

Hop water extract inhibits double-stranded RNA-induced thymic stromal lymphopoietin release from human nasal epithelial cells

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ABSTRACT

Background: Thymic stromal lymphopoietin (TSLP) acts as a master switch for allergic inflammation and plays a key role in allergic diseases, including allergic rhinitis. Double-stranded RNA (dsRNA) recognized by Toll-like receptor 3 (TLR3) strongly activates TSLP release from human nasal epithelial cells (HNECs). Hop (*Humulus lupulus* L.) extracts have been shown to have potent pharmacologic effects on inflammation.

Methods: To investigate whether a hop water extract (HWE) prevents TSLP release from HNECs, human telomerase reverse transcriptase (hTERT)-transfected HNECs, used as a model of normal HNECs, were pretreated with HWE before treatment with the TLR3 ligand Polyinosine-polycytidylic acid (poly[I:C]).

Results: In the hTERT-transfected HNECs, treatment with HWE significantly reduced poly(I:C)-induced production and release of TSLP in a dose-dependent manner, as well as dexamethasone. Treatment with the protein kinase C (PKC) inhibitor GF109203X and NF- κ B inhibitor IMD-0354 also reduced poly(I:C)-induced TSLP release from hTERT-transfected HNECs. Treatment with HWE efficiently prevented up-regulation of PKC activity by 12-O-tetradecanoyl phorbol-13-acetate but not NF- κ B activity induced by IL-1 β in hTERT-transfected HNECs.

Conclusion: Our results clearly indicated that HWE inhibited dsRNA-induced production and release of TSLP via a PKC signal pathway in HNECs and it may have potent preventive effects against allergic rhinitis.

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The nasal epithelium, which is the first site of exposure to inhaled antigens, may play an essential role in innate immunity to allergic rhinitis. Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that is expressed by epithelial cells and activates dendritic cell-mediated Th2-type allergic inflammatory responses.^{1,2} TSLP is thought to be a master switch for allergic inflammation and thus to play a key role in the pathogenesis of allergic inflammation in asthma, atopic dermatitis, and allergic rhinitis.^{1,3,4} Our previous study reported high expression of mRNA and protein of TSLP in human nasal epithelia from patients with allergic rhinitis, and TSLP in human nasal epithelial cells (HNECs) is induced by treatment with a Toll-like receptor 2 (TLR2) ligand, Pam₃Cys-Ser-(Lys)₄, a TLR3 ligand, polyinosine-polycytidylic acid (poly[I:C]); and a mixture of IL-1 β and TNF- α .⁵ Thus, it is possible that blocking TSLP release from nasal epithelial cells may be effective in the treatment of allergic rhinitis.

Glucocorticoids and a synthetic analog of capsaicin can attenuate dsRNA-induced TSLP release from human bronchial epithelial cells and keratinocytes.^{6–8} Berberine and naringenin can suppress the production of TSLP in mast cells.^{9,10} However, little is known about whether natural components can inhibit double-stranded RNA (dsRNA)-induced TSLP production in epithelial cells.

Female hop flowers (*Humulus lupulus* L.) are used in the brewing industry to add bitterness and aroma to beer. Hops have a wide range of pharmacologic roles such as the estrogenic property of phytoestrogen, an inhibitory effect on bone resorption, and anti-inflammatory effects *via* the inhibition of cyclooxygenase-1 activity and nitric oxide production.^{11–15} Furthermore, we previously reported that a hop water extract (HWE) inhibited antigen-induced nasal rubbing and sneezing in albumin-sensitized BALB/c mice and the release of chemical mediator from basophilic cells.^{16,17} However, the effects of HWE on HNECs remain unclear.

In the present study, we investigated whether HWE could prevent TSLP release from HNECs induced by treatment with the TLR3 ligand poly(I:C). We found that treatment with HWE significantly reduced poly(I:C)-induced production and the release of TSLP, as well as dexamethasone. HWE also inhibited dsRNA-induced production and the release of TSLP *via* a protein kinase C (PKC) signal pathway in HNECs.

MATERIALS AND METHODS

Reagents and Antibodies

The poly(I:C) was purchased from InvivoGen (San Diego, CA). Phospho-extracellular signal-regulated kinase (ERK) 1/2 (MAPK; Thr202 /Tyr204) inhibitor PD98059, p38 MAPK inhibitor SB203580, phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002, and pan-PKC inhibitor GF109203X were purchased from Calbiochem-Novabiochem (San Diego, CA). The 12-O-tetradecanoylphorbol 13-acetate (TPA), dexamethasone, and NF- κ B inhibitor IMD-0354 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-1 β was purchased from PeproTech (London, U.K.). Polyclonal rabbit anti-phospho-pan-PKC, anti-pan-PKC, anti-phospho-NF- κ B, and anti-NF- κ B antibodies were purchased from Cell Signaling (Beverly, MA). A polyclonal rabbit antiactin antibody was obtained from Sigma-Aldrich.

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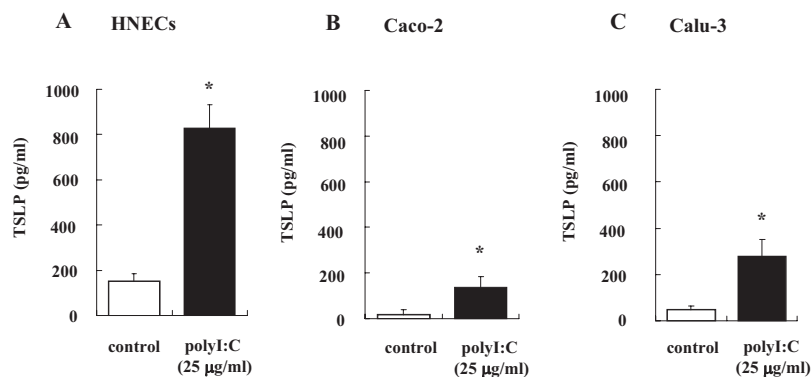


Figure 1. Induction of thymic stromal lymphopoietin (TSLP) release from polyinosine-polycytidylic acid (poly(I:C))-treated epithelial cells. Expression of TSLP in the cultured medium of (A) human telomerase reverse transcriptase (hTERT)-transfected human nasal epithelial cells (HNECs), (B) Caco-2 and (C) Calu-3 after treatment with 25 µg/mL of poly(I:C) for 24 hours was analyzed by ELISA. Error bars represent means $\pm$ SEM ($n = 3$ of three different experiments; * $p < 0.05$ compared with control).

Sample Preparation of HWE

Pellets made from hops cultivated in the Saaz district of the Czech Republic were extracted in water overnight. The hop pellet suspension was filtered through filter paper. The aqueous solution was lyophilized.¹⁷

Isolation and Culture of HNECs

Cultured HNECs were derived from the mucosal tissues of patients with hypertrophic rhinitis or chronic sinusitis who underwent inferior turbinatectomy at Sapporo Medical University, the Sapporo Hospital of Hokkaido Railway Company, or the KKR Sapporo Medical Center Tonan Hospital. Informed consent was obtained from all patients and this study was approved by the ethics committees of the aforementioned institutions.

The methods for primary culture of HNECs were as reported previously.¹⁸ Some primary cultured HNECs were transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase (hTERT) gene, as described previously.^{19–22} The cells were plated on 35- or 60-mm culture dishes (Corning Glass Works, Corning, NY), coated with rat tail collagen (500 µg of dried tendon/mL 0.1% acetic acid). The cells were cultured in serum-free bronchial epithelial cell basal medium (Lonza Walkersville, Walkersville, MD) supplemented with bovine pituitary extract (1% v/v), 5 µg/mL of insulin, 0.5 µg/mL of hydrocortisone, 50 µg/mL of gentamycin, 50 µg/mL of amphotericin B, 0.1 ng/mL of retinoic acid, 10 µg/mL of transferrin, 6.5 µg/mL of triiodothyronine, 0.5 µg/mL of epinephrine, 0.5 ng/mL of epidermal growth factor (Lonza Walkersville), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma-Aldrich) and incubated in a humidified, 5% CO₂/95% air incubator at 37°C. In this experiment, second and third passaged cells were used. The optimal concentration of 25 µg/mL poly(I:C) was chosen to be used in combination with other compounds in this study.^{5,21} The cells were treated with 0.1–50 µg/mL of HWE for 24 hours. Some cells were pretreated with 1–10 µg/mL of HWE, 0.1–1 µM of dexamethasone, 10 µM of LY294002, 10 µM of PD98059, 10 µM of SB203580, 10 µM of GF109203X and 1 µM of IMD-0354 30 minutes before treatment with 25 µg/mL of poly(I:C). Some cells were pretreated with 10 µg/mL of HWE, 10 µM of GF109203, and 1 µM of IMD-0354 at 30 minutes before treatment with 10 nM of TPA or 10 ng/mL of IL-1β for 4 hours.

Culture of Cell Lines

Human colonic carcinoma cell line Caco-2 and airway epithelial cell line Calu-3 (American Type Culture Collection, Rockville, MD), were cultured in DMEM (Sigma-Aldrich) supplemented with 20% FBS and RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS, respectively. The medium contained 100 U/mL of penicillin and 100 µg/mL of streptomycin and the cells were plated on 60-mm culture dishes (Corning Glass Works), coated with rat tail collagen (500 µg of dried

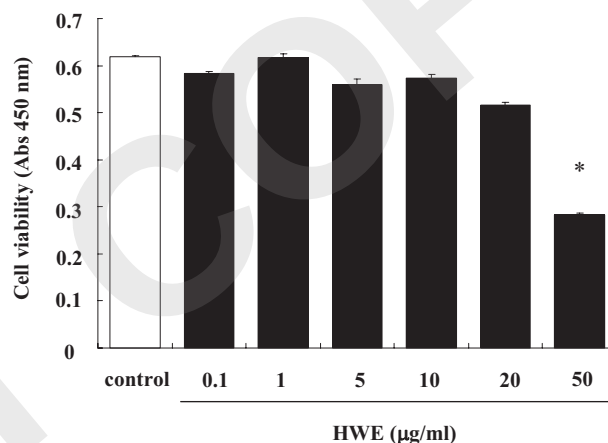


Figure 2. Effect of hop water extract (HWE) on cell viability of human telomerase reverse transcriptase (hTERT)-transfected human nasal epithelial cells (HNECs). Survival rates of hTERT-transfected HNECs after treatment with 0.1, 1, 5, 10, 20 or 50 µg/mL of HWE for 24 hours were determined by MTT assay. Error bars represent means $\pm$ SEM ($n = 3$ of three different experiments; * $p < 0.01$ compared with control).

tendon/mL 0.1% acetic acid), and incubated in a humidified, 5% CO₂/95% air incubator at 37°C. Caco-2 cells and Calu-3 cells were treated with 25 µg/mL of poly(I:C) for 24 hours.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) Assay

The cells plated on 24-well tissue culture plates (BD Labware, Franklin Lakes, NJ) were treated with 0.1–50 µg/mL of HWE for 24 hours. The cell survival was evaluated by using an MTT Cell Growth Assay Kit (Millipore, Billerica, MA) according to the manufacturer's recommendations. We performed three different experiments and measured even triplicates of one sample. The ratio of absorbance was calculated and presented as the mean $\pm$ SEM.

RNA Isolation and Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted and purified from hTERT-transfected HNECs using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (1 µg) was reverse transcribed into cDNA using a mixture of oligo(dT) and Superscript II RTase under the recommended conditions (Invitrogen). Each cDNA synthesis was performed in a total volume of 20 µL for 50 minutes at 42°C and terminated by incubation for 15 minutes at 70°C. Real-time polymerase chain reaction detection was performed by using a TaqMan Gene Expression Assay kit (Applied Biosystems, Foster City, CA). The amount of 18S ribosomal RNA

(Hs99999901) mRNA in each sample was used to standardize the quality of TSLP mRNA (Hs00263639). We performed three different experiments and measured even triplicates of one sample.

Enzyme-Linked Immunosorbent Assay

The concentrations of TSLP and TNF- α protein in cell culture supernatants of hTERT-transfected HNECs were measured at 24 hours after stimulation with dsRNA using ELISA kits for human TSLP and TNF- α (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. In this study, the minimum detection limits for TSLP and TNF- α in the supernatant were 31.25 and 7.8 pg/mL, respectively. We performed three different experiments and measured even triplicates of one sample.

Western Blot Analysis

HNECs were scraped from a 60-mm dish containing 300 μ L of sample buffer (1 mM of NaHCO₃ and 2 mM of phenylmethylsulfonylfluoride) and the total cell lysates were collected in microcentrifuge tubes and then sonicated for 10 seconds. The protein concentrations of the samples were determined using a bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific, Waltham, MA). Aliquots of 15 μ g of protein/lane for each sample were separated by electrophoresis in 4–20% SDS polyacrylamide gels (Cosmo Bio Co., Tokyo, Japan), and electrophoretic transfer to a nitrocellulose membrane (Immobilon; Millipore) was performed.

The membrane was saturated with blocking buffer (25 mM of Tris, pH 8.0, 125 mM of NaCl, 0.1% Tween 20, and 4% skim milk) for 30 minutes at room temperature and then incubated with anti-phospho-pan-PKC, anti-pan-PKC, anti-phospho-NF- κ B, anti-NF- κ B, and anti-actin antibodies (1:1000) at room temperature for 1 hour or at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Dako A/S, Copenhagen, Denmark) at room temperature for 1 hour. The immunoreactive bands were detected using an Enhanced Chemiluminescence Western blotting system (GE Healthcare, Little Chalfont, U.K.). We performed three different experiments and measured even triplicates of one sample.

Data Analysis

Signals were quantified using Scion Image Beta 4.02 Win (Scion Co., Frederick, MA). Each set of results shown is representative of at least three separate experiments. Results are given as means $\pm$ SEM. Differences between groups were tested by analysis of variance followed by a *post hoc* test and an unpaired two-tailed Student's *t*-test and considered to be significant when $p < 0.05$.

RESULTS

dsRNA Induces TSLP Release from HNECs

It is reported that the synthetic dsRNA analog poly(I:C) significantly induces TSLP from keratinocytes,⁷ bronchial epithelial cells,^{8,23} and intestinal epithelial cells.³

By ELISA, we investigated whether poly(I:C) induced TSLP release from hTERT-transfected HNECs, compared with intestinal epithelial cells Caco-2 cells and bronchial epithelial cells Calu-3 cells. As shown in Fig. 1, by treatment with 25 μ g/mL of poly(I:C) for 24 hours, TSLP release from hTERT-transfected HNECs was strongly increased compared with the control, and the values were higher than those from Caco-2 cells and Calu-3 cells.

Cell Viability of HNECs Treated with HWE

To investigate the influence of HWE on hTERT-transfected HNECs, we measured the survival rates of HNECs after treatment with 0.1–50 μ g/mL of HWE for 24 hours by MTT assay. As shown in Fig. 2, 50 μ g/mL of HWE significantly reduced the viability of HNECs, whereas <20 μ g/mL of HWE did not. There were no signs that HWE at the concentrations used in the subsequent evaluation was cytotoxic.

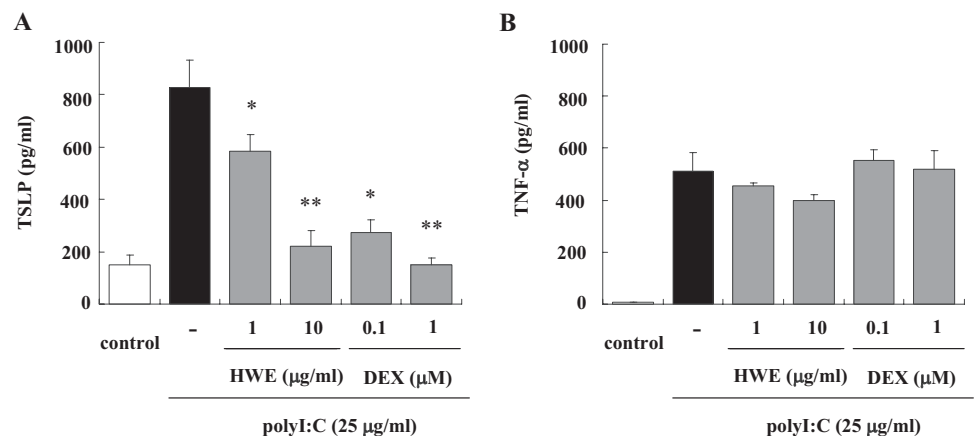
HWE and Dexamethasone Inhibit dsRNA-Induced Release and Production of TSLP but not TNF- α in HNECs

Dexamethasone has many anti-inflammatory effects and inhibits dsRNA-induced production of TSLP from keratinocytes.⁷ In the present experiments, dexamethasone was used as a positive control.

To investigate whether HWE inhibited dsRNA-induced release of TSLP and TNF- α in hTERT-transfected HNECs, the cells were pretreated with 1–10 μ g/mL of HWE and 0.1–1 μ M of dexamethasone 30 minutes before treatment with 25 μ g/mL of poly(I:C) for 24 hours. ELISA revealed that treatments with HWE inhibited dsRNA-induced TSLP release from hTERT-transfected HNECs in a dose-dependent manner as well as dexamethasone (Fig. 3 A), whereas treatment with both HWE and dexamethasone did not prevent dsRNA-induced TNF- α release (Fig. 3 B).

Furthermore, to confirm whether the inhibitory effects of HWE and dexamethasone on dsRNA-induced TSLP release occurred at the mRNA level, we performed real-time polymerase chain reaction for TSLP mRNA using hTERT-transfected HNECs pretreated with 10 μ g/mL of HWE and 1 μ M of dexamethasone before treatment with 25 μ g/mL of poly(I:C) for 24 hours. As shown in Fig. 4, both HWE and dexamethasone significantly inhibited dsRNA-induced mRNA expression of TSLP in hTERT-transfected HNECs.

Figure 3. Effects of hop water extract (HWE) and dexamethasone (DEX) on polyinosine-polycytidylic acid (poly[I:C])–induced thymic stromal lymphopoietin (TSLP) and TNF- α release from human telomerase reverse transcriptase (hTERT)–transfected human nasal epithelial cells (HNECs). (A) TSLP and (B) TNF- α in the cultured medium of hTERT-transfected HNECs pretreated with 1 or 10 μ g/mL of HWE and 0.1 or 1 μ M of DEX before treatment with 25 μ g/mL of poly(I:C) for 24 h were detected by ELISA. Error bars represent means $\pm$ SEM ($n = 3$ of three different experiments; * $p < 0.05$, ** $p < 0.01$ compared with control).



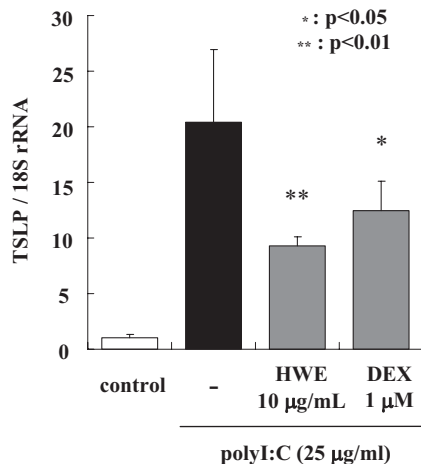


Figure 4. Effects of hop water extract (HWE) and dexamethasone (DEX) on polyinosine-polycytidylic acid (poly(I:C))-induced thymic stromal lymphopoietin (TSLP) mRNA in human telomerase reverse transcriptase (hTERT)-transfected human nasal epithelial cells (HNECs). TSLP mRNA in hTERT-transfected HNECs pretreated with 10 µg/mL of HWE and 1 µM of DEX before treatment with 25 µg/mL of poly(I:C) for 24 hours were determined by real-time polymerase chain reaction (PCR). Error bars represent means $\pm$ SEM ($n = 3$ of three different experiments; * $p < 0.05$, ** $p < 0.01$ compared with control).

Effects of Signal Inhibitors on dsRNA-Induced TSLP Release from HNECs

It is unclear which signal transduction pathways HWE used to inhibit dsRNA-induced TSLP release from HNECs. It is thought that the production and release of TSLP are regulated *via* distinct signal transduction pathways. We first investigated the signal transduction pathways in dsRNA-induced TSLP release from HNECs, by using the various inhibitors. hTERT-transfected HNECs were pretreated with 10 µM of PD98059, 10 µM of SB203580, 10 µM of LY98059, 10 µM of GF109203X, and 1 µM of IMD-0354 before treatment with 25 µg/mL of poly(I:C) for 24 hours. As shown in Fig. 5, dsRNA-induced TSLP release from HNECs was significantly prevented by the pan-PKC inhibitor GF109203X and the NF-κB inhibitor IMD-0354 in ELISA. In the present study, the TSLP release induced by dsRNA in HNECs was regulated *via* PKC and NF-κB pathways.

HWE Regulates the Activity of PKC and NF-κB Induced by TPA and IL-1β in HNECs

To investigate whether HWE could prevent the activity of PKC and NF-κB in hTERT-transfected HNECs, the cells were pretreated with HWE, pan-PKC inhibitor GF109203X, and NF-κB inhibitor IMD-0354 before treatment with TPA and IL-1β, respectively, for 4 hours. Western blotting revealed that the treatment with HWE efficiently prevented phosphorylation of pan-PKC induced by treatment with a PKC activator TPA as well as the pan-PKC inhibitor (Fig. 6 A). However, HWE did not prevent phosphorylation of NF-κB induced by treatment with the NF-κB activator IL-1β, whereas the NF-κB inhibitor prevented the phosphorylation (Fig. 6 B).

DISCUSSION

Epithelial cell-derived cytokines, including TSLP, IL-25, and IL-33 are critical regulators of innate and adaptive immune responses associated with Th2 cytokine-mediated inflammation at nasal mucosal sites.^{24,25} Overexpression of TSLP is observed in allergic rhinitis, and TSLP is thought to play a key role in allergic rhinitis.^{4,5} The present study, for the first time, indicated that treatment with HWE signifi-

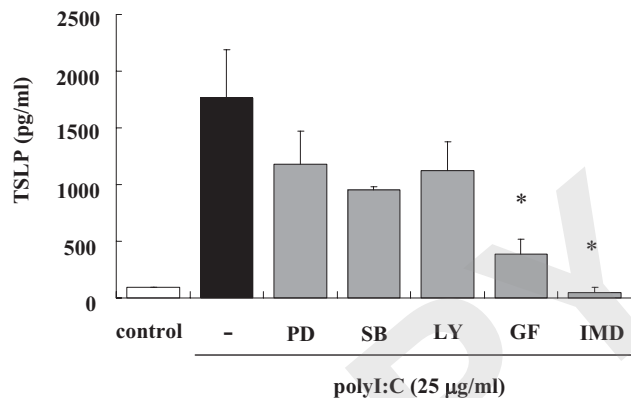


Figure 5. Effects of inhibitors of signal transduction pathways on polyinosine-polycytidylic acid (poly(I:C))-induced thymic stromal lymphopoietin (TSLP) release from human telomerase reverse transcriptase (hTERT)-transfected human nasal epithelial cells (HNECs). TSLP in the cultured medium of hTERT-transfected HNECs pretreated with PD98059 (10 µM), SB203580 (10 µM), LY98059 (10 µM), GF109203X (10 µM), or IMD-0354 (1 µM) before treatment with poly(I:C) (25 µg/mL) for 24 hours were detected by ELISA. Error bars represent means $\pm$ SEM ($n = 3$ of three different experiments; ** $p < 0.01$ compared with control).

cantly suppressed dsRNA-induced production and release of TSLP *via* a PKC signal pathway in HNECs.

Glucocorticoids are known to have highly anti-inflammatory effects through multiple mechanisms.²⁶ They significantly suppress dsRNA-induced TSLP production in human bronchial epithelial cells and keratinocytes.^{6,7} In the present study, HWE significantly suppressed dsRNA-induced TSLP, but not TNF-α, in HNECs, as well as the glucocorticoid dexamethasone. It is reported that hop bitter acids efficiently block inflammation independent of glucocorticoid receptor α.²⁷ In the present study, HWE may have inhibited dsRNA-induced TSLP in HNECs independent of the glucocorticoid receptor.

It is thought that production of TSLP is regulated *via* distinct signal transduction pathways. Primary human airway smooth muscle cells pretreated with inhibitors of p38 mitogen-activated protein kinase (MAPK) or p42/44 MAPK, but not PI3K, show a significant decrease in TSLP release after IL-1β and TNF-α treatments.²⁸ In human nasal fibroblasts, up-regulation of TSLP protein after treatment with IL-1β is prevented by inhibitors of PKC and p38 MAPK, whereas up-regulation of TSLP protein after treatment with TNF-α is prevented by inhibitors of PKC, PI3K, and JNK.²⁹ On the other hand, NF-κB is a transcription factor that regulates production of cytokines and chemokines in various inflammatory diseases. IL-1β and TNF-α regulate human TSLP gene expression in an NF-κB-dependent manner in human airway epithelial cells.³⁰ The induction of TSLP by dsRNA is also dependent on NF-κB in human bronchial epithelial cells and keratinocytes.^{6,31} In the present study, the release and production of TSLP induced by dsRNA in HNECs were prevented by inhibitors of PKC and NF-κB, but not p42/44 MAPK, p38 MAPK, or PI3K. When we investigated whether HWE prevents the activation of PKC by TPA or the activation of NF-κB by IL-1β, we found that it efficiently suppressed up-regulation of PKC activity by TPA, but not NF-κB activity induced by IL-1β in HNECs. These findings suggested that HWE inhibited dsRNA-induced release and production of TSLP by blocking a PKC signal transduction pathway in HNECs.

TSLP is produced by not only epithelial cells but also stromal cells, smooth muscle cells, lung fibroblasts, and mast cells.³² Nasal polyp fibroblasts produce TSLP in response to stimulation by TNF-α.³³ TSLP expression in human nasal fibroblasts is induced together with IL-33 by treatment with IL-1β or TNF-α.³³ Treatment with HWE also prevented up-regulation of TSLP and IL-33 by treatment with IL-1β or

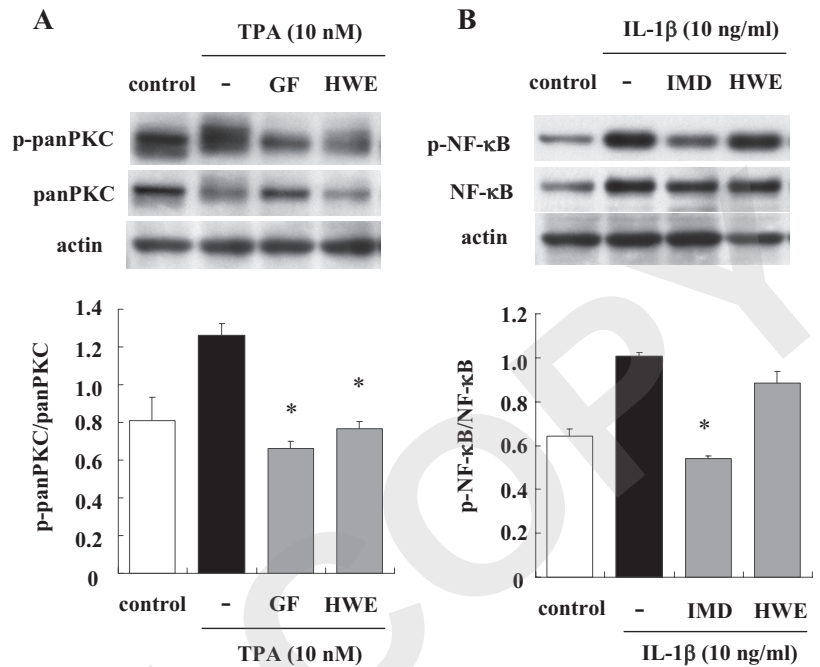


Figure 6. Effects of hop water extract (HWE) on activity of protein kinase C (PKC) and NF- κ B in human telomerase reverse transcriptase (hTERT)-transfected human nasal epithelial cells (HNECs). Expression of (A) phospho-pan-PKC and pan-PKC, (B) phospho-NF- κ B and NF- κ B in hTERT-transfected HNECs pretreated with (A) GF109203X (10 μ M) or (B) IMD-0354 (1 μ M) and HWE (10 μ g/mL) before treatment with (A) 12-O-tetradecanoylphorbol 13-acetate (TPA; 10 nM) or (B) IL-1 β (10 ng/mL) for 4 hours was detected by Western blot analysis. Bar graphs of panels A and B: error bars represent means $\pm$ SEM (three different experiments; * p < 0.05 compared with control).

TNF- α in human nasal fibroblasts (data not shown). This suggested that HWE could prevent release of TSLP from human nasal mucosal epithelial cells and fibroblasts during allergic rhinitis.

In conclusion, we found that HWE inhibited dsRNA-induced production and release of TSLP in HNECs at concentrations that did not cause a direct cytotoxic effect of the compound. HWE has previously been shown to exhibit effectiveness in the treatment against nasal allergy *in vitro* and *in vivo*.^{16,17,34} HWE may have potent preventive effects against allergic rhinitis through the inhibition of TSLP.

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