure 6. UA represses the phosphorylation of Akt in antigen-treated RBL-2H3 cells.
RBL-2H3 cells were incubated with IgE for 2 h in DMEM (without FBS) and then washed with MT buffer. Test compounds were added to each well containing MT buffer and incubated for 10 min. Then, cells were treated with DNP-HSA for 20 min. Cell lysates were prepared and used for western blot analysis. The compounds tested were blank (B), control (C), wortmannin (W), and urolithin A (UA). Experiments were performed three times. (A) shows a representative pattern of detected bands. Each band in Figure 6A was analyzed by a densitometrical analyzer and calculated the ratio of p-Akt and Akt. All results shown are means ± S.E. from three independent experiments. (B).

DISCUSSION

Ellagic acid has long been known as a polyphenol contained in fruits such as pomegranates, strawberries, and walnuts [31]. However, although the antioxidant activity of ellagic acid *in vitro* has been reported [32], there have been few reports of its other biological effects or pharmacological effects *in vivo*. Recently, it has been reported that UA in the metabolites of ellagic acid generated by intestinal bacteria has various physiological effects [33, 34]. Some of those reports suggested the anti-allergic effects of UA [19]. However, the effect of UA on degranulation reactions in obese cells, which have an important role in allergic reactions, has not been reported.

In this study, we purchased UA from urolithins available as reagents and examined its inhibitory effect on degranulation in rat obese cells (RBL-2H3) caused by an antigen-antibody reaction.

Wortmannin, used as a positive inhibitor of degranulation in this evaluation system [35], completely inhibited β-hexosaminidase and histamine secretion at 0.1 μM in the present study. UA showed a concentration-dependent inhibitory effect on the release of β-hexosaminidase and histamine up to 10 μM. However, existing anti-allergy drugs such as tranilast, ketotifen, and cromoglicate, did not show repressive effects against antigen-antibody-triggered degranulation when compared with the effect of UA. In this study, the drug was allowed to act on the cells for less than 10 min before DNP IgE stimulation, and it is possible that a longer preincubation time was required for these existing drugs to be effective. In addition, these drugs have anti-inflammatory effects, which might be linked to the clinical effect rather than the degranulation inhibitory effect.

Wortmannin is a multi-target inhibitor of PI3K and MLCK with IC_{50}s of 3 nM and 200 nM, respectively [36]. Therefore, this degranulation system might be mainly controlled by PI3K-Akt signaling. Indeed, it has been reported that PI3K/Akt is involved in the signal transduction pathway leading to the degranulation reaction induced by IgE and DNP IgE [29].

Therefore, we tried to detect phosphorylated Akt by western blotting to investigate whether UA suppressed the phosphorylation of PI3K/Akt. We found that UA almost completely suppressed the phosphorylation of Akt at a concentration of 10 μM, suggesting UA suppressed the degranulation reaction by blocking the signaling pathway associated with the phosphorylation of Akt. In the future, it will be necessary to investigate factors upstream of the signal transmission pathway.

This study is the first to report the strong inhibitory effect of UA on mast cell degranulation compared with conventional anti-allergic agents. Additionally, we revealed that UA suppressed IgE-mediated PI3K-Akt signaling pathway, suggesting a novel mechanism related to its anti-allergic properties. These findings provide new insights into UA as a potential therapeutic compound for allergic reactions.

In Japan, UA was recently produced from pomegranate peel using a fermentation method [22], but it is still difficult for many people to take it daily. If the constituent bacteria of the intestinal flora that efficiently metabolize ellagic acid to UA and their properties are clarified, the effect of ingredients that suppress allergic reactions to the daily diet might be effective. Because research on the function of such intestinal flora has begun recently [37-38], future developments in this field are expected.

CONCLUSION

UA suppressed the IgE-induced the release of β-hexosaminidase and histamine to a greater degree than that of other anti-allergic drugs such as tranilast, ketotifen, and sodium cromoglicate. This suggests that UA suppresses the degranulation of mast cells by inhibiting the IgE-induced signal pathway. In the future, when UA is produced more efficiently, or when the

optimal intestinal microbiota population that efficiently produces UA from ellagic acid is clarified, UA might be an effective treatment for lifestyle-related diseases.

List of Abbreviations: UA: urolithin A, TNF: Tumor Necrosis Factor, LPS: Lipopolysaccharide, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, DNP-HSA: human serum albumin conjugated with 2,4-dinitrophenyl hapten, MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, PBS: Phosphate Buffered Saline, TBST: Tris buffered saline containing 0.05% Tween 20, PI3K: phosphoinositide 3-kinase, MLCK: myosin light-chain kinase. The use of the term 'functional food' complies with FFC standards [39, 40].

Competing Interests: There are no conflicts of interest to declare.

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Authors' Contributions: KT designed the study, analyzed data, and contributed fundamental conceptualization for the research. DH and MH performed the degranulation assay and western blotting. YM critically revised the manuscript. All authors read and approved the final version of the manuscript.

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