ORIGINAL

Identification of pyrogallol from Awa-tea as an anti-allergic compound that suppresses nasal symptoms and IL-9 gene expression

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Abstract : As the expression level of allergic disease sensitive genes are correlated with the severity of allergic symptoms, suppression of these gene expressions could be promising therapeutics. We demonstrated that protein kinase Cδ/heat shock protein 90-mediated H1R gene expression signaling and nuclear factor of activated T-cells (NFAT)-mediated IL-9 gene expression signaling are responsible for the pathogenesis of pollinosis. Treatment with Awa-tea combined with wild grape hot water extract suppressed these signaling and alleviated nasal symptoms in toluene-2,4-diisocyanate (TDI)-sensitized rats. However, the underlying mechanism of its anti-allergic activity is not elucidated yet. Here, we sought to identify an anti-allergic compound from Awa-tea and pyrogallol was identified as an active compound. Pyrogallol strongly suppressed ionomycin-induced up-regulation of IL-9 gene expression in RBL-2H3 cells. Treatment with pyrogallol in combination with epinastine alleviated nasal symptoms and suppressed up-regulation of IL-9 gene expression in TDI-sensitized rats. Pyrogallol itself did not inhibit calcineurin phosphatase activity. However, pyrogallol suppressed ionomycin-induced dephosphorylation and nuclear translocation of NFAT. These data suggest pyrogallol is an anti-allergic compound in Awa-tea and it suppressed NFAT-mediated IL-9 gene expression through the inhibition of dephosphorylation of NFAT. This might be the underlying mechanism of the therapeutic effects of combined therapy of pyrogallol with antihistamine. J. Med. Invest. 67 : 289-297, August, 2020

Keywords **:** *allergic rhinitis, calcineurin, histamine H_1 receptor gene, Interleukin-9 gene, nuclear factor of activated T-cells*

INTRODUCTION

Pollinosis is a seasonal allergic rhinitis (AR) 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 and its action is mainly mediated through the activation of histamine H_1 receptor (H1R). Therefore, antihistamines that antagonize H1R or suppress H1R constitutive activity as inverse agonist are widely used as the first-line medicine for pollinosis. Previously, we have demonstrated that H1R gene expression is correlated with the severity of symptoms in toluene-2,4-diisocyanate (TDI)-sensitized rats and patients with pollinosis (2-4). We also showed that protein kinase Cδ (PKCδ) signaling was involved in H1R gene expression, and that suppression of the H1R gene up-regulation alleviated nasal symptoms in TDI-sensitized rats (5-11).

In addition to histamine, Th2 cytokines, including Interleukin (IL)-4, IL-5, IL-9, and IL-13 are also involved in the pathogenesis of allergic symptoms (12). We have shown that Th2 cytokine gene expressions were up-regulated in TDI-sensitized rats, and suplatast, which did not suppress PKCδ signaling, suppressed TDI-induced up-regulation of Th2 cytokine gene expressions in TDI-sensitized rats (13). In addition, combination of suplatast with antihistamine markedly alleviated nasal symptoms in TDI-sensitized rats (14). Recently, we have reported that IL-9 gene is an additional allergy sensitive gene in TDI-sensitized rats and nuclear factor of activated T-cells (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).

It is well known that fermentation of food produces many bioactive compounds such as antioxidant, anti-inflammatory, anti-diabetic, and anti-atherosclerotic activity (15). Previously, we showed that treatment with green-tea improved nasal symptoms and suppressed up-regulated H1R gene expression in TDI-sensitized rats (10). Awa-tea is a unique traditional tea in Tokushima, which consists of leaves fermented with *Lactobacillus pentosus and Lactobacillus plantarum*. It was reported that Awa-tea has anti-obesity activity (16). It was also reported that resorcinol was an active compound for its anti-oxidant activity (17). Furthermore, Awa-tea suppressed mono- and disaccharides-induced increase in the blood glucose level in mice (18). Awa-tea has been traditionally used for the improvement of allergic symptoms in Tokushima, Japan. We have reported that treatment with Awa-tea combined with wild grape hot water extract that suppresses PKCδ signaling, markedly alleviated TDI-induced nasal symptoms and up-regulation of IL-9 gene expression in

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TDI-sensitized rats (19). However, the underlying mechanism of its anti-allergic activity is not elucidated yet.

In this paper, we sought to identify an anti-allergic compound from Awa-tea. We investigated the effect of anti-allergic compound on ionomycin-induced IL-9 gene expression in RBL-2H3 cells. Then, we evaluated the effect of this compound on nasal symptoms in TDI-sensitized rats. We isolated and identified pyrogallol as an active compound in Awa-tea. Pyrogallol suppressed up-regulation of Th2 cytokine genes including IL-4, IL-9, and IL-13. Treatment with pyrogallol improved nasal symptoms in TDI-sensitized rats. Combined treatment with pyrogallol with epinastine, an antihistamine, also markedly alleviated nasal symptoms in TDI-sensitized rats. Pyrogallol suppressed dephosphorylation and nuclear translocation of NFAT in response to ionomycin stimulation in BHK cells overexpressing NFAT. These results suggest that pyrogallol improved nasal symptoms through the suppression of NFAT-mediated IL-9 gene up-regulation in TDI-sensitized rats.

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 the control, sensitized with TDI (Wako Pure Chemical, Osaka), and test groups, with 4 rats in each group. All animal experiments were approved by the Ethical Committee for Animal Research of Tokushima University.

Sensitization and provocation with TDI

Rats were sensitized with TDI by the method described by Kitamura *et al*. with slight modifications (20). 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). Awa-tea leaves (10 g) were boiled for 10 min in 1 L of water, and then extract was centrifuged, filtered, and freeze-dried extract was kept at -30°C until use. Awa-tea extract, re-dissolved in water on the day of the experiments, pyrogallol, and epinastine were 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*. (21). 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.

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

Nasal response	Score			
	0	1	2	3
Watery rhinorrhea	(-)	At nostril	Between 1 and 3	Drops of discharges from nose
Swelling and redness	(-)	Slightly swollen	Between 1 and 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. RBL-2H3 cells were cultured in Minimum Essential Medium Eagle containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/ml streptomycin. The cells were cultured to 70% confluency in six-well dishes. The cells were treated with Awa-tea or pyrogallol for 24 h before treatment with 1 μM of iomomycin. After the stimulation with ionomycin for 2 h, the cells were harvested, and total RNA was prepared using RNAiso Puls reagent. In the case of IgE/antigen stimulation, RBL-2H3 cells were treated with Awa-tea or pyrogallol for 6 h, then, treated with 100 ng/ml of monoclonal anti-dinitrophenyl (DNP)-IgE (Sigma-Aldrich) for 12 h. After the stimulation with anti-DNP-IgE, the cells were stimulated with 100 ng/ml of DNP-albumin (Sigma-Aldrich) for 1 h, then the cells were harvested and total

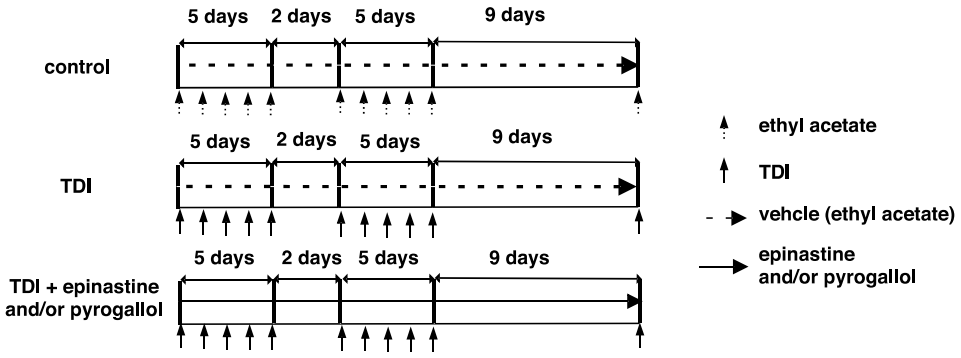

Fig 1. Experimental protocol.
The rats were sensitized by intranasal application of 10 μl of 10% TDI for 2 weeks. After 1 week, the nasal symptoms were provoked using 10 μl of 10% TDI. The control group was sensitized with ethyl acetate only. Water extract of tea leaves, epinastine, and pyrogallol were administered orally once a day for 3 weeks. The drugs were administered 1 h before the rats were treated with TDI.

RNA were prepared. The RNA samples (2 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 used in this study are summarized in Table 2. Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). To standardize the starting material, Rodent GAPDH Control Reagents (VIC™ Probe ; Applied Biosystems) were used, and the data were expressed as the ratio to GAPDH mRNA.

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

Primer/probe name	Sequence
Rat IL-4 mRNA	
Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
Anti-sense primer	5'-CACCGAGAACCCCAGACTTG-3'
Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
Rat IL-9 mRNA	
Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
Anti-sense primer	5'- AGCGGAGCCTCTTCCAAGTAA -3'
Probe	FAM-CTTCTCTCGAACGGACTCAGATACC ACCT-TAMRA

For measuring rat IL-13 mRNA, primer probe kit from Applied Biosystems (Rn00587615) was used.

Isolation and identification of active compound from Awa-tea

Awa-tea leaves (10 g) was mixed with 1 L of pure water at 90°C for 10 min. The extract was centrifuge at 3000xg for 10 min, resulting supernatant was passed through a filter paper, and the filtrate was freeze-dried (730 mg). The extract was re-dissolved in water and extracted using ethyl acetate. The water layer was then fractionated using butanol. The yield of each fraction was as follows ; ethyl acetate fraction (249 mg), butanol fraction (213 mg), and water fraction (257 mg). Suppressive activity of IL-4 gene expression in obtained fractions were analyzed. Next, active fraction was applied on a Sephadex LH-20 column (2.5 ϕ × 21 cm), and the column was eluted isocratically using acetone. According the developmental profiles of thin layer chromatography, fractions were classified 3 fractions and the suppressive activity of IL-9 gene expression of each fraction was assayed. The yield of each fraction was as follows ; fraction-1 (52.7 mg), fraction-2 (64.1 mg), and fraction-3 (98.7 mg). The structure of the constituents in each fraction was identified using ^{1}H-NMR and ^{13}C-NMR.

Calcineurin assay

Phosphatase activity of calcineurin was calorimetrically assayed using calcineurin cellular activity assay kit (Enzo, Farmingdale, NY, USA). Briefly, recombinant calcineurin (40 U/assay) was incubated with or without pyrogallol, INCA-6, an NFAT inhibitor (Tocris, Bristol, UK), or phosphatase inhibitor mix (PhosSTOP, Sigma-Aldrich) for 30 min at 30°C. RII phosphopeptide was used as a substrate. The reaction was stopped by the addition of 100 μl of BIOMOL reagent and further incubated for 30 min at room temperature. Then, absorbance at 620 nm derived phosphates released by phosphatase reaction was measured using the Infinite M200 microplate reader (Tecan Japan, Kanagawa, Japan).

Immunoblot analysis

For immunoblot analysis, we used BHK-21 cells overexpressing EGFP-fused NFAT (22). BHK-21 cells were treated with pyrogallol (50-300 μM) for 10 min. Then, the cells were stimulated with 1 μM ionomycin for 30 min. After the stimulation with ionomycin, the cells were harvested in Tris-buffered saline (TBS) containing proteinase inhibitors (cOmplete, Mini, Sigma-Aldrich) and phosphatase inhibitors (PhosSTOP), and whole cell extracts were prepared by sonication. For the immunoblot analysis, 30 μg of each protein sample was separated by 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was briefly rinsed in TBS containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco). The membrane was then incubated with monoclonal anti-GFP antibody (JL-8) (BioRad) overnight at 4 °C. To detect β-actin, β-actin (8H10D10) mouse monoclonal antibody (Cell Signaling Technology Japan, Tokyo). Goat anti-mouse-HRP conjugate (Bio-Rad) was used as the secondary antibody, and proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

Subcellular localization of NFAT

BHK-21 cells were cultured in Dulbecco Modified Eagle Medium, high glucose containing 10% fetal bovine serum (FBS), 5 mg/ml puromycin (Nacalai Tesque, Kyoto Japan), 100 IU/mL penicillin, and 100 μg/ml streptomycin. The cells were placed onto 35-mm glass-bottomed dishes (AGC Techno Glass, Shizuoka, Japan). The cells were treated with pyrogallol (100 μM) for 24 h before stimulation with 1 μM of ionomycin. After stimulation, the cells were washed once with Ca^{2+}-free PBS (PBS(-)) and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at 4°C. Nuclear stain was conducted by the addition of 0.25 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS(-) containing 1% BSA and 0.1% Tween 20 for 10 min. The subcellular localization of EGFP-tagged NFAT was determined using a confocal laser microscope (LSM510 ; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The results are indicated as means ± S.E.M. The statistical analysis was performed by One-way ANOVA with Dunnett's test. P values less than 0.05 were considered significant.

RESULTS

Effect of fermentation of tea leaves on IgE/antigen (Ag)-stimulated up-regulation of IL-4 gene expression in RBL-2H3 cells and TDI-induced nasal symptoms in TDI-sensitized rats

It is well known that fermentation of food produces many bioactive compounds (15). As Awa-tea is tea fermented by lactic acid bacteria, it is speculated that Awa-tea has additional bioactive compounds. It is also known that Th2 cytokines are involved in the pathogenesis of allergic symptoms and IL-4 plays a central role in allergic inflammation (23). Thus, we investigated the effect of fermentation of tea leaves on IgE/Ag-stimulated IL-4 gene up-regulation in RBL-2H3 cells. The extract from fermented leaves dose dependently suppressed IgE/Ag-stimulated up-regulation of IL-4 gene expression in RBL-2H3 cells, while, the extract from non-fermented leaves showed weaker suppression (Fig. 2). Application of TDI for 3 weeks caused nasal symptoms in TDI-sensitized rats. Treatment with Awa-tea extract suppressed TDI-induced nasal symptoms in TDI-sensitized rats (Fig. 3). These finding suggest that fermentation produced new anti-allergic compounds that suppress IL-4 gene up-regulation.

Isolation and identification of active compound in Awa-tea extract

Water extract of Awa-tea leaves was firstly extracted using ethyl acetate. Then, the water layer was fractionated using butanol. Ethyl acetate fraction showed suppression of IgE/Ag-stimulated IL-4 gene up-regulation in RBL-2H3 cells (Fig. 4A). Either water fraction and butanol fraction showed no suppressive activity (Fig. 4 B and C). Next, active fraction was applied on a Sephadex LH-20 column, and the column was eluted isocratically using acetone. According the developmental profiles of thin layer chromatography, fractions were classified 3 fractions. All fractions showed suppression of IgE/Ag-stimulated up-regulation of IL-4 gene expression in RBL-2H3 cells (Fig. 4 D-F). Structural studies showed major constituent of fraction-1, fraction-2, and fraction-3 was pyrogallol, epicatechin (EC), and epigallocatechin (EGC), respectively. Therefore, we investigated the effect of these compound on IL-4 gene up-regulation. Pyrogallol suppressed IgE/Ag-stimulated up-regulation of IL-4 gene expression (IC_{50} = 103 μM, Fig. 5A). EC and EGC also suppressed IL-4 gene up-regulation, but less effective (IC_{50} = 480 μM and 147 μM, respectively, Fig. 5B and C). Among these three compounds, EC and EGC were detected in the extracts from both fermented and non-fermented tea leaves. On the other hand, pyrogallol was detected only in the extract from fermented tea leaves (Awa-tea).

Effect of pyrogallol on the expression of Th2 cytokine genes in RBL-2H3 cells

Data suggest that anti-allergic compound in Awa-tea is pyrogallol. Thus, we investigated the effect of pyrogallol on gene up-regulation of Th2 cytokine genes. Pyrogallol suppressed ionomycin-induced up-regulation of IL-4 and IL-9 gene expressions in RBL-2H3 cells (IC_{50} = 152 μM and 33.3 μM, respectively, Fig. 6 A and B). Pyrogallol also suppressed IgE/Ag-stimulated IL-13 gene up-regulation in RBL-2H3 cells (IC_{50} = 96.5 μM, Fig. 6C).

Effect of pyrogallol on TDI-induced nasal symptoms and IL-9 gene up-regulation in TDI-sensitized rats

Application of TDI for 3 weeks caused nasal symptoms in TDI-sensitized rats. Pretreatment with pyrogallol in combination with epinastine for 3 weeks improved TDI-induced nasal symptoms compared with epinastine alone (Fig. 7A and B). Application of TDI for 3 weeks also up-regulated IL-9 gene expression. Treatment with pyrogallol alone suppressed TDI-induced IL-9 gene up-regulation in nasal mucosa of TDI-sensitized rats

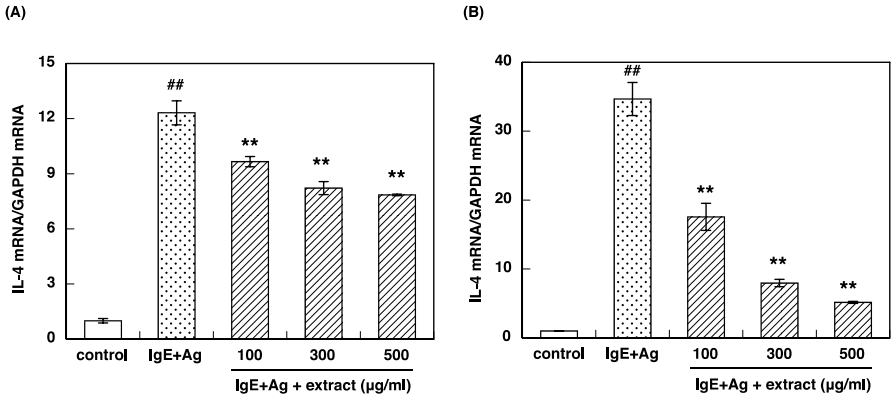

Fig 2. Effect of fermentation of tea leaves on the suppression of IL-4 gene up-regulation in RBL-2H3 cells. RBL-2H3 cells were treated with the extract from non-fermented (A) or fermented tea leaves (B) for 6 h, then, treated with 100 ng/ml of monoclonal anti-DNP-IgE for 12 h. After the stimulation with anti-DNP-IgE, the cells were stimulated with 100 ng/ml of DNP-albumin for 1 h, then the cells were harvested, and total RNA were prepared. IL-4 mRNA was determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). ##, $p < 0.01$ vs. control. **, $p < 0.01$ vs. IgE+Ag.

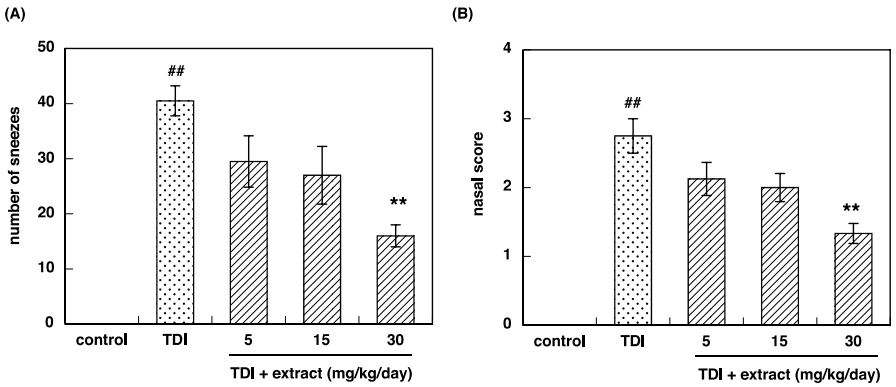

Fig 3. Effect of the extract from fermented tea leaves (Awa-tea) on TDI-induced nasal symptoms in TDI-sensitized rats.
The rats were sensitized and provoked as described in the Materials and Methods. The numbers of sneezes (A) and the nasal score (B) were measured over the 10-min period immediately after TDI provocation. The data were expressed as means ± S.E.M. (n = 4). ##, $p < 0.01$ vs. control. **, $p < 0.01$ vs. TDI.

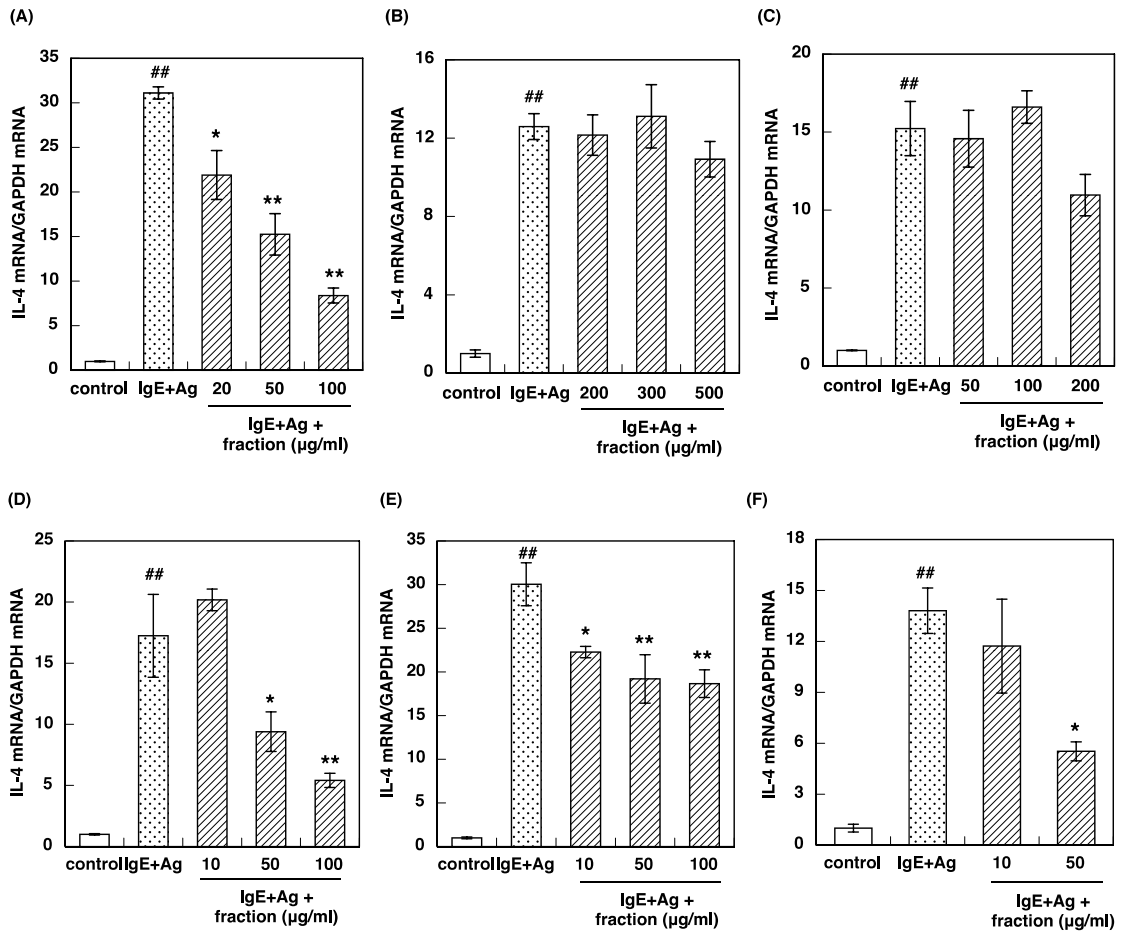

Fig 4. Isolation of pyrogallol from Awa-tea extract.
Awa-tea leaves was extracted with 1 L of water at 90°C for 10 min. The extract was fractionated using ethyl acetate followed by butanol, and suppressive activity of IL-4 gene expression in ethyl acetate fraction (A), butanol fraction (B), and water fraction (C) were analyzed. (D), (E), and (F) ; Suppressive activity of IL-4 gene expression in fraction-1 (D), fraction-2 (E), and fraction-3 (F) obtained by the elution with acetone from a Sephadex LH20 column. RBL-2H3 cells were treated with fractions as described in Fig. 2, and IL-4 mRNA was determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}$, $p < 0.01$ vs. control. **, $p < 0.01$; *, $p < 0.05$ vs. IgE+Ag.

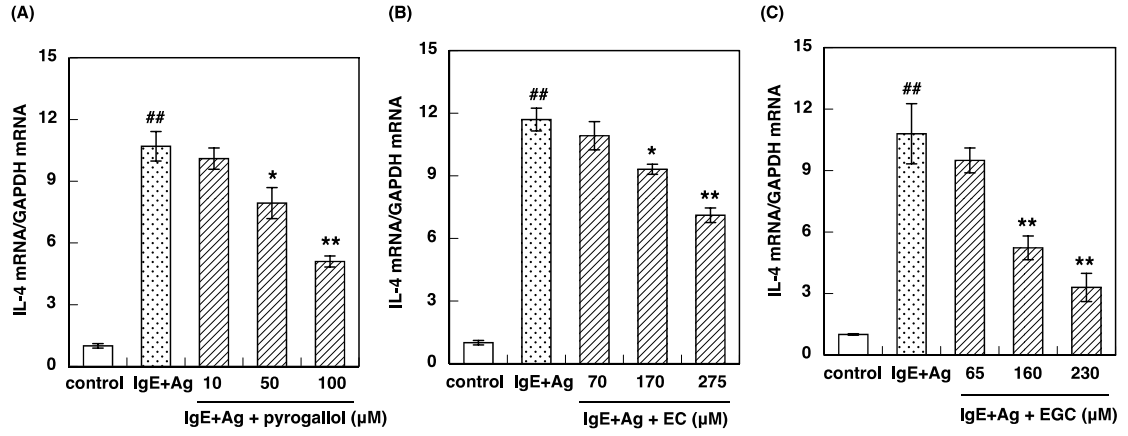

Fig 5. Effect of pyrogallol, epicatechin, and epigallocatechin on the suppression of IL-4 gene up-regulation in RBL-2H3 cells.
RBL-2H3 cells were treated with pyrogallol (A), epicatechin (B), and epigallocatechin (C) as described in Fig. 2, and IL-4 mRNA was determined by real-time quantitative RT-PCR. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}$, $p < 0.01$ vs. control. **, $p < 0.01$; *, $p < 0.05$ vs. IgE+Ag.

(Fig. 7C). Treatment with pyrogallol combination with epinastine also suppressed TDI-induced up-regulation of IL-9 gene expression in nasal mucosa of TDI-sensitized rats. On the other hand, treatment with epinastine alone didn't show any suppression of IL-9 gene up-regulation (Fig. 7C).

Mechanism of suppression of NFAT signaling-mediated IL-9 gene expression by pyrogallol

Our data suggest that pyrogallol suppresses NFAT signaling. NFAT is activated through its dephosphorylation by calcineurin. Thus, we investigated the effect of pyrogallol on calcineurin phosphatase activity. As we expected, phosphatase inhibitor inhibited phosphate release from the substrate RII phosphopeptide (Fig. 8). INCA-6, a calcineurin-NFAT interaction inhibitor, does not indiscriminately block calcineurin substrates other than NFAT, thus, it slightly inhibited calcineurin phosphatase activity. On the other hand, pyrogallol did not inhibit phosphatase activity of calcineurin (Fig. 8). However, immunoblot analysis revealed that pyrogallol suppressed iomomycin-induced dephosphorylation of NFATc1 and NFATc2 by calcineurin (Fig. 9A). Pyrogallol also inhibited ionomycin-induced nuclear translocation of NFATc1 and NFATc2 (Fig. 9B).

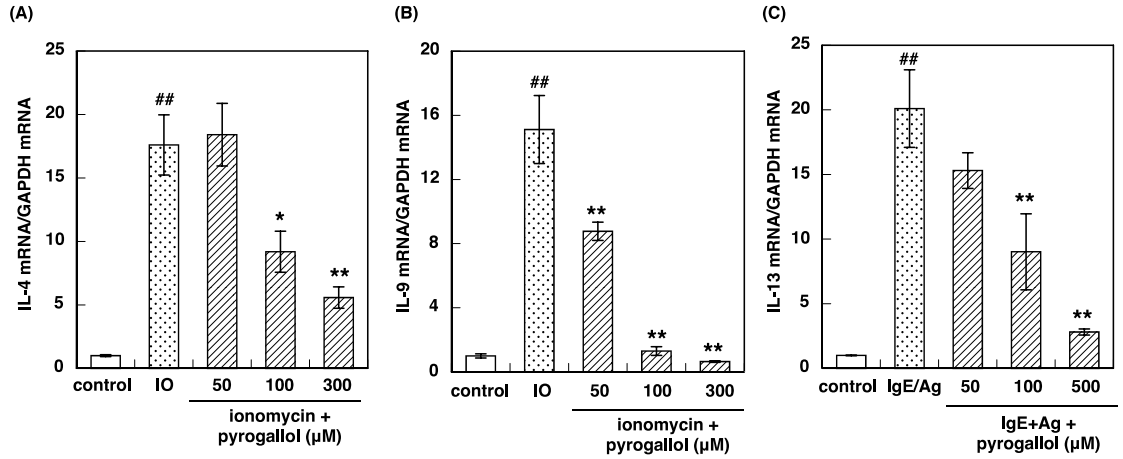

Fig 6. Effect of pyrogallol on the expression of Th2 cytokine genes in RBL-2H3 cells.
RBL-2H3 cells were treated with pyrogallol as described in Fig. 2. In the case of ionomycin stimulation, the cells were treated with pyrogallol for 24 h before treatment with 1 μM of iomomycin. After the stimulation with ionomycin for 2 h, the cells were harvested and total RNA was prepared. The mRNA levels of IL-4 (A), IL-9 (B), and IL-13 (C) were determined by real-time quantitative RT-PCR. IO, ionomycin. The data are expressed as means ± S.E.M. (n = 4). $^{\#\#}$, $p < 0.01$ vs. control. **, $p < 0.01$; *, $p < 0.05$ vs. ionomycin or IgE+Ag.

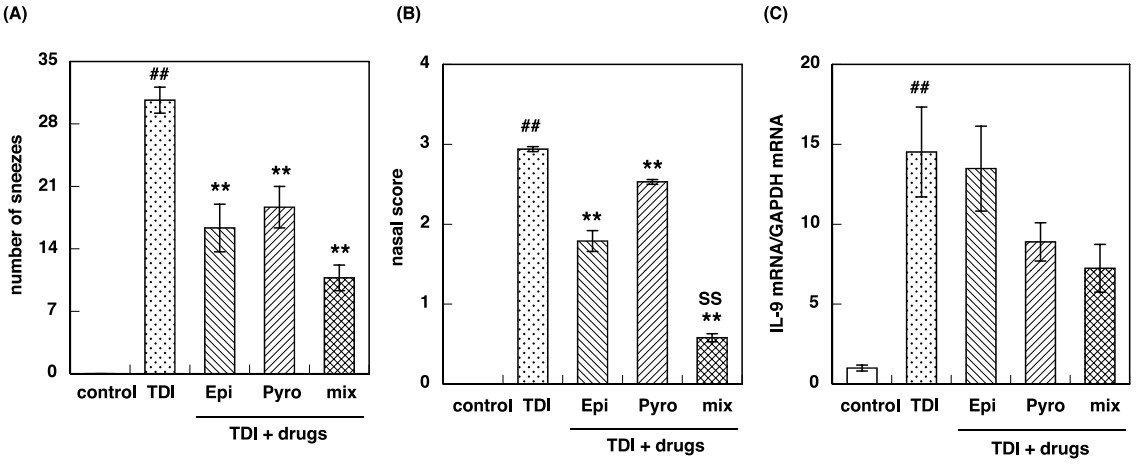

Fig 7. Effect of pyrogallol in combination with epinastine on TDI-induced nasal symptoms and IL-9 mRNA up-regulation in TDI-sensitized rats.
The rats were sensitized and provoked as described in the Materials and Methods. Pyrogallol (24 mg/kg/day) and/or epinastine (24 mg/kg/day) were administered orally 1 h before the TDI sensitization for 3 weeks. The numbers of sneezes (A) and the nasal score (B) were measured over the 10-min period immediately after TDI provocation. The rats were sacrificed 4 h after provocation with TDI, and total RNA was isolated. The mRNA levels of IL-9 (C) was determined by real-time quantitative RT-PCR. Epi, epinastine. Pyro, pyrogallol. Mix, epinastine + pyrogallol. The data were expressed as means ± S.E.M. (n = 4). $^{\#\#}$, $p < 0.01$ vs. control. **, $p < 0.01$ vs. TDI. $^{\$\$}$, $p < 0.01$ vs epinastine.

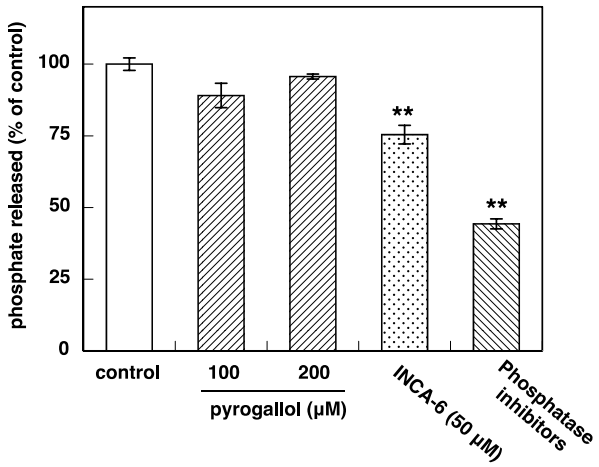

Fig 8. Effect of pyrogallol on calcineurin phosphatase activity.
Recombinant calcineurin and RII phosphopeptide were incubated with pyrogallol or INCA-6, or phosphatase inhibitors for 30 min at 30°C. The reaction was stopped by the addition of 100 μl of BIOMOL reagent and further incubated for 30 min at room temperature. Then, absorbance at 620 nm derived phosphates released by phosphatase reaction was measured. The data are expressed as means ± S.E.M. (n = 4). **, $p < 0.01$ vs. control.

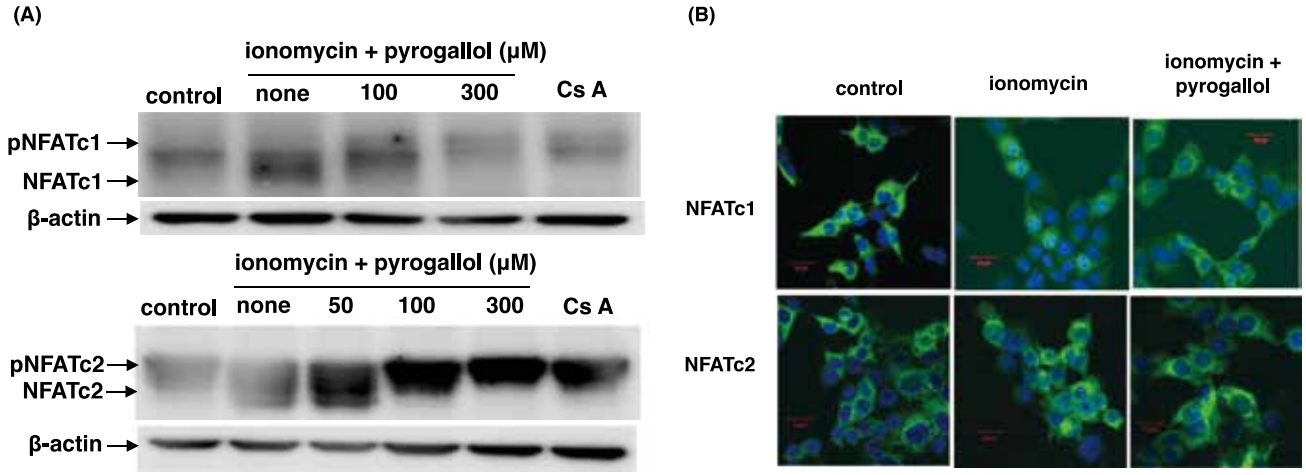

Fig 9. Effect of pyrogallol on dephosphorylation (A) and nuclear translocation (B) of NFAT in response to ionomycin stimulation.
(A), BHK-21 cells that overexpress EGFP-tagged NFATc1 or NFATc2 were treated with or without pyrogallol (50-300 μM) for 10 min. Then, the cells were stimulated with 1 μM ionomycin for 30 min. After the stimulation with ionomycin, total cell lysates were isolated and subjected to immunoblot analysis. Cyclosporin A (2 μM) was used as a positive control. pNFATc1; phospho-NFATc1, pNFATc2; phospho-NFATc2, Cs A; cyclosporin A. In (B), RBL-2H3 cells were plated onto 35-mm glass-bottomed dishes. The cells were treated with pyrogallol (100 μM) for 10 min before stimulation with 1 μM of ionomycin. After the stimulation with ionomycin for 30 min, the cells were fixed with 4% paraformaldehyde for 10 min at 4°C. Nuclear stain was conducted by the addition of 0.25 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS(-) containing 1% BSA and 0.1% Tween 20 for 10 min. The subcellular localization of EGFP-tagged NFAT was determined using a confocal laser microscope. Scale bars = 20 μm.

DISCUSSION

In the present study, we isolated anti-allergic compound from hot water extract of Awa-tea leaves and identified pyrogallol as an active compound. Accumulating evidences suggest the biological effectiveness of fermented foods, especially fermented by lactic acid bacteria (15). During fermentation, lactic acid bacteria synthesize vitamins and minerals, and produce many biologically active compounds. In addition to histamine, Th2 cytokines, including IL-4, IL-5, IL-9, and IL-13 also involve in the pathogenesis of allergic symptoms (12). Among them, IL-4 plays a central role in allergic inflammation, which is associated with the development of T lymphocytes, acts as a growth factor for Th2 cells, stimulates IgE synthesis, and is involved in mast cell activation (23). Fermented tea extract (Awa-tea) showed stronger suppression of IL-4 gene expression than non-fermented tea extract in RBL-2H3 cells, suggesting that Awa-tea contains additional compounds that do not exist in non-fermented tea extract. Therefore, we tried to purify the active compound in Awa-tea by monitoring IL-4 suppressive activity, and pyrogallol was identified as an active compound in Awa-tea. Besides suppression of IL-4 and IL-13 gene up-regulation, pyrogallol strongly suppressed

ionomycin-induced up-regulation of IL-9 gene expression. Furthermore, treatment with pyrogallol combined with epinastine that suppresses H1R gene expression signaling, markedly alleviated TDI-induced nasal symptoms and IL-9 up-regulation in TDI-sensitized rats. Previously, we showed that suplatast strongly suppressed ionomycin-induced IL-9 mRNA up-regulation in RBL-2H3 cells, in which NFAT signaling is involved (14). We also showed that treatment with suplatast in combination with antihistamines markedly alleviated TDI-induced nasal symptoms and suppressed TDI-induced up-regulation of IL-9 gene expression in TDI-sensitized rats (14). These data suggest that, similar to suplatast, pyrogallol suppresses NFAT-mediated IL-9 gene expression signaling, the 2nd intracellular signaling responsive for the pathogenesis of pollinosis.

It was reported that IL-9 increased the expression of mast cell proteases and pro-allergic cytokines in cultured mast cells (24). It was also reported that neutralization of IL-9 by anti-IL-9 antibody improved nasal symptoms and reduced the infiltration of eosinophils in the nasal mucosa, in which treatment with anti-IL-9 antibody suppressed Th17 response and increased Treg response (25). These findings suggest that the IL-9 signaling plays an important role in the pathogenesis of pollinosis, and suppression of IL-9 gene up-regulation by pyrogallol is effective for alleviating nasal symptoms in patients with pollinosis.

NFAT is first discovered as a transcription factor that binds to IL-2 promoter to activate cytokine production after T-cell activation (26). It is now well-known that NFAT regulates not only T-cell activation and differentiation but also the function of other immune cells including dendritic cells and B-cells (27). NFAT consists of five isoforms including NFATc1, NFATc2, NFAT3, NFAT4, and NFAT5 (28). Among them, NFATc1 and NFATc2 are most prominent NFAT family member in immune response. In NFAT signaling, NFAT is activated through its dephosphorylation by calcineurin. Then, activated NFAT is translocated into the nucleus, where it regulates the expression of genes that are involved in cell survival, proliferation and secretion of inflammatory mediators in cooperation with multiple transcriptional partners such as AP-1 (29). Pyrogallol itself did not inhibit protein phosphatase activity of calcineurin. However, pyrogallol inhibited NFATc1 and NFATc2 dephosphorylation by calcineurin, followed by their translocation into the nucleus, suggesting its suppression of NFAT signaling.

In literature, anti-inflammatory activity of pyrogallol have been reported. For example, pyrogallol inhibited histamine release from RBL-2H3 cells stimulated with a calcium ionophore, A23187 (30). In our hand, pyrogallol as well as cyclosporin A suppressed IgE/Ag-stimulated β-hexosaminidase release in RBL-2H3 cells (Sawada and Mizuguchi unpublished data). These finding suggest the involvement of NFAT signaling in mast cell degranulation. It was reported that pyrogallol also inhibited the *P. aeruginosa*-dependent expression of the pro-inflammatory cytokine IL-6 (31). In TDI-sensitized rats, pyrogallol suppressed TDI-induced up-regulation of IL-6 gene expression in the nasal mucosa of rats (unpublished data). As IL-6 leads to an acute severe systemic inflammatory response known as cytokine storm (32), pyrogallol might be useful for suppressing cytokine storm although precise molecular mechanism for its suppression of IL-6 gene expression is not clear at present.

In conclusion, we identified pyrogallol as an active compound from Awa-tea extract. Pyrogallol alleviated the nasal symptoms and suppressed IL-9 gene up-regulation in TDI-sensitized rats, possibly through the inhibition of NFAT signaling. This might be the underlying mechanism of the therapeutic effects of combined therapy of pyrogallol with antihistamine.

CONFLICT OF INTERESTS

All authors declare no financial conflicts of interest.

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AUTHORS' CONTRIBUTIONS

Tomohiro Nakano and Mitsuhiro Ikeda contributed equally to this work.

HM, NT, and HF designed the study and wrote the manuscript. TN, MI, TW, and YK contributed to data collection and analysis. HM performed the statistical analysis. HM, OK, NK, MY, HF, YK, and HF performed interpretation of the results. All authors read and approved the final manuscript.

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