ORIGINAL ARTICLE

Sho-seiryu-to Suppresses Histamine Signaling at the Transcriptional Level in TDI-Sensitized Nasal Allergy Model Rats

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ABSTRACT

Background: The therapeutic use of Kampo medicine, Sho-seiryu-to (SST) in allergic disorders is well known. As histamine plays a central role in allergic diseases, it is possible that SST affects the allergy-related histamine signaling. In this study, we investigated the effect of SST on allergy-related histamine signaling in the nasal mucosa of toluene 2, 4-diisocyanate (TDI)-sensitized nasal allergy model rats.

Methods: Six-week-old male, Brown Norway rats were sensitized for 2 weeks with 10 μl of 10% TDI, and after a 1 week interval, provocation was initiated with the same amount of TDI. SST (0.6 g/rat) was given orally 1 hour before TDI treatment began for a period of 3 weeks. Nasal symptoms were scored for 10 minutes immediately after TDI-provocation. The genes expression in nasal mucosa was determined using real-time quantitative RT-PCR.

Results: SST significantly suppressed TDI-induced nasal allergy-like symptoms. TDI provocation showed a significant up-regulation of histamine H_1 receptor (H1R) and histidine decarboxylase (HDC) gene expressions. Prolonged pre-treatment of SST significantly suppressed the mRNA levels of H1R and HDC that was up-regulated by TDI. SST also suppressed TDI-induced interleukin (IL)-4 and IL-5 mRNA elevation. However, SST showed no significant effect for TDI-induced mRNA elevation of IL-13.

Conclusions: These results demonstrate that SST alleviates nasal symptoms by the inhibition of histamine signaling through suppression of TDI-induced H1R and HDC gene up-regulation. SST also suppresses cytokine signaling through suppression of IL-4 and IL-5 gene expression. Suppression of histamine signaling may be a novel mechanism of SST in preventing allergic diseases.

INTRODUCTION

The prevalence of allergic diseases has increased significantly around the world today[1,2] where 1 in every 5 children develops an allergy during their childhood. Thus, the satisfactory diagnosis and treatment of allergy is still a challenge in modern medicine. Synthetic as well as natural resources are being investigated for this purpose and the potent therapeutic plants mentioned in ancient literature, as well as traditional medicine, can be a credible area to be explored for the establishment of improved therapy for allergic conditions.

Histamine is a key biogenic amine responsible for allergy and inflammatory reactions mediated through 4 distinct receptors such as H_1, H_2, H_3 and H_4 receptors.[3,4] Among them, the histamine H_1 receptor (H1R) plays an essential role in the development of

allergic pathogenesis. In eukaryotes, histidine decarboxylase (HDC) is the sole enzyme responsible for synthesizing histamine and consequently a crucial regulatory step for histamine signaling. The allergic reaction is also characterized by a disruption of Th1/Th2 balance toward a pronounced Th2 profile. Th2 cytokines, especially interleukin (IL)-4, IL-5 and IL-13, may play a vital role in the development and maintenance of allergic responses.[5-9] Increasing experimental evidence suggest the existence and importance of the 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.[10-13]

Toluene 2,4-diisocyanate (TDI) is a recognized human irritant and one of the leading causes of occupational allergic diseases in industrialized countries.[14,15] We previously reported that TDI-sensitized animals share several features of nasal allergic diseases, including elevation of H1R mRNA levels and its protein level,[15] increase in HDC mRNA levels, HDC activity and histamine content.[16] TDI also induces respiratory allergy with Th2-dominated responses such as increase in the levels of IL-4, IL-5 and IL-13, total and specific serum IgE levels, airway inflammation characterized by activated CD4+ T cells, eosinophils and mast cells, and airway remodeling.[17-20]

Sho-seiryu-to (SST), also known as Xiao-quing-long-tang in China and So-cheong-ryong-tang in Korea, is a traditional Kampo medicine that has been used as a natural cure for allergy symptoms for hundreds of years in Asian countries. Several studies have been conducted recently to evaluate its effect clinically and experimentally, as well as its pharmacological properties. SST showed suppressive effects on histamine release from rat peritoneal mast cells,[21] reduced serum IgE levels in allergic rhinitis patients,[22,23] repressed allergen-induced bronchial inflammation in mite-sensitized mice,[24] modulation of Th1- and/or Th2-cytokine in CD4+ T cells in mice.[25-27] However, the therapeutic mechanisms of this Kampo medicine are still unclear.

In the present study, we investigated the effect of SST on allergic symptoms as well as on the expressions of the allergy-sensitive genes involved in histamine signaling such as H1R and HDC using TDI-sensitized nasal allergy model rats. We also investigated the effect of SST on TDI-induced elevation of the Th2-cytokine mRNAs including IL-4, IL-5, and IL-13.

METHODS

PREPARATION AND ADMINISTRATION OF SST

Thirty-six grams of SST (Tsumura & Co., Tokyo, Japan) was added to 500 ml of distilled water and left to stand for 1 hour at room temperature. The SST suspension was then boiled for 2 hours filtered twice to remove insoluble materials, and. the extract was concentrated to 126 ml. The rats were orally given the extract once a day at a dosage of 0.6 g/rat for 3 weeks, 1 hour before TDI application. This dosage corresponds to that of the original SST used for extraction. In literature, Sakaguchi et al. used 100 mg/kg–1000 mg/kg SST[28] and Umesato et al. administered SST orally once a day at a dosage of 1 g/kg.[29] Therefore the amount of SST used in this study is comparable with the amount that has been used in previous studies.

TDI SENSITIZATION AND PROVOCATION

We used 6-week-old male, Brown, Norway rats, weighing 200–250 g (Japan SLC, Hamamatsu, Japan) for the present study. Rats were given free access to water and food, and kept in a room where constant temperature (25 ± 2)℃ and humidity (55 ± 10%) were maintained, and a 12/12 hour light/dark cycle was kept. Rats were divided into 3 groups each containing 4 rats. Sensitization by TDI was performed by the method previously reported.[16] In brief, 10 μl of 10% solution of TDI (Wako Chemical, Tokyo, Japan) in ethyl acetate (Wako) was applied bilaterally in the nasal vestibules 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 applied again to the nasal vestibules to provoke nasal allergy-like behaviors in the TDI-sensitized rats. Nasal allergy-like behavior was measured by counting the number of sneezes during the 10 minutes immediately after TDI provocation. Among the 3 groups, rats in groups 2 and 3 were sensitized with TDI solution. SST was given orally to each rat in group 3. Group 1 was treated with ethyl acetate and phosphate buffer as a control (Fig. 1). All animal experiments were approved by the Ethical Committee for Animal Studies of School of Medicine, the University of Tokushima, Japan.

MEASUREMENT OF mRNAs BY REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (REAL-TIME RT-PCR)

Rats were sacrificed 4 hours after provocation, and nasal mucosa was removed from the nasal septum. Samples were collected in RNA*later*® (Applied Biosystems, Foster City, CA) and stored at −80℃ until used. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) as reported previously.[16] In short, nasal mucosa was homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of ice-cold TRIzol reagent until completely disrupted. The homogenates were mixed with chloroform and centrifuged at 15,000 rpm for 15 minutes at 4℃. The RNA was precipitated by addition of isopropanol in the aqueous phase and centrifuged. The RNA pellet was washed
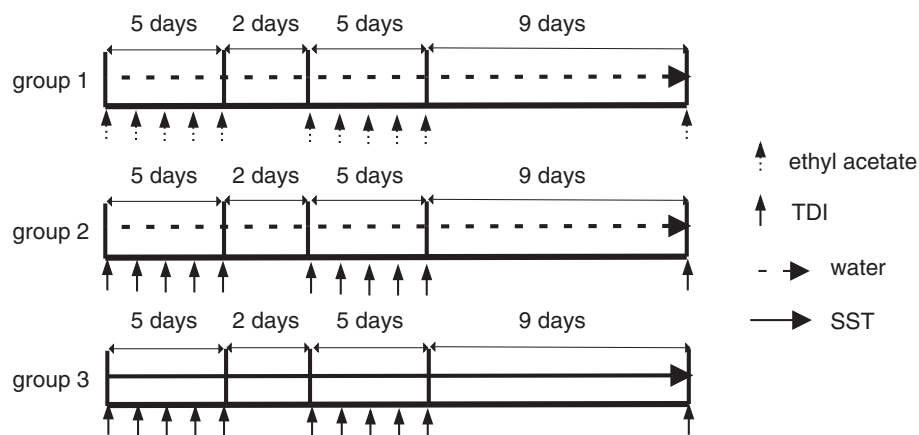
Fig. 1 Protocol for TDI sensitization and provocation. Rats were sensitized with 10 μl of 10% TDI in ethyl acetate for 5 consecutive days. This was repeated after a 2- day interval. Provocation was initiated 9 days after the last sensitization with 10 μl of 10% TDI in ethyl acetate. Control rats were sensitized and provocated with ethyl acetate under the same procedure.

with 70% ice-cold ethanol, air-dried and dissolved in 20 μl of diethylpyrocarbonate-treated water. RNA samples (8 μg) were reverse-transcribed to cDNA in a 40 μl reaction volume in the presence of a first-strand buffer [250 mM Tris-HCl, pH 8.3, at room temperature containing 375 mM KCl, 15 mM MgCl$_2$, and 0.8 mM of each of deoxyribonucleoside phosphates (dNTPs), 40 μM oligo (dT) primers, 0.004 units of RNase inhibitor, and 8 units of reverse transcriptase (Superscript II, Invitrogen)]. After samples were incubated at 37℃ for 60 minutes, 2 μl of 2 N NaOH was added before they were further incubated at 65℃ for 30 minutes. Subsequently, 12.8 μl of 1 M Tris-HCl, pH 8.0, was added before samples were heated at 95℃ for 10 minutes and then chilled to 4℃ for 5 minutes. TaqMan primers and probe were designed using Primer Express software (Applied Biosystems). The sequences of the primers and probes used for H1R, HDC, IL-4, IL-5, and IL-13 mRNA measurement are listed in Table 1. The primers and probe of rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems) were used for the internal standard. 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, pH 8.4, containing 50 mM KCl, 3 mM MgCl$_2$, 200 μM dNTPs, 900 nM each of the primer, and 0.25 units of Platinum Taq. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. The identity of the PCR products was verified by sequencing using a genetic analysis system (Beckman CEQ 8000, Beckman Coulter, Fullerton CA, USA).

STATISTICAL ANALYSIS

The results are presented as means ± standard errors (SE). Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). One-way ANOVA followed by Dunnet's multiple comparison test were used for statistical analysis. P values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

NASAL ALLERGY LIKE BEHAVIOR

Intranasal application of TDI induced nasal allergic symptoms. We counted the number of sneezes for 10 minutes immediately after provocation as a parameter of allergy intensity. In TDI-sensitized rats, the total number of sneezes was 29 ± 1.4 (Fig. 2). Pretreatment of SST suspension significantly suppressed sneezing (7.7 ± 2.7). Control rats sensitized and provocated with ethyl acetate failed to show any allergic symptoms.

H1R GENE EXPRESSION

Allergic pathogenesis is mediated mainly through H1R. This led us to monitor the effect of SST on H1R mRNA. H1R mRNA expression significantly increased after TDI provocation (3.19 ± 0.22) compared to the control group (1.0 ± 0.13, Fig. 3A). Oral administration of SST prior to sensitization almost completely attenuated this up-regulation (1.06 ± 0.24).

HDC mRNA EXPRESSION

To examine whether SST has any effect on histamine synthesizing enzymes, we investigated the effect of SST suspension on HDC gene expression. Oral administration of SST was able to significantly suppress the elevation of HDC mRNA expression (2.16 ± 0.06) compared to that of TDI-sensitized allergy model rats (6.05 ± 0.38, Fig. 3B).

Table 1 Primer and probe sequences used for real-time RT-PCR

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'-CACCGAGAACCCCAGACTTG-3'
	Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
IL-5 mRNA	Sense primer	5'-CAGTGGTGAAAGAGACCTTGATACAG-3'
	Anti sense primer	5'-GAAGCCTCATCGTCTCATTGC-3'
	Probe	FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA

For measuring IL-13 mRNA, primer probe kit from Applied Biosystems (Rn00587615-A1 1113) was used.
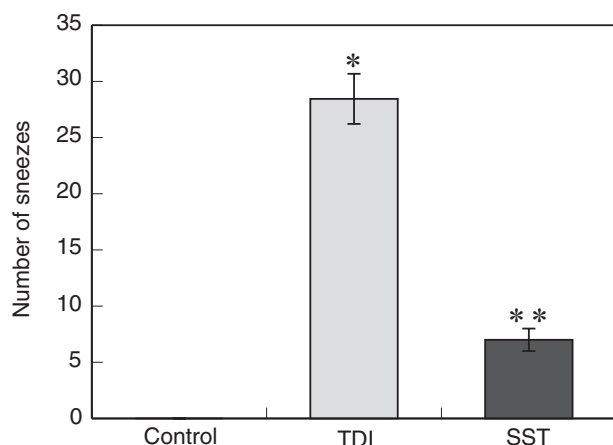

Fig. 2 SST reduces number of sneezes in TDI-sensitized nasal allergy model rats. Rats were sensitized and provocation induced as described in the Methods section. SST was given orally 1 hour before sensitization and provocation. The number of sneezes was measured for 10 minutes immediately after provocation. $*P < 0.01$ compared with control; $**P < 0.01$ compared with TDI ($n = 4$).

EFFECT OF SST ON TDI-INDUCED IL-4, IL-5 AND IL-13 mRNA ELEVATION IN RAT NASAL MUCOSA

As Th2-cytokines play an important role in allergic hyperresponses, we investigated the effect of SST on the elevation of IL-4, IL-5 and IL-13 mRNA in nasal mucosa of TDI-sensitized rats. Real-time RT-PCR analysis showed that SST suppressed IL-4 mRNA significantly (2.5 ± 0.30, Fig. 4A) compared to that of TDI-sensitized allergic rats (5.5 ± 0.76), and almost completely suppressed the expression of IL-5 mRNA (1.37 ± 0.46, Fig. 4B). However, SST failed to show any significant effect on IL-13 mRNA expression (Fig. 4C).

DISCUSSION

SST has been used for treating allergic diseases for a long time in Asian countries. Previous studies demonstrated the various pharmacological evidence behind the anti-allergic effect of SST including the decrease in antigen-induced eosinophil infiltration in guinea pigs,[21] suppression of allergen-induced bronchial inflammation in mite-sensitized mice,[24] and decrease in serum IgE level in allergic rhinitis patients.[23,30] The primary objective of this study was to evaluate the effect of SST on the gene expression of 2 important parameters in histamine signaling responsible for allergic pathogenesis, i.e. H1R mRNA and HDC mRNA. We also investigated the role of SST in the expression level of the Th2-cytokines, as histamine signaling is closely related with Th2-cytokine signaling.

For this purpose, we used rats sensitized with TDI as subjects in this study. Allergic rhinitis is an IgE mediated disease and is distinct from other rhinitis.[31] In more than 90% of patients, the allergens responsible for the cause of disease could be clarified. Nevertheless, since rhinitis caused by TDI is a non-IgE mediated disease,[30] nasal symptoms and pathogenesis observed in TDI-sensitized rats could be different. However, intranasal application of TDI caused the neuropeptide-mediated release of histamine from mast cells in the nasal mucosa and led to the development of nasal allergy-like symptoms such as sneezing and water rhinorrhea in TDI-sensitized guinea pigs.[14] In this respect, these TDI-induced nasal allergy-like symptoms are similar to those observed in allergic rhinitis patients. In addition to the nasal allergy-like symptoms, TDI-sensitized rats exhibit many of the characteristical features of allergic rhinitis in humans including infiltration of eosinophils and mast cells,[32] increase in the level of cytokines,[17-20] elevation of H1R mRNA and protein level,[15] increase in HDC mRNA level, and HDC activity and histamine con-
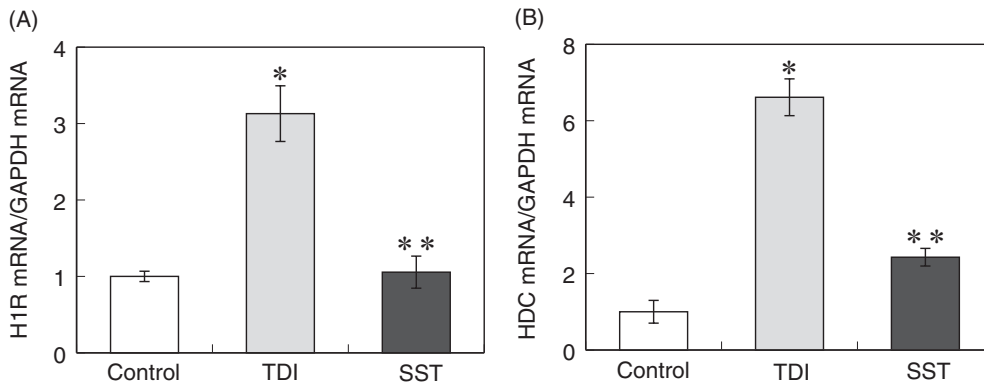
Fig. 3 SST blocks TDI-induced up-regulation of H1R mRNA (**A**) and HDC mRNA (**B**). Rats were sensitized and provocated as described in the Methods section. SST was given orally 1 hour before sensitization and provocation. Rats were sacrificed 4 hours after the TDI-provocation and the mRNA levels of H1R (**A**) and HDC (**B**) in the nasal mucosa were determined by quantitative real-time RT-PCR. Data were normalized by rodent GAPDH mRNA and expressed as mean ± standard error (SE). *$P < 0.01$ compared with control; **$P < 0.01$ compared with TDI ($n = 4$).
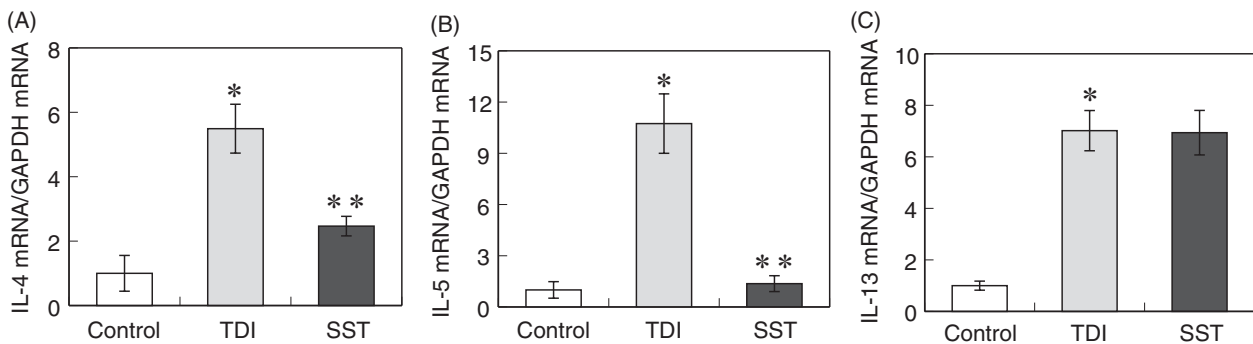
Fig. 4 SST suppresses the TDI-induced IL-4 (**A**) and IL-5 (**B**) but not IL-13 mRNA elevations in TDI-sensitized allergy model rats. Rats were sensitized and provocated as described in the Methods section. SST was given orally 1 hour before sensitization and provocation. Rats were sacrificed 4 hours after provocation and the mRNA levels of IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) in the nasal mucosa were determined by quantitative real-time RT-PCR. Data were normalized by rodent GAPDH mRNA and expressed as mean ± standard error (SE). *$P < 0.01$ compared with control; **$P < 0.01$ compared with TDI ($n = 4$).

tent,[16] although neuropeptide, not IgE, triggered histamine release in TDI-sensitized animals. Accordingly it is difficult to differentiate between immunological sensitization and irritation by low molecular weight chemicals such as TDI and it is likely that the underlying mechanisms driving TDI-induced rhinitis share similarities with allergic rhinitis caused by ubiquitous airborne protein allergens.

Pre-treatment with SST suppressed sneezing (Fig. 2). This finding corroborates the results of our previous studies that showed the trigger of such behaviors after TDI stimulation.[16,33] Nasal allergy-like behaviors during the 10 minutes following provocation was studied to examine the effect of SST in the early phase which is derived from the release of preformed

histamine during the sensitization process. We did not investigate the eosinophil infiltration in this study. However, Irifune has reported on infiltration of eosinophil and mast cells in tissue following TDI-provocation in TDI-sensitized guinea-pigs.[32] We have observed a similar eosinophil infiltration following TDI-provocation in TDI-sensitized rats (unpublished observation). Thus, the suppressive effect of SST on allergic symptoms also shows its effect in the induction phase.

Previously, we demonstrated that H1R is the key factor for histamine signaling involved in allergic responses.[15] In TDI-sensitized rats, neurogenic inflammation caused the release of histamine from mast cells in the nasal mucosa in IgE-independent man-

ner[30] and lead to the development of nasal hypersensitivity.[34] Pretreatment with *d*-chlorpheniramine significantly reduced TDI-induced nasal hypersensitivity behavior.[35,36] Many other studies have also shown the involvement of H1R in the pathogenesis of allergy and high efficacy of H1R antagonists in controlling nasal hypersensitivity symptoms in the eliciting/effecter phase of nasal allergy.[33,37-39] Our study demonstrated that the SST pre-treated group showed a significant repression of TDI-induced H1R mRNA up-regulation in rat nasal mucosa (Fig. 3A). This result may contradict the findings of Sakaguchi *et al*. who reported no effect of SST on [³H] mepyramine binding activity in guinea pig cerebral cortex[21] and lung and rat brain.[28] They dissolved SST in distilled water and administrated it orally. We, on the other hand, created an extract of SST with hot water. As SST consists of *Pineliale Tuber* (22.2%) and 11.1% of each of *Glycyrrhizae Radix*, *Cinnamomi Cortex*, *Schisandrae Fructus*, *Asiasari Radix*, *Paeoniae Radix*, *Ephedrae Herba*, and *Zingiberis sciccatum Rhizoma*, hot water extraction may have caused some changes in the ingredients. This may explain the conflict between our results. Sakaguchi *et al*. also administrated SST to animals for 1 week, while we treated rats with the SST extract for 3 weeks. We have found that a 3-week treatment of epinastine is more effective on the suppression of H1R gene expression than a 1-week treatment (unpublished data). Therefore it is possible that this difference in duration of drug treatment can result in a different level of H1R gene expression.

HDC is the sole enzyme for the biosynthesis of histamine.[40] In this study, the expression of HDC mRNA in the nasal mucosa significantly increased after TDI provocation in TDI-sensitized rats (Fig. 3B). This finding is in line with studies in which the HDC mRNA level increased in patients with allergic rhinitis and bronchial asthma.[41,42] We examined the effect of SST on this up-regulation of HDC gene expression in TDI-sensitized rats. Our result demonstrated that SST significantly suppresses the increase of HDC mRNA expression (Fig. 3B). Since pretreatment of SST also suppressed TDI-induced nasal allergy-like behaviors, it is suggested that the SST reduced nasal hypersensitivity by its inhibitory effect on the up-regulation of HDC mRNA expression thereby suppressing histamine synthesis. This result indirectly supports the finding of Sakaguchi *et al*. showing the suppression of histamine release from rat peritoneal mast cells.[21]

Cytokines also play an important role in allergic pathogenesis. Allergic diseases are associated with an increase in the number of Th2 cells and Th2 cytokines and a decrease in the number of Th1 cells and Th1 cytokines. Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13 are involved in the initiation and maintenance of the allergic inflammation by promoting eosinophil infiltration and IgE responses.[5-9] In TDI-

sensitized animals, 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, therefore the inflammation induced by TDI is associated with a mixed Th1/Th2 immune response.[48] Johnson *et al*. have also shown that the inflammation and gene expression changes caused by TDI-exposure were isolated to the upper airways and that the lower airways showed no evidence of inflammation. In addition, the changes observed in the nasal mucosa were most likely due to local antigen recognition, processing, and presentation to effector cells at the site of exposure.[48] In the present study, we found that SST significantly suppressed the up-regulation of IL-4 and IL-5 but had no effect on IL-13 mRNA in the nasal mucosa of TDI-sensitized allergy model rats (Fig. 4). This finding is parallel to the reports describing the suppressive effect of SST on Th2 differentiation in CD4+ T cells in mice,[25-27,42,43] although some contradicting effects of SST on Th2 cytokines have been reported in allergic patients.[23,30] IL-4 shares many biological properties with IL-13 and the redundancy between two cytokines is explained by their sharing of the common receptor subunit.[44] However, many reports have suggested that IL-4 and IL-13 may be under differential regulation under some level of independent regulation.[45,46] Ghaffar *et al*. showed that the percentage of the IL-13 mRNA positive cells co-expressing the IL-4 mRNA was 66.6 ± 10.5%, although 100% of the IL-4 mRNA positive cells expressed IL-13 transcript.[47] It was also reported that the expression pattern of IL-13 was different from that of IL-4 in the nasal mucosa of TDI-induced allergic rhinitis mice.[48] Therefore, it is possible that SST showed the opposite effect on IL-4 and IL-13 gene expression in TDI-sensitized rats.

Increasing experimental evidences suggest the existence 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.[10-13,20] It has been reported that pre-treatment of IL-4 primes for the release of histamine, prostaglandins, leukotrienes, and cytokines in response to FcεRI.[49,50] On the contrary, reports have indicated that histamine modulates the release of IL-4 and IFN-γ from T cells[51] and induces the release of IL-5.[52] Our data suggest that pre-treatment with SST suppresses histamine-cytokine network.

In conclusion, pre-treatment with SST improves TDI-induced nasal allergic symptoms by suppressing TDI-induced elevation of H1R and HDC gene expression, the key components of histamine signaling in rat nasal mucosa, in addition to gene expression of some Th2 cytokines including IL-4 and IL-5.

ACKNOWLEDGEMENTS

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