ORIGINAL

Effect of wild grape on the signaling of histamine H_1 receptor gene expression responsible for the pathogenesis of allergic rhinitis

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Abstract : As expression level of allergic disease-sensitive genes are correlated with allergic symptom severity, suppression of these gene expressions could be good therapeutics. We have demonstrated that PKCδ signaling and NFAT signaling, involve in histamine H_1 receptor (H1R) and IL-9 gene expressions, respectively, are responsible for the pathogenesis of allergic rhinitis. We explore anti-allergic compounds that suppress these signaling pathways and found that wild grape (WG) contains such compounds. Here, we investigated the effect of WG hot water extract (WGE) on the signaling pathways for PKCδ-mediated H1R and NFAT-mediated IL-9 gene expressions. WGE suppressed histamine/PMA-induced H1R gene up-regulation in HeLa cells. Toluene-2,4-diisocyanate (TDI)-induced H1R mRNA elevation in TDI-sensitized rats was also suppressed by WGE treatment. Treatment with WGE in combination with Awa-tea, suppresses NFAT signaling-mediated IL-9 gene, markedly alleviated nasal symptoms. Furthermore, WGE suppressed PMA-induced IL-33 gene up-regulation in Swiss 3T3 cells. Data suggest that combination of WGE, suppresses PKCδ signaling with Awa-tea, suppresses NFAT signaling would have distinct clinical and therapeutic advantages as a substitute for anti-allergic drugs. In addition, as the expression level of IL-33 mRNA was correlated with the blood eosinophils number in patients with pollinosis, WG could alleviate eosinophilic inflammation through the suppression of IL-33 gene expression. J. Med. Invest. 65 : 242-250, August, 2018

Keywords **:** *Allergic rhinitis, histamine H_1 receptor, Interleukin-33, nuclear factor of activated T-cells, protein kinase Cδ*

INTRODUCTION

Pollinosis is a seasonal allergic rhinitis caused by hypersensitivity to tree or grass pollens and affects approximately 40% of the Japanese population (1). Histamine is one of the major chemical mediators of the allergic reaction, especially of the early-phase reaction and its action is mainly mediated through the activation of histamine H_1 receptor (H1R). Previously, we demonstrated that H1R gene expression is correlated with the severity of nasal acute symptoms such as sneezing and watery rhinorrhea in toluene-2,4-diisocyanate (TDI)-sensitized rats and patients with pollinosis (2, 3). We also showed that PKCδ signaling was involved in H1R gene expression, and that suppression of the H1R gene up-regulation alleviated these nasal symptoms in TDI-sensitized rats (4-7).

Th2 cytokines are also considered to be important for development of allergy. It was reported that interleukin (IL)-4, IL-5, and IL-13 are involved in the initiation and maintenance of allergic reaction (8-10). We also reported that anti-histamines suppressed Th2 cytokine gene up-regulations in TDI-sensitized rats, and that the expression level of H1R mRNA was correlated with those of IL-4 and IL-5 in patients with pollinosis (3, 11). In addition, accumulating evidence suggests the existence and important role of the histamine–cytokine network in allergic inflammation (12, 13). Recently, we have demonstrated that IL-9 gene is an additional allergy sensitive gene in TDI-sensitized rats and NFAT signaling is involved in IL-9 gene expression and that suppression of both PKCδ signaling and NFAT signaling remarkably improved nasal symptoms in allergy model rats (14).

The important role of eosinophils for late phase reaction of allergic rhinitis and other eosinophilic inflammations such as asthma and eosinophilic sinusitis has been reported (1). Release of leukotrienes from eosinophils cause nasal mucosal swelling, observed in a late phase (15, 16). Genome-wide association studies uncovered IL-33 gene is susceptible for asthma onset (17, 18). IL-33 is a cytokine belonging to the IL-1 superfamily and is known to induce helper T cells, mast cells, eosinophils, and basophils to produce Th2 cytokines (19). Thus, IL-33 is considered to be crucial for the induction of Th2-dominant immune responses (20). However, relationship between the level of IL-33 gene expression and eosinophils was not elucidated yet.

Wild grape (*Ampelopsis glandulosa,* WG), common name porcelain berry, is a species of plant native to Japan, China, India, Nepal, Myanmar, Vietnam, and the Philippines. It was reported that *A. brevipedunculata*, synonymous to *A. glandulosa* has anti-inflammatory, anti-hepatotoxic, and anti-osteoclastogenesis activity (21-24). However, effect of WG on the PKCδ and NFAT signaling remains unknown.

Awa-tea is a unique traditional tea in Tokushima, which consists of leaves fermented with *Lactobacillus pentosus and Lactobacillus plantarum*. Anti-obesity activity of Awa-tea was reported (25).

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Anti-oxidant activity of Awa-tea also reported and resorcinol was identified as anti-oxidant (26). Furthermore, Awa-tea suppressed mono- and disaccharides- induced increase in the blood glucose level in mice (27). In Tokushima, Awa-tea has been traditionally used to treat allergic diseases. However, its pathological mechanism remains unknown.

In the present study, we investigated the effect of WG hot water extract (WGE) on PKCδ-mediated H1R and NFAT-mediated IL-9 gene expressions responsible for the pathogenesis of acute symptoms of allergic rhinitis in TDI-sensitized rats. We also investigated the effect of WGE on the gene expression of histamine signaling related Th2 cytokines, IL-4 and IL-5. In addition, we examined the effect of WGE on the IL-33 gene up-regulation that could be closely related to chronic symptoms of allergic rhinitis and eosinophilic inflammations. We found that WGE suppressed H1R, IL-4, IL-5, and IL-33 gene up-regulations but not IL-9 gene up-regulation. We also showed that treatment with WGE in combination with Awa-tea, suppresses NFAT-mediated IL-9 gene expression was more effective than the treatment with each single suppressant.

MATERIALS AND METHODS

Animals

Six-week-old male Brown Norway rats weighing 180-220 g (Japan SLC, Hamamatsu) were used for the present study. Rats were allowed free access to water and food and kept in a room at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/ dark cycle. The animals were divided into 7 groups comprising of the control, sensitized with TDI (Wako Pure Chemical, Osaka), and test groups, with 5 rats in each group. All animal experiments were approved by the Ethical Committee for Animal Research of Tokushima University.

Sensitization and provocation with TDI and administration of wild grape and Awa-tea

Rats were sensitized with TDI by the method described by Kitamura *et al.* with slight modifications (28). Briefly, 10 μl of 10% TDI in ethyl acetate (Wako Pure Chemical) was applied bilaterally to the nasal vestibule once a day for 5 consecutive days. This sensitization procedure was then repeated after a 2-day interval. Nine days after the second sensitization, 10 μl of 10% TDI solution was again applied to the nasal vestibule to provoke nasal allergic-like symptoms. Control rats were treated with ethyl acetate only according to the same schedule (Fig. 1). WG powder and Awa-tea leaves was supplied from Nab co., ltd. (Kamikatsu, Tokushima). WG powder (50 g) or Awa-tea leaves (10 g) was boiled for 90 min or 10 min, respectively, in 1liter of water, and then extracts were centrifuged and filtered. Finally, freeze-dried extracts were kept at -30°C until use. WGE (25 mg/kg and 50 mg/kg) and/or Awa-tea (40 mg/kg) were dissolved in water at the concentration of 10 mg/ml on the day of the experiments and administered orally 1 h before the TDI sensitization for 3 weeks (Fig. 1). The number of sneezes and the extent of watery rhinorrhea considered as the indicator of nasal allergic-symptoms and were determined using the protocol of Abe *et al* (6). After TDI provocation, the number of sneezes and watery rhinorrhea severity were examined for 10 min. Scaling from 0 to 3 was used as the basis to estimate the level of watery rhinorrhea described in the Table 1. For standardization of WGE, gallic acid (1 mg/ml in water) was used as a standardization marker. Freeze-dried WGE (1 mg) was dissolved in 1 ml of water and applied on a HPLC system (Hitachi High-Tech Science, Tokyo) equipped with Cosmosil 5C18-MS-II column (4.6 ID x 250 mm ; Nacalai Tesque, Kyoto) at room temperature under constant flow rate of 1 ml/min using the solvent system of 20% acetonitrile in 0.05% trifluoroacetic acid. The HPLC chemical fingerprint was recorded by UV-VIS L-2420 detector (Hitachi High-Tech Science) at 254 nm. Standardization analyzes revealed that hot water extract of WGE contains 12.2% of gallic acid.

Cell culture

HeLa cells were cultured in MEM-α medium containing 8% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. RBL-2H3 cells were cultured in EMEM containing 10% FBS, 100,000 U/mL penicillin, and 10 mg/mL streptomycin. Swiss 3T3 cells were cultured in DMEM medium containing 10% FBS, 100,000 U/mL penicillin, and 10 mg/mL streptomycin.

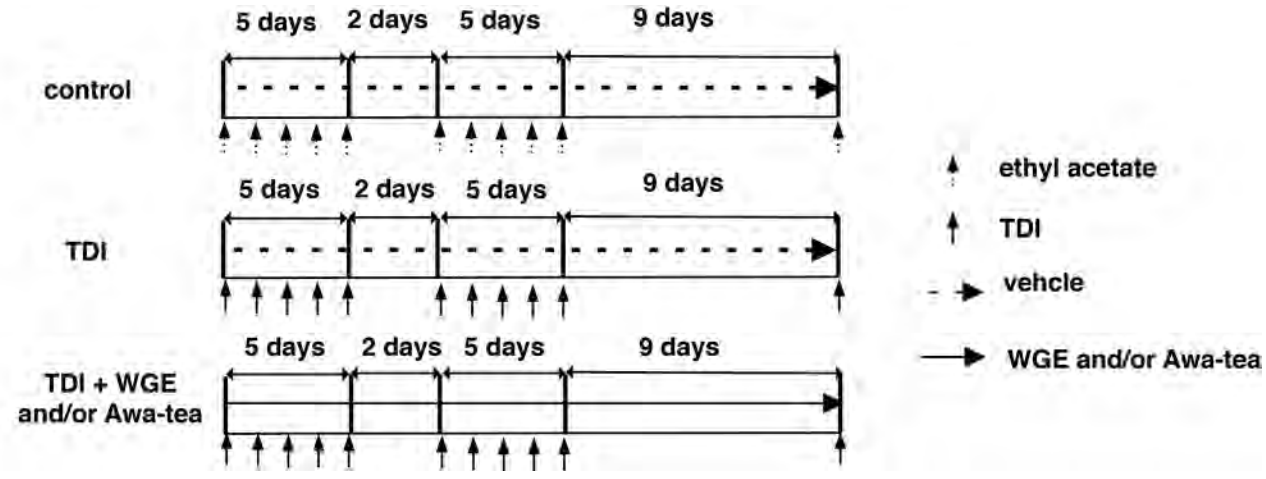

Fig. 1. **Experimental protocol.**
Rats were sensitized by intranasal application of 10 μl of 10% TDI in ethylacetate for 2 weeks. After a 1-week interval, provocation was done with 10 μl of 10% TDI. The control group was sensitized with ethyl acetate only. The drug-treated group was orally treated with WG hot water extract (WGE, 25 and 50 mg/kg/day) and/or Awa-tea (40 mg/kg/day) once a day for 3 weeks. Drugs were administrated 1 h before rats were sensitized with TDI.

Table 1. Criteria for grading the severity of TDI-induced nasal responses in rats.

	Score			
Nasal response	0	1	2	3
Watery rhinorrhea	(-)	At the nostril	Between 1and 3	Drops of discharges from nose
Swelling and redness	(-)	Slightly swollen	Between 1and 3	Strong swelling with redness

Real-time quantitative RT-PCR

Nasal mucosa of rats was separated from the nasal septum, collected in RNAlater (Applied Biosystems, Foster City, CA, USA) 4 h after provocation with TDI, and stored at -80°C until used. Nasal mucosa was homogenized using a Polytron (Model PT-K ; Kinematica, AG, Littau/Luzern, Switzerland). Total RNA was isolated using RNAiso Plus reagent (Takara Bio Inc., Kyoto) according to the manufacturer's instructions. HeLa cells were serum-starved and the concentration of FBS in the culture medium for Swiss 3T3 cells were reduced to 0.5% before subject to the mRNA determination. HeLa cells, RBL-2H3 cells, and Swiss 3T3 cells were cultured to 70% confluency in six-well dishes. The cells were treated with WGE or Awa-tea for 24 h before treatment with 100 nM of PMA (for HeLa cells and Swiss 3T3 cells) or 1 μM of iomomycin (for RBL-2H3 cells). After the stimulation with PMA for 3 h or ionomycin for 2 h, the cells were harvested and total RNA was prepared. The RNA samples (5 μg) were reverse transcribed to cDNA using PrimerScript RT Reagent Kit (Takara Bio Inc.). TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The nucleotide sequences of the primers and probes for H1R and IL-9 are listed in Table 2. The IL-33 primers and probe kit (Mm00505403_m1, Applied Biosystems) was used to determine mouse IL-33 mRNA. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, endogenous control human GAPDH and rodent GAPDH control reagents (Applied Biosystems) were used, and data were expressed as a ratio of GAPDH mRNA.

Immunoblot Analysis

For the immunoblot analysis, 10 μg of each protein sample was separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was briefly rinsed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco) or 3% BSA (for detecting phosphoproteins ; Sigma, St. Louis, MO, USA). The membrane was then incubated with a primary antibody [PKCδ(C-20) (sc-937, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho-PKCδ (Tyr^{311}) and β-actin (#2055S and #4697, Cell Signaling Technology Japan, Tokyo)] overnight at 4°C. Proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

Analysis of IL-33 mRNA expression in the nasal mucosa of patients with pollinosis

IL-33 mRNA was determined using the nasal mucosa samples of patients as described previously (11). Patient information, preparations for scraping the nasal mucosa, evaluation of nasal symptoms, and other experimental conditions were previously reported (3). Blood samples were collected and blood cell analysis was conducted to count eosinophils. Nasal mucosa scrapings were obtained from patients by an otolaryngologist as previously described (3). Total RNA was isolated and reverse transcribed using the RNAqueous Micro Kit (Applied Biosystems) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), respectively. The nucleotide sequences of the primers and probes for human IL-33 are listed in Table 2. Universal Probe Library #27 (Roche Diagnostics, Basel, Switzerland) were used for the IL-33 probes. The pumilio RNA binding family member 1 (PUM1) primer and probe kit (Hs 00206469-m1, Applied Biosystems) was used to generate a standard (3). The data are expressed as the ratio of IL-33 mRNA to PUM1 mRNA as previously described. The ethics committee of Tokushima University Hospital and Yashima General Hospital approved this study ; written informed consent was obtained from each patient before the study commenced.

Table 2. Nucleotide sequences of primers and probes used in this study.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
Anti-sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
Human IL-33 mRNA	
Sense primer	5'-AGGCCTTCACTGAAAACAGG-3'
Anti-sense primer	5'-TACCAAAGGCAAAGCACTCC-3'
Rat H1R mRNA	
Sense primer	5'-TATGTGTCCGGGCTGCACT-3'
Anti-sense primer	5'-CGCCATGATAAAACCCAACTG-3'
Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
Rat IL-4 mRNA	
Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
Anti-sense primer	5'-CACCGAGAACCCCAGACTTG-3'
Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
Rat IL-5 mRNA	
Sense primer	5'-CAGTGGTGAAAGAGACCTTGATACAG-3'
Anti-sense primer	5'-GAAGCCTCATCGTCTCATTGC-3'
Probe	FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA
Rat IL-9 mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
Anti-sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACCT-TAMRA

Universal Probe Library #27 (Roche) were used for the IL-33 probes.

Statistical Analysis

The results are shown as the mean ± S.E.M. Statistical analyses were performed with analysis of variance with Dunnett's test and the Spearman's rank correlation method using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of wild grape hot water extract (WGE) on PMA- and histamine-induced up-regulation of H1R mRNA in HeLa cells

Stimulation of HeLa cells with PMA or histamine induced significant and transient increase in H1R mRNA with a maximum 3 h after PMA or histamine stimulation (29). We investigated the effect of WGE on PMA- or histamine-induced up-regulation of H1R mRNA expression in HeLa cells. Pretreatment with WGE suppressed

PMA- and histamine-induced up-regulation of H1R mRNA in a dose-dependent manner (Fig. 2A and 2B).

Effect of WGE and Awa-tea on ionomycin-induced IL-9 gene expression in RBL-2H3 cells

Previously, we have shown that stimulation with ionomycin increased the level of IL-9 mRNA and NFAT signaling is involved in this transcription (14). We investigated the effect of WGE on NFAT-mediated IL-9 gene expression in RBL-2H3 cells. Treatment with WGE showed no inhibition for ionomycin-induced up-regulation of IL-9 gene expression (Fig. 3A).

Although Awa-tea has been used to treat allergic diseases, its pathological mechanism is not fully studied. Recently, we have found that treatment with Awa-tea in combination with anti-histamine markedly improved allergic symptoms in TDI-sensitized rats (Nakano and Mizuguchi, manuscript in preparation). This effect of Awa-tea is similar to that of suplatast that suppresses NFAT-mediated IL-9 gene expression (14). Therefore, we investigated the effect of Awa-tea on NFAT-mediated IL-9 gene expression in RBL-2H3 cells. Treatment with Awa-tea dose-dependently suppressed ionomycin-induced IL-9 gene up-regulation in RBL-2H3 cells (Fig. 3B), suggesting that Awa-tea suppressed NFAT signaling.

Effect of WGE and/or Awa-tea on TDI-induced nasal symptoms in TDI-sensitized rats

Application of TDI caused nasal symptoms characterized by sneezing and watery rhinorrhea in TDI-sensitized rats. Pre-treatment with WGE (25 and 50 mg/kg/day) for 3 weeks significantly reduced TDI-induced sneezing and nasal core (Fig. 4A and 4B). Control rats showed no nasal symptoms after provocation with ethyl acetate. In the previous study, we have demonstrated that

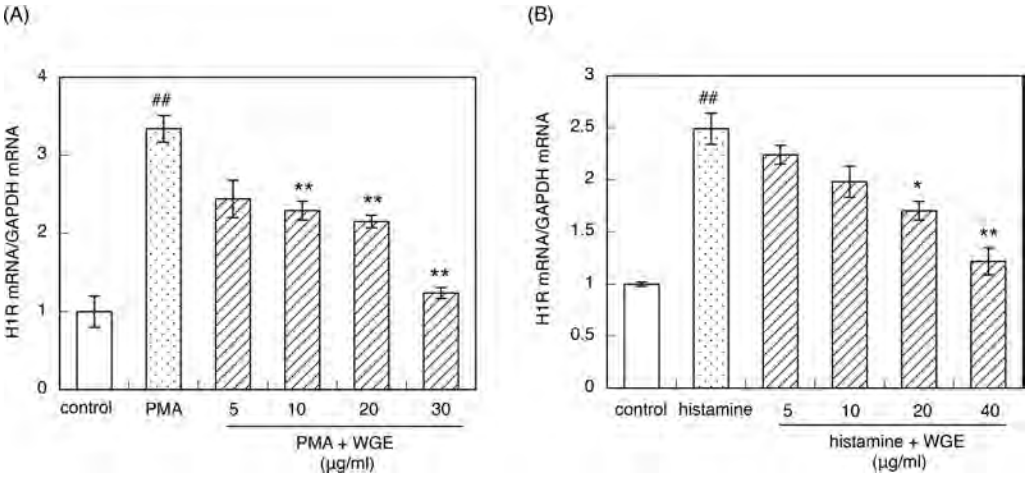

Fig. 2. **Effect of WGE on PMA- and histamine-induced up-regulation of H1R mRNA in HeLa cells.**
HeLa cells were serum-starved for 24 h at 37°C before treatment with 100 nM PMA for 3 h (A) or 100 μM histamine for 3 h (B). WGE were incubated for 24 h before PMA and histamine stimulation. H1R mRNA was determined by quantitative RT-PCR. Data are expressed as means ± S.E.M. (n=3). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PMA or histamine.

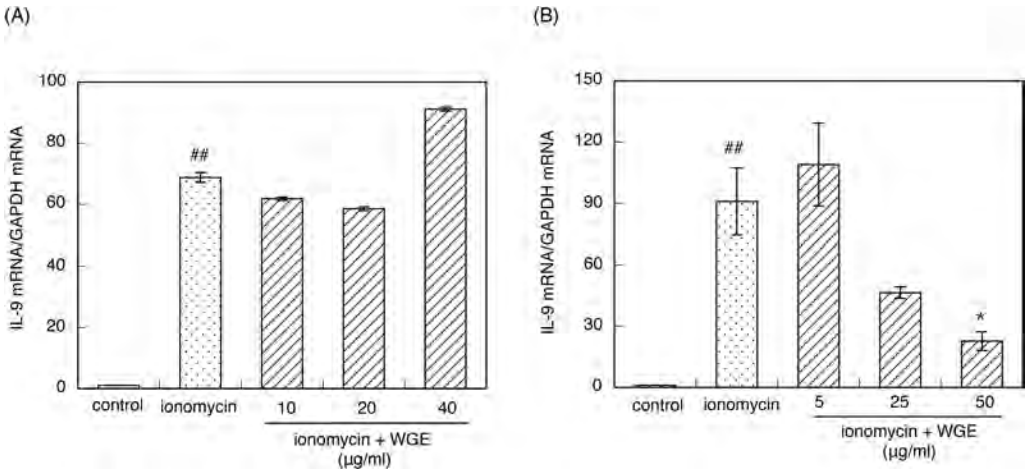

Fig. 3. **Effect of WGE and Awa-tea on ionomycin-induced up-regulation of IL-9 mRNA in RBL-2H3 cells.**
RBL-2H3 cells were treated with 10-40 μg/ml of WGE (A) or 5-50 μg/ml of Awa-tea for 24 h before ionomycin (1 μM) stimulation. After 2 h of stimulation with ionomycin, the RBL-2H3 cells were harvested and IL-9 mRNA was determined by real-time quantitative RT-PCR. The data are expressed as the mean ± S.E.M. (n=3). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, vs. ionomycin.

suppression of both PKCδ signaling and NFAT signaling markedly alleviated TDI-induced nasal symptoms in TDI-sensitized rats (14). The *in vitro* experiments data suggest that WGE and Awa-tea suppress PKCδ signaling and NFAT signaling, respectively. Thus, we investigated the effect of WGE and/or Awa-tea on TDI-induced nasal symptoms in TDI-sensitized rats. Combined treatment with WGE (50 mg/kg/day) and Awa-tea (40 mg/kg/day) significantly reduced TDI-induced sneezing and the nasal score (Fig. 4A and 4B).

Effect of WGE and/or Awa-tea on TDI-induced up-regulations of H1R and IL-9 gene expression in the nasal mucosa of TDI-sensitized rats

Sensitization with TDI increased H1R and IL-9 mRNA expression in the nasal mucosa of TDI-sensitized rats (14, 30). Our previous data found that H1R and IL-9 mRNA expression reached a maximum after 4 h of TDI provocation. Pretreatment with WGE (50 mg/kg/day) significantly reduced H1R and IL-9 gene expression in the nasal mucosa of the TDI sensitized rats (Fig. 5). Treatment with WGE (50 mg/kg/day) combined with Awa-tea showed significant suppression of H1R and IL-9 gene up-regulation than single treatment with Awa-tea.

Effect of WGE and/or Awa-tea on TDI-induced up-regulations of Th2 cytokine gene expressions in the nasal mucosa of TDI-sensitized rats

Repeated sensitization with TDI also up-regulated gene expressions of Th2 cytokines such as IL-4 and IL-5 (2, 14). Treatment with WGE (50 mg/kg/day) significantly suppressed TDI-induced up-regulation of IL-4 and IL-5 (Fig. 6A and 6B). Treatment with Awa-tea also showed significant suppression of up-regulation of IL-4 gene expression but not IL-5 gene expression. However, significant suppression of TDI-induced up-regulation of gene expression of these cytokines was observed in combination of WGE with Awa-tea.

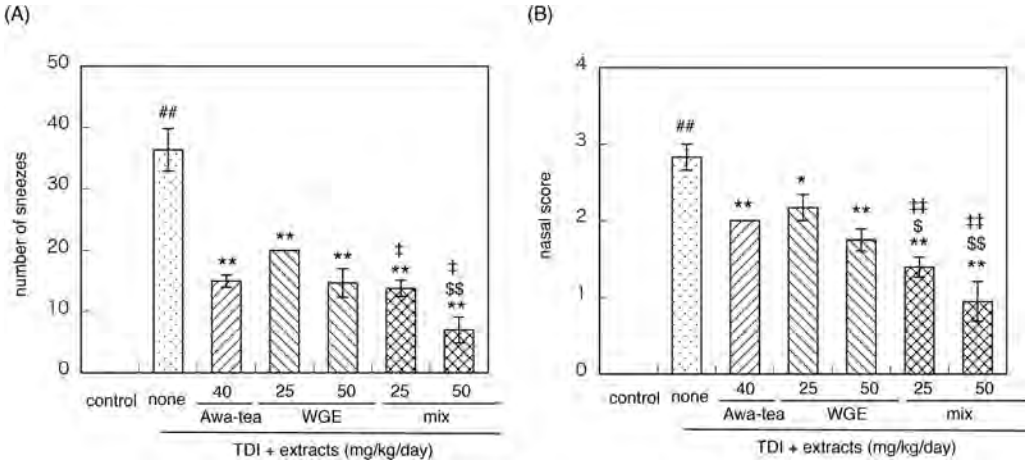

Fig. 4. **Effect of WGE and/or Awa-tea on toluene-2,4-diisocyanate (TDI)-induced nasal symptoms in TDI-sensitized rats.** The rats were sensitized and provoked as described in the Materials and Methods. The numbers of sneezes (A) and the nasal score (B) were measured over the 10-min period immediately after TDI provocation. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TDI ; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Awa-tea ; $^{\ddagger}p < 0.05$ vs. WGE at the corresponding concentration.

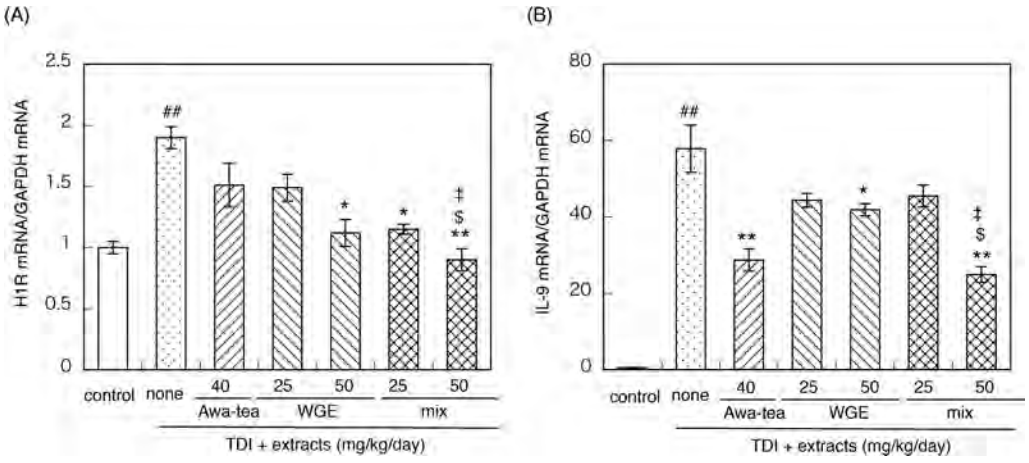

Fig. 5. **Effect of WGE and/or Awa-tea on toluene-2,4-diisocyanate (TDI)-induced up-regulation of H1R and IL-9 gene expression in TDI-sensitized rats.**
The rats were sensitized and provoked as described in the Materials and Methods. The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of H1R (A) and IL-9 (B) were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TDI ; $^{\$}p < 0.05$ vs. Awa-tea ; $^{\ddagger}p < 0.05$ vs. WGE at the corresponding concentration.

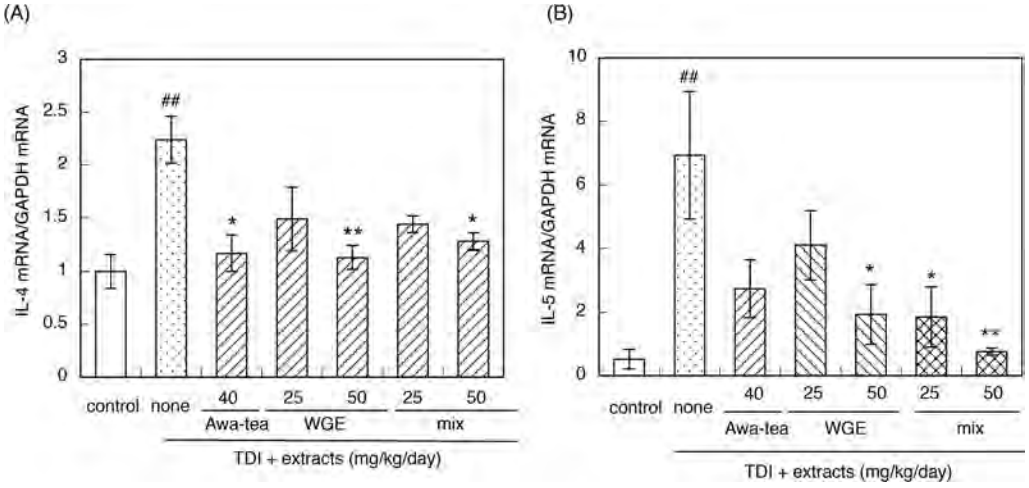

Fig. 6. **Effect of WGE and/or Awa-tea on toluene-2,4-diisocyanate (TDI)-induced up-regulation of IL-4 and IL-5 gene expression in TDI-sensitized rats.**
The rats were sensitized and provoked as described in the Materials and Methods. The rats were sensitized and provoked as described in the Materials and Methods. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of IL-4 (A) and IL-5 (B) were determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. TDI.

Effect of WGE on PMA-induced phosphorylation of PKCδ at Tyr^{311} in HeLa cells

Phosphorylation and dephosphorylation of PKCs regulate their activities, stabilities, and functions, and PKCδ is reportedly activated by phosphorylation at Tyr^{311} (31). Previously, we showed that stimulation with histamine or PMA accelerate to increased phosphorylation of PKCδ at Tyr^{311} in HeLa cells (4). We investigated the effect of WGE on PMA-induced phosphorylation of PKCδ at Tyr^{311}. Pretreatment with WGE suppressed PMA-induced phosphorylation of PKCδ at Tyr^{311} (Fig. 7).

IL-33 mRNA level was correlated with the number of blood eosinophils in patients with polinosis

Although it was reported that serum levels of IL-33 protein were significantly higher in patients with pollinosis than in controls (32), our previous data showed that no suppression of IL-33 mRNA in the nasal mucosa was observed, suggesting that IL-33 is not involved in the therapeutic effect of pre-seasonal treatment with antihistamine for patients with pollinosis (11). As IL-33 gene was reported to be the gene related to asthma that was characterized by eosinophilic airway inflammation, we investigated the relationship between the IL-33 mRNA level and the number of blood eosinophils in patients with pollinosis. The IL-33 mRNA level was not correlated with that of H1R (Fig. 8A). On the other hand, the blood eosinophil number was well correlated with the expression level of IL-33 gene ($r = 0.74$, $p < 0.01$ by the Spearman's rank correlation method ; Fig. 8B).

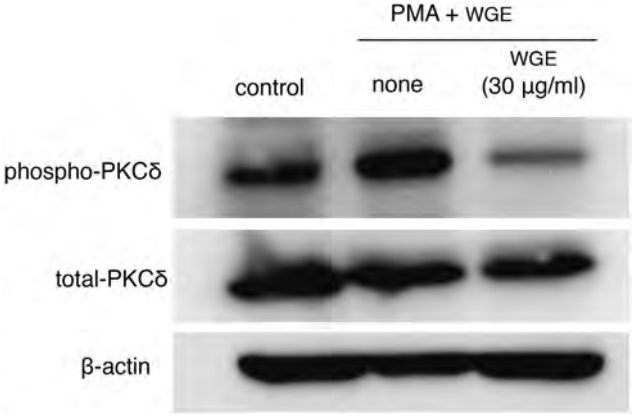

Fig. 7. **Effect of WGE on PMA-induced Tyr^{311} phosphorylation of PKCδ.**
HeLa cells were serum-starved and treated with WGE (30 μg/ml each) for 24 h before stimulation with 100 nM PMA for 10 min. Total cell lysates were prepared, and phosphorylation of PKCδ at Tyr^{311} was determined using immunoblot analysis.

Effect of WGE on PMA-induced IL-33 gene expression in Swiss 3T3 cells

In Swiss 3T3 cells, stimulation with PMA up-regulates IL-33 gene expression. Thus, we investigated the effect of WGE on the PMA-induced up-regulation of IL-33 gene expression in Swiss 3T3 cells. Treatment with WGE dose dependently suppressed PMA-induced IL-33 gene up-regulation (Fig. 9).

DISCUSSION

In the present study, we revealed that hot water extract of WGE suppressed H1R and IL-33 gene expressions in HeLa cells and Swiss 3T3 cells, respectively. In addition, treatment with WGE alleviated TDI-induced acute nasal symptoms and suppressed TDI-induced H1R, IL-4, IL-5 gene up-regulations in the nasal mucosa of TDI-sensitized rats. Histamine plays an important role for the pathogenesis of acute allergic symptoms (1). WGE suppressed histamine signalling through the inhibition of histamine-induced up-regulation of H1R gene expression. We reported that PKCδ signalling was involved in the expression of H1R gene. As phosphorylation of Tyr^{311} is crucial for the activation of PKCδ, western blot analysis suggests that inhibition of phosphorylation is the underlying mechanism of suppressive effect of WGE on the up-regulation of H1R gene expression.

Treatment with WGE showed the suppression of TDI-induced

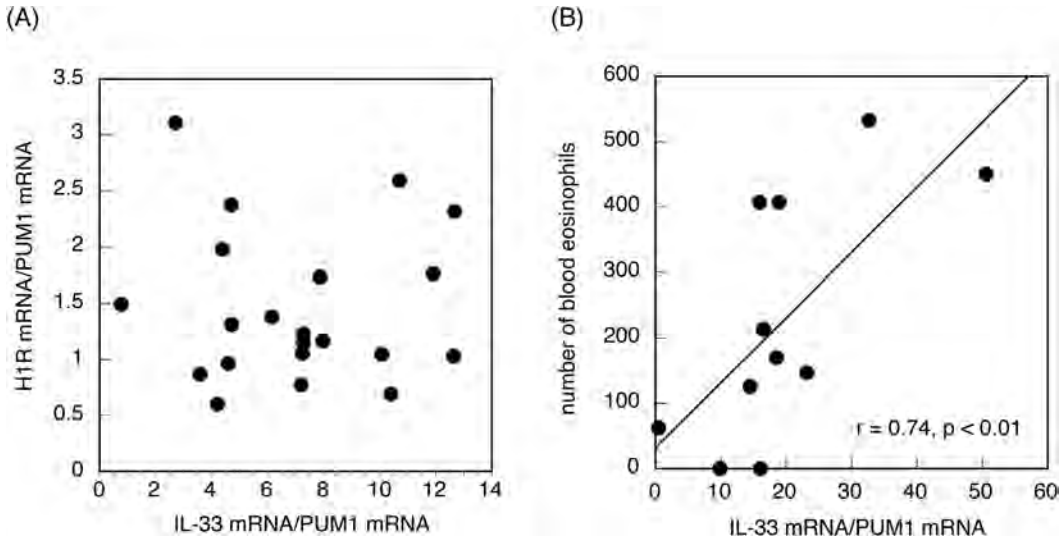

Fig. 8. **Relationship between the expression levels of IL-33 mRNA and that of H1R mRNA and the number of blood eosinophils.**
The scraping of the nasal mucosa from the patients with pollinosis were collected and total RNA was isolated using the RNAqueous Micro Kit as described in (3, 11). IL-33 and H1R mRNA was determined by quantitative real-time RT-PCR. Data for the expression of H1R mRNA were obtained from Mizuguchi *et al*., (3). Correlation was analyzed by Spearman's rank correlation test.

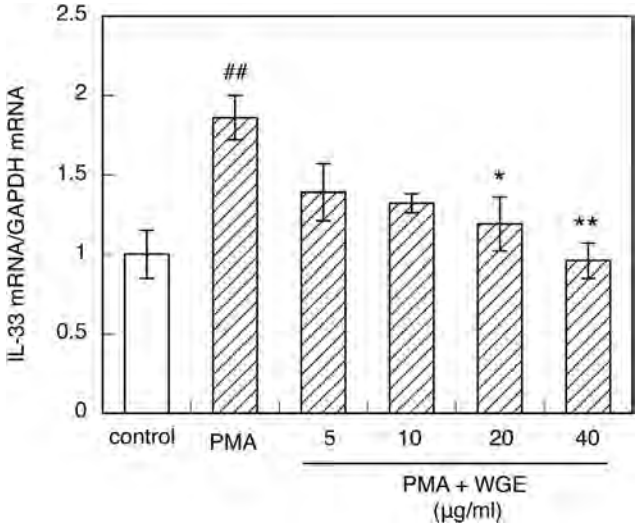

Fig. 9. **Effect of WGE on PMA-induced up-regulation of IL-33 mRNA in Swiss 3T3 cells.**
The concentration of FBS in the culture medium for Swiss 3T3 cells were reduced to 0.5% before subject to the mRNA determination. WGE (5-40 μg/ml) was incubated for 24 h before 100 nM of PMA stimulation. After 3-h stimulation with PMA, the cells were harvested and total RNA was prepared. IL-33 mRNA was determined by quantitative RT-PCR. Data are expressed as means ± S.E.M. (n=3). $^{\#\#}p < 0.01$ vs. control ; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PMA.

IL-9 gene expression although it could not suppress ionomycin-stimulated IL-9 gene up-regulation in RBL-2H3 cells. WGE also suppressed TDI-induced up-regulation of IL-4 and IL-5 genes, which expression levels were shown to be correlated with that of H1R gene (2, 11). The histamine-cytokine network plays important role in allergic inflammation, in which histamine influences the expression and action of several cytokines and some cytokines modulate the production and release of histamine (12, 13, 33). Pretreatment with IL-4 primes the release of histamine in response to FceRI (8, 34). On the other hand, histamine affects the production of IL-4 and IL-5 (35). Furthermore, IL-9 promotes Th2-specific allergic responses and upregulates IL-4 and IL-5 (36, 37). Accordingly, suppression of IL-9 expression affects the expression levels of these Th2 cytokines. IL-4 together with TGF-β was shown to enhance IL-9 production from activated T cells (38). Therefore, it is likely that suppressive effect of IL-9 gene up-regulation by WGE was through the inhibition of histamine–cytokine network.

Our previous report demonstrated that NFAT-mediated IL-9 gene expression is additional signalling responsible for the pathogenesis of acute nasal symptoms (14). And we also showed that suppression of both PKCδ signaling and NFAT signaling markedly alleviated the acute nasal symptoms in TDI-sensitized rats (14). The *in vitro* experiments data showed that WGE and Awa-tea suppressed PKCδ signaling and NFAT signaling, respectively. And treatment with WGE in combination with Awa-tea significantly improved TDI-induced nasal symptoms in TDI-sensitized rats, suggesting the effectiveness of these plants on the acute symptoms.

Eosinophils play an important role for the pathogenesis of the chronic nasal symptoms (1). We showed here that the expression level of IL-33 mRNA was not correlated with that of H1R but correlated with the number of blood eosinophils in the patients with pollinosis. This suggests that suppression of up-regulation of IL-33 gene expression decreases the number of eosinophils, resulting in the improvement of symptoms caused by eosinophilic inflammation. Therefore, the suppression of PMA-induced IL-33 up-regulation by WGE in Swiss 3T3 cells suggests that WG could be effective to alleviate eosinophilic inflammation.

In conclusion, WGE alleviated acute nasal symptoms by the suppression of PKCδ signalling through the inhibition of PKCδ activation. In addition, WGE suppressed IL-33 gene up-regulation that is correlated with the number of blood eosinophils, suggesting that WG could be useful for the improvement of chronic nasal symptoms and eosinophilic inflammation. Our data also suggest that

WG combined with Awa-tea could be useful to alleviate nasal symptoms of allergic rhinitis.

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CONFLICT OF INTEREST

H.I. is an employee of Nab co., ltd. and other authors declare no financial conflicts of interest.

REFERENCES

1. Okubo K, Kurono Y, Ichimura K, Enomoto T, Okamoto Y, Kawauchi H, Suzaki H, Fujieda S, Masuyama K ; Japanese Society of Allergology : Japanese guidelines for allergic thinitis. Allrgol Int. 66 : 205-219, 2017
2. Mizuguchi H, Hatano M, Matsushita C, Umehara H, Kuroda W, Kitamura Y, Takeda N, Fukui H : Repeated pre-treatment with antihistamines suppresses transcriptional upregulations of histamine H1 receptor and interleukin-4 genes in toluene-2,4-diisocyanate-sensitized rats. J Pharmacol Sci 108 : 480-486, 2008
3. Mizuguchi H, Kitamura Y, Kondo Y, Kuroda W, Yoshida H, Miyamoto Y, Hattori M, Fukui H, Takeda N : Preseasonal prophylactic treatment with antihistamines suppresses nasal symptoms and expression of histamine H1 receptor mRNA in the nasal mucosa of patients with pollinosis. Methods Find Exp Clin Pharmacol 32 : 745-748, 2010
4. Mizuguchi H, Terao T, Kitai M, Ikeda M, Yoshimura Y, Das AK, Kitamura Y, Takeda N, Fukui H : Involvement of protein kinase Cδ/extracellular signal-regulated kinase/poly(ADP-ribose) polymerase-1 (PARP-1) signaling pathway in histamine- induced up-regulation of histamine H1 receptor gene expression in HeLa cells. J Biol Chem 286 : 30542-30551, 2011
5. Matsushita C, Mizuguchi H, Niino H, Sagesaka Y, Masuyama K, Fukui H : Identification of epigallocatechin-3-O-gallate as an active constituent in tea extract that suppresses transcriptional up-regulation of the histamine H1 receptor and interleukin-4 genes. J Transl Med 25 : 133-142, 2008
6. Hattori M, Mizuguchi H, Baba Y, Ono S, Nakano T, Zhang Q, Sasaki Y, Kobayashi M, Kitamura Y, Takeda N, Fukui H : Quercetin inhibit transcriptional up-regulation of histamine H1 receptor via suppressing kinase C-δ/ extracellular signal-regulated kinase/poly(ADP-ribose) polymerase-1 signaling pathway in HeLa cells. Int Immunopharmacol 15 : 232-239, 2013
7. Mizuguchi H, Nariai Y, Kato S, Nakano T, Kanayama T, Kashiwada Y, Nemoto H, Kawazoe K, Takaishi Y, Kitamura Y, Takeda N, Fukui H : Maackiain is a novel antiallergic compound that suppresses transcriptional upregulation of the histamine H1 receptor and interleukin-4 genes. Pharmacol Res Perspect 3 : e00166, http : //dx.doi.org/10.1002/prp2.166, 2015
8. Bischoff SC, Sellge G, Lorentz A, Sebald W, Raab R, Manns MP : IL-4 enhances proliferation and mediator release in mature human must cells. Proc Natl Acad Sci USA 96 : 8080-8085, 1999
9. Tepper RI, Levinson DA, Stanger BZ, Campos-Torres J, Abbas AK, Leder P : IL-4 induces allergy like inflammatory disease and alters T cell development in transgenic mice. Cell 62 : 457-467, 1990
10. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD : IL-13 central mediator of allergic asthma. Science 282 : 2258-2261, 1998
11. Kitamura Y, Mizuguchi H, Ogishi H, Kuroda W, Hattori M, Fukui H, Takeda N : Preseasonal prophylactic treatment with antihistamines suppresses IL-5 but not IL-33 mRNA expression in the nasal mucosa of patients with seasonal allergic rhinitis caused by Japanese ceder pollen. Acta OtoLalyngol 132 : 434-438, 2012
12. Marone G, Granata F, Spadaro G, Genovese A, Triggiani M : The histamine-cytokine network in allergic inflammation. J Allergy Clin Immunol 112 : S83-S88, 2003
13. Igaz P, Novak I, Lazar E, Horvath B, Heninger E, Faauls A : Bidirectional communication between histamine and cytokines. Inflamm Res 50 : 123-128, 2001
14. Mizuguchi H, Orimoto N, Kadota T, Kominami T, Das AK, Sawada A, Tamada M, Miyagi K, Adachi T, Matsumoto M, Kosaka T, Kitamura Y, Takeda N, Fukui H : Suplatast tosilate alleviates nasal symptoms through the suppression of nuclear factor of activated T-cells-mediated IL-9 gene expression in toluene-2,4-diisocyanate-sensitized rats. J Pharmacol Sci 130 : 151-158, 2016
15. Okubo K, Okuda M : Time-course changes in nasal airway resistance after house dust antigen challenge : with special reference to late phase response. Allergol Int 47 : 225-232, 1998
16. Ichikawa K, Okuda M, Naka F, Yago H : The sources of chemical substances in allergic nasal fluid. Rhinology 29 : 143-149, 1991
17. Oboki Allergy Asthma Immunol Res 3 : 81-88, 2011
18. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, von Mutius E, Farrall M, Lathrop M, Cookson WO, GABRIEL Consortium : A large-scale, consortium-based genome wide association study of asthma. N Engl J Med 363 : 1211-1221, 2010
19. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Goman DM, Bazan JF, Kastelein RA : IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23 : 479-490, 2005
20. Oboki K, Ohno T, Kajiwara N, Saito H, Nakae S. IL-33 and IL-33 receptors in host defense and diseases. Allergol Int 59 : 143-160, 2010
21. Ali K, Maltese F, Choi YH, Verpoorte R : Metabolic constituents of grapevine and grape-derived products. Phytochem Rev 9 : 357-378, 2010
22. Kim JY, Park SH, OH HM, Kwak SC, Baek JM, Lee MS, Rho MC, Oh J : Ampelopsis brevipedunculata extract prevents bone loss by inhibiting osteoclastogenesis in vitro and in vivo. Molecules 19 : 18465-18478, 2014
23. Yabe, N, Matsui, H : Ampelopsis brevipedunculata (Vitaceae) extract inhibits a progression of carbon tetrachloride-induced hepatic injury in the mice. Phytomedicine 7 : 493-498, 2000
24. Wu MJ, Yen JH, Wang L, Weng CY : Antioxidant activity of Porcelainberry (Ampelopsis brevipedunculata (Maxim.) Trautv.). Am J Chin Med 32 : 681-693, 2004
25. Sogawa M, Seura T, Kohno S, Hirasaka K, Yamaguchi Y, Takayanagi R, Harada A, Okumura Y, Yamamoto S, Kishi K, Nikawa T : Awa (Tokushima) lactate-fermented tea as well as green tea enhance the effect of diet restriction on obesity in

rats. J Med Invest 56 : 42-48, 2009
26. Hiasa M, Kurokawa M, Ohta K, Esumi T, Akira H, Niki K, Yagi Y, Echigo N, Hatakeyama D, Kuzuhara T : Identification and purification of resorucinol, an antioxidant specific to Awaban (pickled and anaerobically fermented) tea. Food Research International 54 : 72-80, 2013
27. Hiasa M, Kurokawa M, Akira H, Harada M, Niki K, Ohta K, Shoji M, Echigo N, Kuzuhara T : Suppression of increased blood glucose levels in mice by Awa-ban tea following oral administration of mono- and disaccharides. Journal of Functional Foods 8 : 188-192, 2014
28. Kitamura Y, Das AK, Murata Y, Maeyama K, Dev S, Wakayama Y, Kalubi B, Takeda N, Fukui H : Dexamethasone suppresses histamine synthesis by repressing both transcription and activity of HDC in allergic rats. Allergol Int 55 : 279-286, 2006
29. Das AK, Yoshimura S, Mishima R, Fujimoto K, Mizuguchi H, Dev S, Wakayama Y, Kitamura Y, Horio S, Takeda N, Fukui H : Stimulation of histamine H1 receptor up- regulates histamine H1 receptor itself through activation of receptor gene transcription. J Pharmacol Sci 103 : 374-382, 2007
30. Kitamura Y, Miyoshi A, Murata Y, Maeyama K, Takeda N, Fukui H : Effect of glucocorticoid on upregulation of histamine H1 receptor mRNA in nasal mucosa of rats sensitized by exposure to toluene diisocyanate. Acta Otolaryngol 124 : 1053-1058, 2004
31. Steinberg SF : Structural basis of protein kinase C isoform function. Physiol Rev 88 : 1341-1378, 2008
32. Sakashita M, Yoshimoto T, Hirota T, Harada M, Okubo K, Osawa Y, Fujieda S, Nakamura Y, Yasuda K, Nakanishi K, Tamari M : Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. Clin Exp Allergy 38 : 1875-1881, 2008
33. Abdelaziz M, Devalia J, Khair O, Bayram H, Prior A, Davies R : Effect of fexofenadine on eosinophil-induced changes in epithelial permeability and cytokine release from nasal epithelial cells of patients with seasonal allergic rhinitis. J Allergy Clin Immunol 101 : 410-420, 1998
34. Yamaguchi M, Sayama K, Yano K, Lantz CS, Noben-Trauth N, Ra C, Costa JJ, Galli SJ : IgE enhances Fcε receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells : synergistic effect of IL-4 and IgE on human mast cell Fcε receptor I expression and mediator release. J Immunol 162 : 5455-5465, 1999
35. Krouwels FH, Hol BE, Lutter R, Bruinier B, Bast A, Jansen HM, Out TA : Histamine affects interleukin-4, interleukin-5, and interferon-g production by human T cell clones from airways and blood. Am J Respir Cell Mol Biol 18 : 721-730, 1998
36. Hauber HP, Bergeron C, Hamid Q : IL-9 in allergic inflammation. Int Arch Allergy Immunol 134 : 79-87, 2004
37. Temann UA, Ray P, Flavell RA : Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. J Clin Invest 109 : 29-39, 2002
38. Schmitt E, Germann T, Goedert S, Hoehn P, Huels C, Koelsch S, Kühn R, Müller W, Palm N, Rüde E : IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. J Immunol 153 : 3989-3996, 1994