Full Paper

Kujin Suppresses Histamine Signaling at the Transcriptional Level in Toluene 2,4-Diisocyanate-Sensitized Rats

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Abstract. *Kujin*, the dried root of *Sophorae flavescens*, has been used in Chinese folklore medicine against allergy. Evaluation of its anti-allergic potential as well as its mechanism of action has rarely been established. We investigated the effect of *Kujin* on toluene-2,4-diisocyanate (TDI)-induced allergic behavior and related histamine signaling including mRNA levels of histamine H₁ receptor (H1R) and histidine decarboxylase (HDC), H1R and HDC activities, and histamine content in rat nasal mucosa. We also investigated the effect of *Kujin* on the mRNA levels of helper T cell type 2 (Th2)-cytokine genes closely related to histamine signaling. TDI provocation caused acute allergic symptoms accompanied with up-regulations of H1R and HDC mRNAs and increases in HDC activity, histamine content, and [³H]mepyramine binding activity in the nasal mucosa, all of which were significantly suppressed by pretreatment with *Kujin* for 3 weeks. *Kujin* also suppressed the TDI-induced IL-4 and IL-5 mRNA elevations. These data suggest that oral administration of *Kujin* showed anti-allergic activity through suppression of histamine signaling by the inhibition of TDI-induced H1R and HDC mRNA elevations followed by decrease in H1R, HDC protein level, and histamine content in the nasal mucosa of TDI-sensitized rats. Suppression of Th2-cytokine signaling by *Kujin* also suggests that it could affect the histamine-cytokine network.

Keywords: allergy, histamine H₁ receptor, histamine signaling, histidine decarboxylase, *Kujin*

Introduction

Kujin is the dried root of *Sophorae flavescens* AITON (English: *Sophorae radix*, Chinese: *Kushen*) of the *Leguminosae* family. This Chinese herb is used extensively in the treatment of allergic diseases and many other pathological conditions for many years in Asian countries. *Kujin* and its active components have been reported to possess anti-microbial (1, 2), anti-inflammatory (3), anti-glycemic (4), anti-asthmatic (5), anti-tumor (6), anti-diarrheal (7), anti-malarial (8), anti-hepatitis-B (9), and anti-pruritic (10) effects. *Kujin* also showed a preventive effect on cardiovascular anaphylaxis

(11) and inhibition of p38 mitogen-activated protein kinase (MAPK) in acute lung injury mice (12). Phytochemical studies of *S. flavescens* have reported the isolation of quinolizidine alkaloids, flavonoids, and triterpenoids (13–16). However, in spite of its extensive use in allergy, little work has been done to justify the usefulness and to elucidate the mechanism behind its effect.

Allergy is defined as hypersensitivity or hyperactivity of the immune system towards undefined foreign objects. People can be allergic to a wide variety of substances. Toluene 2,4-diisocyanate (TDI) is one of the leading causes of occupational allergic diseases in industrialized countries (17). Intranasal application of TDI caused neuropeptide-mediated release of histamine from mast cells in the nasal mucosa and led to the development of nasal allergy-like symptoms such as

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sneezing and watery rhinorrhea in TDI-sensitized guinea pigs and rats (18–23). Although allergic rhinitis that is defined as an IgE-mediated disease could be different from TDI-induced rhinitis that is a non-IgE-mediated disease, nasal allergy-like symptoms induced by TDI are similar to those observed in allergic rhinitis patients (24, 25). In addition to the symptoms, TDI-sensitized rats also display many of the characteristic features of allergic rhinitis in humans including infiltration of eosinophils and mast cells (26); increase in the levels of cytokines (17, 27–29); elevations of histamine H₁-receptor (H1R) mRNA and protein level (22); and increases in histidine decarboxylase (HDC) mRNA level, HDC activity, and histamine content (30), even though histamine release was triggered by neuropeptide, but not IgE in TDI-sensitized animals. Expressions of interleukin (IL)-4 and IL-5 mRNAs were also up-regulated in the nasal mucosa of TDI-sensitized rats after provocation with TDI (31).

Histamine is an important mediator in the initiation and the development of allergic reactions. It is the amine produced by the decarboxylation of histidine, stored in the secretory granules of all mature mast cells including connective tissues, mucosal membranes, skin, and basophils; and its action is mediated through four distinct receptors including H₁, H₂, H₃, and H₄ receptors (32, 33). Many studies have shown that activation of H1R by histamine is responsible for the symptoms of allergic rhinitis, including sneezing, watery rhinorrhea, and nasal itching, and the high efficacy of antihistamines in controlling nasal hypersensitivity symptoms in the eliciting/effector phase of nasal allergy (34–37). In eukaryotes, HDC is the sole enzyme responsible for synthesizing histamine and consequently regulation of the HDC activity is a crucial step for histamine biosynthesis (38).

The allergic reaction is also characterized by a disruption of helper T cell type 1/2 (Th1/Th2) balance toward a pronounced Th2 profile. Th1/Th2 imbalance in the immune system towards the Th2 responses results in the clinical expression of nasal allergy and asthma (39). Th2-cytokines, especially IL-4, IL-5, and IL-13, may play a vital role in the development and maintenance of allergic responses (40–44). IL-4 that regulates the production of IgE by B cells, and the expression of leukotriene C4 synthase by mast cells enhances the Th2 responses (39, 45). Increasing experimental evidences suggest the existence and important role of the histamine–cytokine network in allergic inflammation, in which histamine influences the expression and actions of several cytokines and some cytokines modulate the production and release of histamine (46–48). Pre-treatment of IL-4 primes the release of histamine,

prostaglandins, leukotrienes, and cytokines in response to FcεRI (44, 49). Histamine, on the other hand, modulates the release of IL-4 and interferon gamma (IFN-γ) from T cells (50) and induces the release of IL-5 (51).

In the present study, we investigated the effect of *Kujin* on allergic symptoms as well as on the expressions of the allergy-sensitive genes such as H1R and HDC genes that are involved in histamine signaling using TDI-sensitized nasal allergy model rats. We also investigated the effect of *Kujin* on TDI-induced up-regulations of IL-4, IL-5, and IL-13 mRNAs. Our data suggest that the inhibitory effect of *Kujin* on allergic symptoms was mediated through the suppression of histamine signaling at the transcriptional level in the nasal mucosa of TDI-induced allergy model rats. Our data also suggest that *Kujin* could alleviate allergic symptoms through suppression of the histamine–cytokine network.

Materials and Methods

Animals

Six-week-old male Brown Norway rats weighing 200–250 g (Japan SLC, Hamamatsu) were used for the present study. Rats were allowed free access to water and food and kept in a room maintained at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. The animals were divided into 5 groups comprising of the control, sensitized with TDI (Wako Pure Chemical, Osaka), and test groups, with 4 rats in each group.

Preparation and administration of *Kujin* extract

Root of *S. radix* was procured and authenticated by a local expert. Sixty grams of *Kujin* (“Kojima *Kujin* M”, Lot 902607; Kojima Kampo, Osaka) was added into 1 L of distilled water, boiled for 1.5 h, and filtered twice to remove insoluble materials. Then, the extract was concentrated and used for this study. The yield of freeze-dried extract was 20% (w/w) with respect to the dried root. For standardization of *Kujin* extract, 1 g of freeze-dried extract was dissolved in 10 ml of methanol, and subjected to TLC (pre-coated silica gel 60 F₂₅₄ aluminum sheets; MERK, Darmstadt, Germany). The solvent system for development of the TLC plate was chloroform-methanol (5:1, v/v). As it was known that matrine is one of the major constituents in *Kujin* extract, matrine (1 mg/ml methanol; Sigma, St. Louis, MO, USA) was used as a marker (52). Spots were visualized using Dragendorff’s Reagent (Wako), and matrine was quantified using ImageJ software (NIH, Bethesda, MD, USA). TLC analysis found that 100 μg of *Kujin* extract contains about 1.9 μg of matrine (Fig. 1). The preliminary dose–response experiment showed that *Kujin* extract suppressed TDI-induced mRNA elevation

Fig. 1. Standardization of *Kujin* extract. *Kujin* extract was prepared as described in Materials and Methods. One gram of freeze-dried extract was dissolved in 10 ml of methanol. Matrine (1 mg/ml methanol) was used as a standardization marker. *Kujin* extract (150 µg) or matrine (1 to 5 µg) was applied on a TLC plate and developed using chloroform-methanol (5:1, v/v). A: Photograph of chromatogram showing matrine visualized with Dragendorff's Reagent. B: Determination of matrine content in *Kujin* extract. The spots in panel A are quantified using ImageJ software. Closed squares represent matrine and the closed circle represents *Kujin* extract.

of IL-5 in a dose (6 mg/kg to 300 mg/kg)-dependent manner (data not shown). Thus, the extract was administered orally once a day at a dose of 300 mg/kg for 3 weeks to obtain a clear effect by *Kujin* extract. At this dose, *Kujin* extract did not inhibit the expression of IL-5 mRNA in the normal (i.e., no treatment with TDI) rats. Also, we could not see any toxicity of *Kujin* extract at this dose in all animals subjected to the experiments. The extract was administered 1 h before TDI application when rats were sensitized with TDI.

TDI sensitization and provocation

Sensitization with TDI was performed by the method described by Tanaka et al. (53) with slight modifications. Briefly, 10 µl of a 10% solution of TDI in ethyl acetate (Wako) was applied bilaterally on the nasal vestibule of each rat once a day for five 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 allergy-like symptoms. The control group was sensitized and provoked with 10 µl of ethyl acetate only by the same procedure (Fig. 2).

Assessment of allergy-like symptoms

Nasal allergy-like symptoms were measured during 10 min just after TDI provocation. It includes the number of sneezes and the nasal score, including the extent of watery rhinorrhea, swelling, and redness, measured on a scale ranging from zero to three (Table 1). All animal experiments were approved by the Ethical Committee for Animal Studies of the School of Medicine, The University of Tokushima.

Measurement of mRNA expression level in the nasal mucosa by real-time quantitative reverse transcriptase polymerase chain reaction (real-time RT-PCR)

For measuring mRNA expression, rat nasal mucosa were collected in RNeasy® (Applied Biosystems, Foster City, CA, USA) 4 h after provocation. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Briefly, nasal mucosa was homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of ice-cold TRIzol Reagent. The homogenates were mixed with chloroform and centrifuged at 15,000 rpm for 15 min at 4°C. The aqueous phase containing RNA was trans-

Fig. 2. Experimental protocol. Rats were sensitized with 10 μ l of 10% TDI in ethyl acetate for 2 weeks. After a 1-week interval, provocation was done with 10 μ l of 10 % TDI. The control group was sensitized with ethyl acetate only. In the drug-treated groups, each rat was treated with *Kujin* extract (300 mg/kg) or dexamethasone (1 mg/kg), *d*-chlorpheniramine (30 mg/kg), or olopatadine (30 mg/kg). *Kujin* extract was administered once a day for 3 weeks. The extract was administered 1 h before TDI application when rats were sensitized with TDI. Dexamethasone and H₁-antihistamines were administered intraperitoneally 24 h or 15 min, respectively, before TDI provocation.

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 1 and 3	Drops of discharges
Swelling and redness	(–)	Slightly swollen	Between 1 and 3	Strong swelling with redness

ferred to a new tube, and then the RNA was precipitated by the addition of isopropanol and centrifugation at 15,000 rpm for 15 min at 4°C. The RNA pellet was washed with 70% ice-cold ethanol, air-dried, and dissolved in 20 μ l of diethylpyrocarbonate-treated water. The purity and the yield of total RNA were determined spectrophotometrically. RNA samples were reverse-transcribed to cDNA by incubating at 37°C for 60 min in a reaction volume of 40 μ l in first-strand buffer [250 mM Tris-HCl, pH 8.3, at room temperature containing 375 mM KCl, 15 mM MgCl₂, 0.8 mM concentrations of each deoxyribonucleoside triphosphate (dNTP), 40 μ M oligo (dT) primers, 0.004 units of RNase inhibitor, and 8 units of reverse transcriptase (Superscript II, Invitrogen)]. Then 2 μ l of 2 N NaOH was added and incubation was continued at 65°C for 30 min. The reaction mixture was then neutralized by the addition of 12.8 μ l of 1 M Tris-HCl, pH 8.0. The samples were then heated at 95°C for 10 min and chilled to 4°C for 5 min. Transcripts were subjected to the quantitative real-time PCR analysis for

H1R, HDC, IL-4, IL-5 and IL-13 mRNA expressions using specific primers and probes. TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The sequences of the primers and probes used in this study are listed in Table 2. To measure the differences in starting material, rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe reagents from Applied Biosystems were used as recommended by the manufacturer. The transcripts were utilized for a 40-cycle 3-step PCR using the Gene Amp 7300 Sequence Detection System (Applied Biosystems) in 20 mM Tris-HCl, pH 8.4 containing 50 mM KCl, 3 mM MgCl₂, 200 μ M dNTPs, 900 nM of each primer, and 0.25 units of platinum Taq polymerase. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Identification of the PCR products was carried out using a genetic analysis system (SEQ8000; Beckman Coulter Inc., Fullerton, CA, USA).

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

Primer/probe name		Sequence
H1R mRNA	Sense primer	5'-TATGTGTCCGGGCTGCACT-3'
	Anti sense primer	5'-CGCCATGATAAAACCCAACTG-3'
	Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
HDC mRNA	Sense primer	5'-GCAGCAAGGAAGAACAAAATCC-3'
	Antisense primer	5'-CAACAAGACGAGCGTTCAGAGA-3'
	Probe	FAM-AAAGCGCATGAGCCCAATGCTGCTGAT-TAMRA
IL-4 mRNA	Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
	Anti sense primer	5'-CACCGAGAACCCAGACTTG-3'
	Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
IL-5 mRNA	Sense primer	5'-CAGTGGTGAAAGAGACCTTGATACAG-3'
	Anti sense primer	5'-GAAGCCTCATCGTCTCATTGC-3'
	Probe	FAM-TGTCACCTACCGAGCTCTGTTGACG-TAMRA

For measuring IL-13 mRNA, a primer probe kit from Applied Biosystems (Rn00587615-A1 1113) was used. GAPDH mRNA was measured using Rodent GAPDH Control Reagents (VIC™ Probe) from Applied Biosystems.

[³H]Mepyramine binding assay

Nasal mucosa collected 24 h after provocation, was subjected to the [³H]mepyramine binding assay as described previously with slight modification (53). In brief, nasal mucosa were collected in 1.5 ml of 50 mM Na₂/K-phosphate buffer containing 37.8 mM Na₂HPO₄ and 12.2 mM KH₂PO₄, pH 7.4 and then homogenized using a Polytron homogenizer. The homogenate was centrifuged at 18,000 rpm for 30 min at 4°C. The pellet was resuspended in ice-cold 50 mM Na₂/K-phosphate buffer and served as membrane sample for radioligand binding assay. Membranes were incubated with 15 nM of [³H]mepyramine in the absence (total binding) or presence (non-specific binding) of 10 μM triprolidine for 60 min at 25°C in a final volume of 500 μl. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters presoaked with 1% polyethyleneimine. Filters were soaked in 10 ml of Aquasol-2, kept overnight in a dark place, and the radioactivity trapped on the filters was counted in a liquid scintillation counter. Specific binding was defined as the radioactivity bound after subtraction of non-specific binding as defined by 10 μM triprolidine.

Measurement of HDC activity and histamine content

For measuring HDC activity and histamine content, nasal mucosa was collected 9 h after TDI-provocation, homogenized with 10 volumes of ice-cold HDC buffer [0.1 M potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 1% polyethylene glycol (average molecular weight 300), and 100 μg/ml phenylmethylsulfonyl fluoride] (54). The homogenates were centrifuged at 10,000 × g for

15 min at 4°C and the supernatant (supernatant A) was collected. Half of supernatant A was dialyzed against an adequate volume of HDC solution three times for 6 h at 4°C (supernatant B). Histamine content in supernatant A was determined by high-performance liquid chromatography (HPLC) with a cation exchanger (Tosoh, Tokyo) and an automated *o*-phthalaldehyde fluorometric detection system (Hitachi, Tokyo) in accordance with the method of Yamatodani et al. (55). HDC activity was determined by incubating supernatant B for 4 h at 37°C with 0.25 mM L-histidine. HDC activities were calculated based on the formation of histamine after the subtraction of the blank value.

Determination of the protein concentration

Protein concentration was determined by the bicinchoninic acid (BCA) protein assay reagent (Sigma) using bovine serum albumin (BSA) as a standard.

Statistical analyses

The results are presented as means ± S.E.M. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA followed by Dunnett's multiple comparison test was used for statistical analysis. *P* values less than 0.05 were considered significant.

Results

Effect of Kujin on allergic symptoms

Intranasal application of TDI induced nasal allergy-like symptoms such as sneezing, watery rhinorrhea, and itching. In TDI-sensitized rats, the total number of

Fig. 3. Suppressive effect of *Kujin* on sneezing (A) and nasal scores (B) 10 min after TDI provocation. Nasal allergy-like symptoms were measured during 10 min just after TDI provocation. As the positive controls, effect of dexamethasone (DEX, 1 mg/kg), *d*-chlorpheniramine (Chlor, 30 mg/kg), and olopatadine (Olo, 30 mg/kg) on the nasal allergy-like symptoms were assayed. Data are each presented as the mean with standard error. ** $P < 0.01$ vs. control and ## $P < 0.01$ vs. TDI ($n = 4$).

sneezes and the nasal score were 28.4 ± 1.8 and 2.3 ± 0.19 , respectively (Fig. 3: A and B). Oral administration of *Kujin* significantly decreased the number of sneezes (10.0 ± 1.9 , Fig. 3A). The efficacy of *Kujin* extract was similar to that of dexamethasone, but less than those of H_1 -antihistamines at the doses we used. *Kujin* also tended to decrease the nasal score (1.5 ± 0.5), but its effect was much weaker than those of steroid and H_1 -antihistamines (Fig. 3B).

Effect of *Kujin* on *H1R* mRNA and *H1R* protein

The *H1R* mRNA level was significantly up-regulated (3.13 ± 0.51) compared to the control (1.0 ± 0.09) 4 h after TDI-provocation (Fig. 3A). Pretreatment with *Kujin* for 3 weeks suppressed this TDI-induced up-

regulation to the base level (0.85 ± 0.14 , Fig. 4A). Next, we examined *H1R* protein level in the nasal mucosa by [3 H]mepyramine binding assay. Saturation studies revealed a single class of high-affinity binding sites in the membranes from rat nasal mucosa, with K_d and B_{max} values of 3.6 ± 0.6 nM and 84.8 ± 4.9 fmol/mg protein ($n = 3$), respectively. Sensitization with TDI significantly increased (>200%) the [3 H]mepyramine binding activity in the nasal mucosa (Fig. 4B) and pretreatment with *Kujin* completely suppressed the TDI-induced elevation of *H1R* protein level (Fig. 4B).

Effect of *Kujin* on *HDC* mRNA, *HDC* activity, and histamine content in the nasal mucosa

HDC mRNA level was significantly up-regulated

Fig. 4. *Kujin* suppressed the TDI-induced up-regulation of *H1R* mRNA (A) and *H1R* (B). Rats were sacrificed and nasal mucosa was collected 4 h after provocation. *H1R* mRNA level was determined using real-time RT-PCR. Number of *H1R* expression was determined 24 h after TDI-provocation by using the [3 H]mepyramine binding assay. Data are each presented as the mean with standard error. ** $P < 0.01$ vs. control and ## $P < 0.01$ vs. TDI ($n = 4$).

Fig. 5. Suppression of TDI-induced HDC mRNA (A), HDC activity (B), and histamine content (C) in the nasal mucosa by *Kujin*. Rats were sacrificed and nasal mucosa was collected 4 h after provocation. HDC mRNA level was determined using real-time RT-PCR. HDC activity and histamine content in the nasal mucosa were determined 9 h after TDI-provocation using the HPLC system. Data are each presented as the mean with standard error. ** $P < 0.01$ vs. control; and # $P < 0.05$, ## $P < 0.01$ vs. TDI ($n = 4$).

(6.6 ± 0.48) compared to the control (1.0 ± 0.3) 4 h after TDI-provocation (Fig. 5A). *Kujin* pretreatment significantly suppressed (2.45 ± 0.24) this up-regulation (Fig. 5A). To determine if suppression of HDC mRNA resulted in decreased HDC activity, we checked the effect of *Kujin* on HDC activity. Previously, we have shown that HDC activity reached the maximum level 9 h after the TDI-provocation (19). Therefore, we measured HDC activity in the nasal mucosa 9 h after the provocation. Sensitization with TDI significantly increased the HDC activity (0.108 ± 0.004 pmol·min⁻¹·mg⁻¹ protein) compared to the control (0.005 ± 0.001 pmol·min⁻¹·mg⁻¹ protein). Pretreatment with *Kujin* significantly suppressed (0.049 ± 0.005 pmol·min⁻¹·mg⁻¹ protein) the TDI-induced elevation of HDC activity (Fig. 5B). As histamine is one of the major chemical mediators in the development of nasal allergic reaction, effect of *Kujin* on histamine content in the nasal mucosa of TDI-sensitized rats was also investigated. TDI markedly increased the histamine content (7.95 ± 0.47 pmol/mg vs. control, 3.18 ± 0.47 pmol/mg) in the nasal mucosa 9 h after the provocation. *Kujin* pretreatment significantly decreased the histamine content (6.31 ± 0.41 pmol/mg) induced by TDI (Fig. 5C).

Effect of *Kujin* on Th2-cytokines gene expression

Th2-cytokines including IL-4, IL-5, and IL-13 up-regulated after TDI-provocation in the rat nasal mucosa, suggesting that these cytokine genes are the allergy-sensitive genes. Oral administration of the *Kujin* significantly suppressed the TDI-induced IL-4 mRNA up-regulation (Fig. 6A). Similarly, provocation of TDI elevated the IL-5 mRNA level and *Kujin* pre-treatment significantly decreased its level (Fig. 6B). *Kujin* tended to suppress TDI-induced IL-13 mRNA elevation (Fig. 6C).

Discussion

Chronic allergic disorders have gotten worldwide attention. Histamine signaling has been identified as one of the major factors for allergic pathogenesis. Researchers have searched for synthetic as well as natural resources that have inhibitory effects on histamine signaling. In this study, we demonstrated that the anti-allergic activity of *Kujin* is mediated through the suppression of histamine signaling using TDI-sensitized allergy model rats. Our findings indicate that *Kujin* suppresses the TDI-induced H1R mRNA and H1R protein level, HDC mRNA and HDC activity, and histamine content in rat nasal mucosa. Nasal allergy is associated with nasal symptoms such as sneezing, rhinorrhea, redness, and swelling. In the present study and also in our previous studies (22, 23), we observed that intranasal application of TDI induced these nasal allergy-like symptoms. Nasal allergic symptoms during the 10 min just after provocation represents the early phase that is derived from the release of preformed histamine during the sensitization process (22). *Kujin* extract significantly suppressed sneezing as shown in Fig. 3. It also tended to decrease the nasal score, but the suppression was not significant. It was reported that histamine caused sneezing and nasal rubbing by its binding to H1R on the sensory nerve endings (56, 57). However, in swelling, rhinorrhea, and congestion, other mediators such as leukotriene and prostaglandin were shown to play major roles (58). We think this is the reason why *Kujin* extract did not significantly inhibit the nasal score that is the average of the scores of watery rhinorrhea and swelling and redness.

Our previous studies demonstrated that H1R plays a key role in histamine signaling involved in the allergic response (59). In TDI-sensitized rats, neurogenic inflam-

Fig. 6. Effect of *Kujin* on TDI-induced elevations of IL-4 (A), IL-5 (B), and IL-13 (C) mRNAs. Rats were sacrificed 4 h after provocation and the mRNA levels of IL-4 (A), IL-5 (B), and IL-13 (C) were determined by real-time RT-PCR. Data are each presented as the mean with standard error. ** $P < 0.01$ vs. control and ## $P < 0.01$ vs. TDI (n = 4).

mation caused release of histamine from mast cells in nasal mucosa and lead to development of nasal hypersensitivity (60). Pretreatment with *d*-chlorpheniramine significantly reduced TDI-induced nasal hypersensitivity behavior (22, 23). Many other studies also suggested the involvement of H1R in the pathogenesis of allergy and high efficacy of H1R antagonists in controlling nasal symptoms in the early phase of nasal allergy (35, 36, 61 – 63). In this study, *Kujin* pretreatment completely suppressed TDI-induced H1R mRNA up-regulation in rat nasal mucosa 4 h after provocation. We also checked its effect on TDI-induced H1R protein level in the nasal mucosa by a [3 H]mepyramine binding study. TDI increased the amount of H1R in the rat nasal mucosa 24 h after TDI-provocation. Pretreatment with *Kujin* suppressed TDI-induced elevation of H1R protein (Fig. 4B). These results supported the idea that *Kujin* decreased the number of H1R in the nasal mucosa by suppressing its transcription and thereby mediated less histamine response.

The synthesis of histamine by HDC is an important regulatory step in histamine signaling. We previously reported that histamine content, HDC activity, and HDC mRNA in the nasal mucosa of allergic rats significantly up-regulated after TDI-provocation (31). It was reported that HDC mRNA also increased in patients with allergic

rhinitis and bronchial asthma (39, 64), suggesting the importance of HDC in histamine signaling in allergic responses. We have no strict evidence for which types of cells are responsible for histamine synthesis and release in our model rats. However, our preliminary histological experiments suggest the possibility that mast cells are mainly responsible for this (data not shown). Expression of HDC mRNA in the nasal mucosa was significantly increased 4 h after TDI provocation, and it was followed by an increase in HDC activity (Fig. 5: A and B). Pretreatment with *Kujin* significantly suppressed the TDI-induced increase in the level of HDC mRNA expression and thereby suppressed the HDC activity. These results suggest that *Kujin* suppressed HDC activity at the transcriptional level. Increase in HDC activity caused the increase in histamine synthesis in the nasal mucosa of TDI-sensitized rat. Therefore suppression of HDC activity by *Kujin* pretreatment acted to restrain the synthesis of histamine in rat nasal mucosa. Accordingly, *Kujin* reduced the amount of histamine available for binding to H1R and thereby less allergic effect was exerted. Inhibition of histamine content in the nasal mucosa by *Kujin* seems not to be parallel to the inhibition of HDC activity (Fig. 5: B and C). Currently, we have no experimental data to explain this result. It might be sufficient to synthesize and store histamine in

the nasal mucosa during the 9 h after the provocation, although about 50% of the HDC activity was inhibited in *Kujin*-treated rats. Alternatively, *Kujin* might inhibit histamine release from nasal mucosal mast cells. To confirm this, it might be helpful to measure histamine content in the nasal lavage fluid.

Previously, we have shown that histamine induced up-regulation of the H1R gene and protein expression via H1R in HeLa cells (65). This agonist-induced up-regulation then increases the amount of H1R protein and makes cells more sensitive to histamine stimulation. So this “positive feedback circuit” between histamine and H1R might exacerbate the allergic symptoms. Decrease in histamine content by *Kujin* pretreatment could shut down this positive feedback circuit of the histamine-induced up-regulations of H1R gene and H1R-protein expressions in the nasal mucosa.

Several other studies suggested that disruption of the Th1/Th2 balance towards a pronounced Th2 profile is also responsible for the pathogenesis of allergic diseases. Elevated levels of CD4⁺ Th2 cell-derived cytokines such as IL-4 and IL-5 are strongly associated with asthma and allergic diseases. These cytokines mediate several key characteristics of the allergic inflammatory process, including tissue eosinophilia, mucus production, and increased IgE levels (66–68). However, Tanaka reported that the inflammation triggered with repeated exposure to TDI is a neurogenic and IgE-independent (24). They observed TDI-specific IgE in only few guinea pigs sensitized with TDI. It was reported that infiltration of eosinophils and mast cells in the histology after TDI-provocation in TDI-sensitized guinea pigs (26). We observed similar eosinophil infiltration after TDI-provocation in TDI-sensitized rats (unpublished observation), so it is expected that eosinophil infiltration should be suppressed in the *Kujin*-pretreated group, although we have no data to support this expectation. In TDI-sensitized mice, it has been reported that TDI treatment increases not only Th2-cytokines including IL-4, IL-5, and IL-13 but also Th1 cytokines such as IFN- γ , so the inflammation induced by TDI is associated with a mixed Th1/Th2 immune response (69). In our model rats, the mRNA levels of both Th2-cytokines such as IL-4, IL-5, and IL-13 (Fig. 6) and Th1-cytokine IFN- γ (unpublished result) were also increased by TDI treatment. This finding suggests that TDI-induced inflammation in our TDI-sensitized rats is also associated with a mixed Th1/Th2 immune response. *Kujin* suppressed TDI-induced mRNA elevations of IL-4 and IL-5 and tended to suppress the TDI-induced IL-13 mRNA, although we have not checked the effect of *Kujin* on TDI-induced IFN- γ mRNA elevation. These data suggest that *Kujin*

suppresses the levels of the Th2-cytokines induced by TDI and resulted in a shift of the Th1/Th2 balance towards a predominance of Th1.

Accumulating evidences suggest that the histamine–cytokine network is important for allergic inflammation, in which histamine influences the expression and actions of several cytokines, and in turn, some cytokines modulate the production and release of histamine (45–47). Pretreatment with *Kujin* suppressed the TDI-induced increase in the level of IL-4 and IL-5 mRNA (Fig. 6: A and B). *Kujin* extract also suppressed TDI-induced elevation of IL-13, but it was not significant (Fig. 6C). Our previous study showed that H1R mRNA up-regulation was partially suppressed by *d*-chlorpheniramine and olopatadine, H₁-antihistamines, in TDI-sensitized rats (70), and this TDI-induced H1R mRNA up-regulation was completely inhibited by a Th2 cytokine inhibitor (unpublished results). These data suggest that there are at least 2 pathways for H1R mRNA up-regulation, one pathway is H1R-mediated and the other is non-H1R-mediated. We think IL-4 may be a candidate responsible for H1R mRNA up-regulation. We have reported that prophylactic treatment with H₁-antihistamines suppressed TDI-induced up-regulations of both H1R and IL-4 mRNAs in TDI-sensitized rats (71). Direct administration of IL-4 into the nasal cavity of non-TDI-treated normal rats up-regulated the expression of H1R mRNA (unpublished result). It was also reported that IL-4 stimulates the expression of H1R mRNA in human rheumatoid synovial fibroblasts (72). On the other hand, direct application of histamine into the nasal cavity for 1 week caused an increase in IL-4 mRNA elevation in normal rats (unpublished observation). Furthermore, we have reported that stimulation of H1R with histamine caused up-regulation of H1R mRNA in HeLa cells (65). Similar H1R up-regulation was found in normal rats (unpublished result). These findings suggest the existence of the “vicious circuit” between histamine and IL-4, in which histamine up-regulates IL-4 gene expression through H1R and IL-4, and in turn, up-regulates the expression of H1R mRNA and the increase in H1R expression makes cells more sensitive to histamine and deteriorates the allergic symptoms. *Kujin* completely suppressed TDI-induced mRNA elevation of histamine H₁ receptor (H1R) as shown in Fig. 4A. As *Kujin* also suppressed TDI-induced IL-4 mRNA elevation, *Kujin* suppresses not only H1R-mediated H1R gene expression but also non-H1R-mediated (possibly IL-4-induced) H1R gene expression, shuts down the histamine-IL-4 “vicious circuit”, and ameliorates allergic symptoms in TDI-sensitized rats.

Allergy is usually considered as a chronic disorder

and its satisfactory diagnosis and treatment is still a challenge to modern medicine. The H₁-antihistamines, pharmacological antagonists of the H₁R, are usually used for the treatment of acute allergic reactions. However, these anti-allergic effects are not uniformly shared among all drugs of this class, and suppression of TDI-induced H₁R mRNA up-regulation by H₁-antihistamines in TDI-sensitized rats is only partial (70). Furthermore, most first-generation H₁-antihistamines have anti-cholinergic, sedative, local anaesthetic, and anti-5-HT effects, which might favorably affect the symptoms of the allergic response but also contribute to side effects (63). Topical glucocorticoids also have been shown to be effective against nasal allergic symptoms. The anti-inflammatory effect of topical glucocorticoids includes stabilization of mast cell membrane, down-regulation of antigen-presenting cells, inhibition of the expression of IL-4 mRNA, reduction of cytokine and chemokine release, and reduction of T cell and eosinophilic infiltration (73, 74). In TDI-sensitized rats, we have shown that dexamethasone reduced allergy-like symptoms and suppressed TDI-induced H₁R mRNA elevation in a dose-dependent manner, in which 1 mg/kg of dexamethasone completely suppressed TDI-induced elevation of H₁R mRNA (22). Although the anti-inflammatory effect of topical glucocorticoids is strong, they may also cause harm in terms of local and systemic side effects. So screening of natural sources to find new and safe chemical entities with anti-allergic potentials is relevant now. The active compound in *Kujin* can be one of the candidates if we identify and characterize it from *Kujin* extract.

In conclusion, prolonged oral administration of *Kujin* during sensitization significantly reduced TDI-induced allergic responses by suppressing histamine signaling including H₁R and HDC gene expression in rat nasal mucosa.

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