Original Research

The β-sitosterol attenuates atopic dermatitis-like skin lesions through down-regulation of TSLP

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Abstract

The compound β-sitosterol (BS) is one of the most common forms of phytosterols and has anti-cancer, anti-oxidant, anti-bacterial, and anti-inflammatory effects. However, the effect of BS on atopic dermatitis (AD) has not been elucidated. Therefore, we investigated whether BS would be an effective treatment against AD. We treated BS on 2,4-dinitrofluorobenzene (DNFB)-induced AD-like skin lesions in NC/Nga mice, anti-CD3/anti-CD28-stimulated splenocytes, and phorbol myristate acetate/calcium ionophore A23187-stimulated human mast cell line (HMC-1) cells. Histological analysis, ELISA, PCR, caspase-1 assay, and Western blot analysis were performed. BS reduced the total clinical severity in DNFB-treated NC/Nga mice. Infiltration of inflammatory cells and number of scratching were clearly reduced in the BS-treated group compared with the DNFB-treated group. BS significantly reduced the levels of inflammation-related mRNA and protein in the AD skin lesions. BS significantly reduced the levels of histamine, IgE, and interleukin-4 in the serum of DNFB-treated NC/Nga mice. The activation of mast cell-derived caspase-1 was decreased by treatment with BS in the AD skin lesions. BS also significantly decreased the production of tumor necrosis factor-α from the stimulated splenocytes. In the stimulated human mast cell line, HMC-1 cells, increased intracellular calcium levels were decreased by treatment with BS. Further, BS inhibited the production and mRNA expression of TSLP through blocking of caspase-1 and nuclear factor-κB signal pathways in the stimulated HMC-1 cells. These results provide additional evidence that BS may be considered an effective therapeutic drug for the treatment of AD.

Keywords: β-Sitosterol, atopic dermatitis, mast cell, TSLP, caspase-1

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Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases affecting up to 20% of children and 1–3% of adults in industrialized countries.[1] AD results from complex interactions among several genetic factors, from skin barrier function, from exposure to various allergens and infectious agents, and from immune responses.[2] The symptoms of AD include erythematous skin lesions, pruritus, altered epidermal barrier, and marked inflammatory cell infiltration in the dermis, leading to a significant impairment in quality of life.[3]

NC/Nga mice are widely studied AD animal models used to develop effective anti-AD drugs. In the NC/Nga mice model of spontaneously developing AD-like skin lesions, environmental allergens contribute to the development of skin lesions and elevation of IgE levels in serum. In contrast, contact hypersensitivity was induced by repeated skin exposure to haptens, such as 2,4-dinitrofluorobenzene (DNFB).[4] Although a hapten primarily induces a Th1 response, repeated exposure to such an agent results in mixed responses of Th1 and Th2.[5]

Numerous inflammatory cell types, including mast cells, eosinophils, lymphocytes, and macrophages, play crucial roles in the pathogenesis of AD.[6] T-cells (IL-4- producing $CD4^+$ T-cells) and mast cells, as well as an increasing number of Th2 cytokines and chemokines, infiltrated the skin lesions of NC/Nga mice.[7] Mast cells play an important role in both adaptive and innate immunity and increase in the presence of immunological skin diseases, including contact dermatitis, AD, immunobullous disease, and scleroderma.[8] A number of studies have reported that activated mast cells infiltrate skin lesions of the AD animal model.[9–11] After allergen challenge, IgE-stimulated mast cells can secrete a large variety of inflammatory mediators, including histamine, tryptase, prostaglandins, or leukotrienes.[12] Recently, we reported that thymic stromal lymphopoietin

(TSLP) as developmental factor of AD was produced by the intracellular calcium/caspase-1/ receptor-interacting protein 2(RIP2)/nuclear factor-κB (NF-κB) pathways on mast cells.[13,14] TSLP exacerbates the pathogenesis of AD through the initiation of allergic inflammatory reactions.[15,16]

Phytosterols are natural constituents of plants and are present in vegetable oils, seeds, fruits, nuts, cereals, and legumes.[17] Among the various phytosterols, β-sitosterol (24-ethyl-5-cholestene-3-ol, BS) is the most common sterol. BS has various types of activity such as anti-oxidant, anti-inflammatory, anti-apoptotic, hypocholesterolemic, and anti-hyperglycemic effects.[18–21] However, the effect of BS on AD has not yet been clarified. Therefore, we investigated the effects and regulatory mechanisms of BS on DNFB-induced AD-like skin lesions in NC/Nga mice, anti-CD3/anti-CD28-stimulated splenocytes, and phorbol myristate acetate/calcium ionophore A23187 (PMACI)-stimulated human mast cell line (HMC-1) cells.

Materials and methods

Reagents

We purchased Isocove's Modified Dulbecco's Medium (IMDM) from Gibco BRL (Grand Island, NY, USA); BS (#C/N = S9889, purity ≥97%), PMA, calcium ionophore A23187, 2-bis(2-aminophenoxy)ethane-N,N,N0,N0-tetraacetic acid acetoxymethyl ester (BAPTA-AM), Fura-2/AM, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co (St. Louis, MO, USA); TSLP antibodies from R&D Systems (Minneapolis, MN, USA); RIP2, caspase-1, NF-κB, Poly-ADP-ribose polymerase (PARP), phosphorylated IκBα (pIκBα), actin, and tubulin antibodies from Santa Cruz Biotechnology (Santacruz, CA, USA); Recombinant caspase-1 from Biovision, Inc. (Mountain View, CA, USA); BS, DNFB, and *o*-phthaldialdehyde (OPA) from Sigma Chemical Co. (St. Louis, MO, USA); IgE, IL-4, IL-6, and TNF-α antibodies from BD Pharmingen (Torreyana Road, San Diego, CA, USA). BS was prepared by dissolving it with carboxylmethyl cellulose buffer including 10% dimethylsulfoxide (DMSO).

Animals

Male NC/Nga mice were obtained from Charles River Laboratories International, Inc. (Yokohama, Japan) and the animals were maintained under conventional condition and performed under approval from the animal care committee of Kyung Hee University [Protocol Number. KHUASP (SE)-11-009]. Mice were sacrificed with CO_2 inhalation.

Sensitization with DNFB

For active sensitization, 100 μL 0.15% DNFB dissolved in acetone was topically challenged to the shaved abdominal skins of NC/Nga mice. A week later, the shaved dorsal skins of NC/Nga mice was challenged with 50 μL 0.15% DNFB. The DNFB challenge was repeated four times every three days, beginning seven days after initial sensitization (thus on days 7, 10, 13, and 16) in accordance with previous report.[22] We selected the concentration of BS (2.5 mg/kg) as a mean dose of 1.7 μg/mL and 3.1 μg/mL in accordance with previous reports.[23–25] BS (2.5 mg/kg) and saline (control group) were orally administrated to DNFB-challenged mice at that time. The same volume of acetone was challenged to the shaved dorsal skin and saline was orally administrated as an unchallenged vehicle group. Dorsal skin samples, serum, and spleen were obtained 4 h after the last DNFB challenge. After anesthetization, blood was withdrawn from the heart of mouse into syringes. Then, serum was prepared by centrifugation at 3400 rpm at 4°C for 10 min.

Histological analysis

Formaldehyde (10%) fixed dorsal skin samples were embedded in paraffin, cut into 4 μm thick sections. After dewaxing and dehydration, sections stained with hematoxylin and eosin (H&E) (for eosinophils), alcian blue and safranin O (for mast cells), CD4 (for T cells; Abbiotec, San Diego, CA), F4/80 (for macrophages; Santa Cruz Biotechnology). It was measured at the same site of the dorsal skin lesions among groups. The epidermal thickness (dimension from the basal membrane to the stratum corneum) was measured with Axiovision 4.8 software (Carl Zeiss, Jena, Germany).[26]

Myeloperoxidase assay

To evaluate the effect of BS on neutrophil infiltration, the activity of tissue myeloperoxidase (MPO) was assessed. A biopsy was placed in 0.75 mL of 80 mM PBS, pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide and homogenized (45 s at 0°C) in a motor-driven homogenizer. The homogenate was decanted into a microcentrifuge (Microfuge; Eppendorf, Westbury, NY, USA) tube, and the vessel was washed with a second 0.75 mL aliquot of hexadecyltrimethylammonium bromide in a buffer. The wash was added to the tube, and the 1.5 mL sample was centrifuged at 12,000 *g* at 4°C for 15 min. Samples of the resulting supernatant were added to 96-well microliter plates in triplicate at a volume of 30 μL. For the MPO assay, 200 μL of a mixture containing 100 μL of 80 mM PBS, pH 5.4, 85 μL of 0.22 M PBS, pH 5.4, and 15 μL of 0.017% hydrogen peroxide were added to the wells. The reaction was started by the addition of 20 μL of 10 mM *O*-dianisidine dihydrochloride in 80 mM PBS, pH 5.4. The plates were incubated at 37°C for 3 min and then placed on ice. The reaction was stopped by the addition of 30 μL of 1.46 M sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using a plate reader set to measure absorbance at 460 nm and is expressed as optical density milligram per tissue.

Cytokines assay

The levels of TSLP, IL-4, IL-6, IgE, and TNF-α were determined using a sandwich ELISA method according to the manufacturer's instructions (R & D system Inc, Minneapolis, MN, USA; Pharmingen, San Diego, CA, USA). The single cells (2.5×10^6/mL) from the spleen of normal NC/Nga mouse were treated with BS and

stimulated with immobilized anti-CD3 (2 μg/mL)/soluble anti-CD28 antibodies (4 μg/mL) in 24-well plates. Cell supernatant was measured by ELISA method.

Reverse transcription-PCR analysis

Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Republic of Korea), we isolated the total RNA from HMC-1 cells in accordance with the manufacturer's specifications. We performed reverse transcription polymerase chain reaction (RT-PCR) with the following primers: TSLP (5′-TGC AAG TAC TAG TAC GGA TGG GG C-3′; 5′-GGA CTT CTT GTG CCA TTT CCT GAG-3′); IL-4 (5′-ACG GAG ATG GAT GTG CCA AA-3′; 5′-CGA GTA ATC CAT TTG CAT GA-3′); IFN-γ (5′-TAC TGC CAC GGC ACA GTC ATT GAA-3′; 5′-GCA GCG ACT CCT TTT CCG CTT CCT-3′); IL-6 (5′-CGG GAT CCA TGT TCC CTA CTT CAC AA-3′; 5′-CCC AAG CTT CTA CGT TTG CC-3′); TNF-α (5′-TAC AGG CTT GTC ACT CGA AT-3′; 5′-ATG AGC ACA GAA AGC ATG AT-3′); thymus and activation-regulated chemokine/CCL17 (TARC) (5′-CAG GAA GTT GGT GAG CTG GTA TA-3′; 5′-TTG TGT TCG CCT GTA GTG CAT A-3′). Reference gene GAPDH (5′-GGC ATG GAC TGT GGT CAT GA-3′; 5′-TTC ACC ACC ATG GAG AAG GC-3′) was used to verify that equal amounts of RNA. The annealing temperature was 62°C for TSLP, IL-4, and IFN-γ; 50°C for IL-6; 60°C for TNF-α and GAPDH; 55°C for TARC. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The levels of each mRNA were normalized to the level of the GAPDH.

Histamine assay

The histamine contents from serum were measured by the OPA spectrofluorometric procedure.[27] The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

Caspase-1 activity assay

Caspase-1 activity was measured according to the manufacturer's instructions by using a caspase-1 assay kit (R & D system Inc.).

Analysis of confocal laser-scanning microscope

The dorsal skin samples were immediately fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with fetal bovine serum (FBS) followed by 1 h of incubation and then stained with anti-rabbit caspase-1 and FITC-conjugated anti-rabbit IgG, (Santa Cruz Biotechnology), PE-conjugated anti-mouse c-Kit antibodies (BD Pharmingen). 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) containing mounting medium was used to counterstain the nuclei. The images of stained cells and specimens were randomly visualized using a confocal laser-scanning microscope.

HMC-1 cells culture

HMC-1 cells were incubated in IMDM supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS at 37°C in 5% CO_2 with 95% humidity.

Fluorescent measurements of the intracellular calcium level

To measure the intracellular calcium in a time-dependent manner, the HMC-1 cells (1×10^5) were pretreated with Fura-2/AM in IMDM containing 10% FBS for 30 min. After being washed twice with a calcium free medium containing 0.5 mM EGTA, the cell suspension was placed into a 96-well plate and pretreated with BS and then stimulated with PMACI. The kinetics of the intracellular calcium was recorded every 10 s at 440 nm (excitation at 360 nm) in a spectrofluorometer.

MTT assay

Cell viability was measured by a MTT assay. Briefly, HMC-1 cell (4×10^5) suspension was treated with BS for 2 h and stimulated with PMACI for 8 h and then harvested. The cell suspension containing MTT solution (5 mg/mL) was incubated at 37°C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, the optical density of 96-well culture plate was determined using an ELISA reader at 540 nm.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using a SYBR Green master mix and the detection of mRNA was analyzed using an ABI StepOne RT-PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences for the reference gene GAPDH and gene TSLP were as follows: GAPDH (5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′; 5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′); TSLP (5′-TAT GAG TGG GAC CAA AAG TAC CG-3′; 5′-GGG ATT GAA GGT TAG GCT CTG G-3′). Typical profile times used were the initial step, 95°C for 10 min followed by a second step at 95°C for 10 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the GAPDH and compared to the control. Data were analyzed using the ΔΔCT method.

Western blot analysis

The stimulated cells were lysed and separated through 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and then the membranes were blocked and incubated with primary and secondary antibodies. Finally, the protein bands were visualized by an enhanced chemiluminesence assay according to the manufacturer's instructions (Amersham Co. Newark, NJ, USA).

Kinetic recombinant caspase-1 assay

The hydrolysis of tetrapeptide WEHD-*p*-nitroaniline (*p*NA) by recombinant caspase-1 was measured by a caspase-1

assay kit. In brief, the mixture (2 × reaction buffer, recombinant caspase-1, and DTT) was seeded in a 96-well microplate and tetrapeptide WEHD-*p*NA (substrate) was added except a blank group. Then, the release of *p*NA from substrate at 37°C was spectrophotometrically measured at 405 nm for 24 h in the presence of BS.

Preparation of nuclear extracts and cytoplasmic extracts

Briefly, after cell activation, the cells were washed with ice-cold PBS and resuspended in 60 μL of buffer A (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The cells were left on ice for 15 min, lysed gently with 2.5 μL of 10% Nonidet P-40, and centrifuged at 2000*g* for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extract. The nuclei pellets were resuspended in 40 μL of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 15,000 *g* for 15 min. The supernatant (nuclear extract) was collected and stored at −70°C until the analysis was conducted.

Statistical analysis

These data result from at least-three experiments and are presented as the mean ± SD. Statistical evaluation between vehicle group and DNFB control group, DNFB control group and DNFB + BS group, or unstimulated cells and PMACI-stimulated cells was performed by an independent *t*-test. The statistical evaluation between PMACI-stimulated cells and PMACI + BS (0.025, 0.25, and 2.5 μg/mL) cells was performed by a one-way ANOVA with a Tukey *post hoc* test in each factor. The results were considered significant at a value of $P < 0.05$.

Results

Inhibitory effect of BS on clinical symptoms of DNFB-treated NC/Nga mice

We examined whether the administration of BS could inhibit clinical symptoms in DNFB-treated NC/Nga mice. The AD-like clinical signs and symptoms of noticeable erythema, hemorrhage, excoriation, dryness, and erosion were present in skin lesions of DNFB-treated NC/Nga mice, whereas the administration of BS (2.5 mg/kg) markedly improved these phenotypes in AD skin lesions (Figure 1a). BS (2.5 mg/kg) had no effect on clinical observations in DNFB-unchallenged mice (Figure 1a). Later, we examined the infiltration of inflammatory cells and the thickness of the epidermis by H&E staining. The infiltration of inflammatory cells (eosinophils, mast cells, $CD4^+$Tcells, macrophages, and neutrophils) and the thickness of the epidermis were reduced in skin lesions of the BS-administered group compared with that of the DNFB-treated group (Figure 1b–d). Furthermore, we measured the number of scratching for 10 min after the last DNFB challenge. BS significantly suppressed scratching behavior ($P < 0.05$, Figure 1d).

Inhibitory effect of BS on the levels of TSLP in skin lesions of DNFB-treated NC/Nga mice

We examined the inhibitory effect of BS on the levels of TSLP, the developmental factor of AD in AD skin lesion. TSLP mRNA and protein levels upregulated by DNFB challenge were reduced by BS administration (Figure 2(a) to (c)). Next, we examined whether BS would reduce mRNA expressions of inflammatory cytokines or chemokine in AD skin lesions. The mRNA expressions of IL-4, IFN-γ, IL-6, TNF-α, and TARC were upregulated by DNFB challenge, but the upregulated mRNA expressions were reduced by BS administration (Figure 2a and b). The protein levels of IL-4 and IL-6 were significantly reduced by BS administration ($P < 0.05$, Figure 2c). BS also significantly reduced the levels of serum histamine, IgE, and of IL-4 ($P < 0.05$, Figure 2(d) and (e)).

Inhibitory effect of BS on the levels of caspase-1 in skin lesions of DNFB-treated NC/Nga mice

Recently, we reported that TSLP was expressed through the activation of caspase-1 in HMC-1 cells.[14] Thus, we examined whether BS would regulate the level of caspase-1 *in vivo*. DNFB challenge increased the caspase-1 activation, whereas BS significantly inhibited the caspase-1 activation in AD skin lesions ($P < 0.05$, Figure 3a). Furthermore, immunohistochemical analysis of the AD skin lesion sections in the DNFB-treated NC/Nga mice demonstrated that the mast cell-derived caspase-1 expression was significantly increased, whereas in the BS treatment mice, it was decreased (Figure 3b).

Inhibitory effect of BS on the productions of TNF-α from the stimulated splenocytes

We examined whether BS could inhibit TNF-α production on the anti-CD3/anti-CD 28 antibodies-stimulated splenocytes. As can be seen in Figure 4, the stimulated splenocytes increased the TNF-α production, whereas BS significantly decreased the TNF-α production on the stimulated splenocytes ($P < 0.05$).

Inhibitory effect of BS on the level of intracellular calcium in the stimulated HMC-1 cells

An increase in concentration of intracellular calcium is a sufficient condition for activation of mast cells and secretion of mediators from the mast cells.[28] Thus, we examined the regulatory effect of BS on the level of intracellular calcium in the PMACI-stimulated HMC-1 cells. BAPTA-AM (calcium chelator) was used as a positive control. The stimulation with PMACI increased the calcium release from intracellular stores (in 0.5 mM EGTA containing media), whereas BS or BAPTA-AM decreased the level of intracellular calcium increased by PMACI (Figure 5a). BS did not affect the cell viability of HMC-1 cells (Figure 5b).

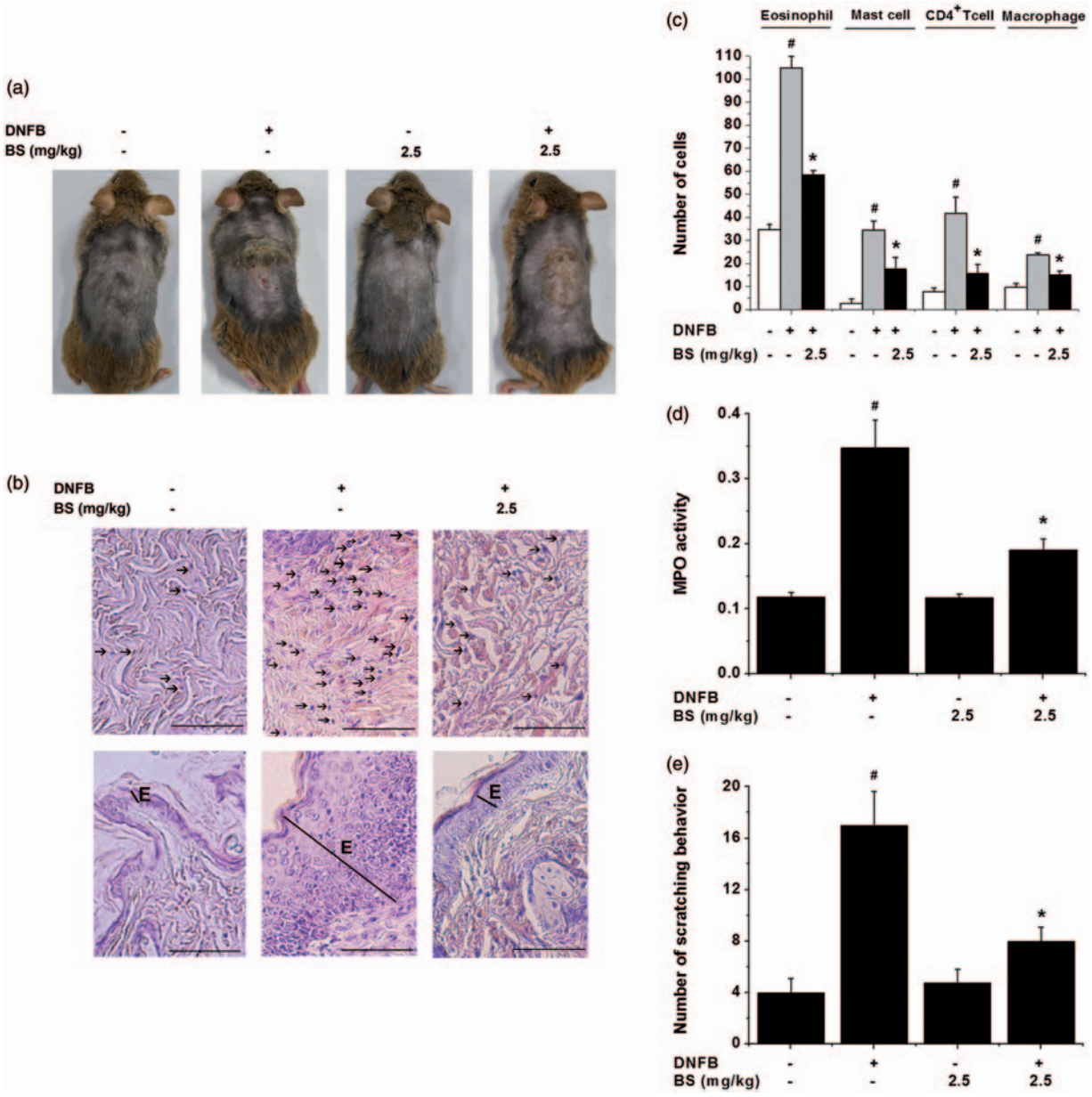

Figure 1 BS reduced clinical symptoms in DNFB-treated NC/Nga mice. (a) The clinical features were observed 4 h after the last DNFB challenge. (b) Histological analysis of skin lesions was examined by H&E staining for eosinophils (upper panel) and epidermal thickness (lower panel). The eosinophils are indicated by arrows. Representative photomicrographs were examined at 400× magnification. E: epidermis. (Scale bar = 5 μm). (c) Five tissue sections ($1.5 \times 10^3\,\mu m^2$) per mouse were randomly selected and inflammatory cells were counted. (d) MPO activity was assayed as described in the Materials and methods section. (e) The number of scratching behavior was measured at 4 h after the last DNFB challenge for 10 min. $^{\#}P < 0.05$, significantly different from vehicle group. $^{*}P < 0.05$, significantly different from control group (DNFB). $n = 5$. (A color version of this figure is available in the online journal.)

Inhibitory effect of BS on the level of TSLP from the stimulated HMC-1 cells

Because increased intracellular calcium induces the production of TSLP from the stimulated mast cells,[13] we examined the regulatory effect of BS on the TSLP production. In the stimulated HMC-1 cells, the production and mRNA expression of TSLP were increased. However, BS reduced the production and mRNA expression of TSLP in the stimulated HMC-1 cells ($P < 0.05$, Figure 6). BS alone had no effect on the production and mRNA expression of TSLP (data not shown). We selected the concentration of dexamethasone (DEX, 3 μg/mL) as a positive control[29] in accordance with previous reports.[30,31] DEX also reduced the production and mRNA expression of TSLP in the stimulated HMC-1 cells ($P < 0.05$, Figure 6).

Inhibitory effect of BS on the activations of RIP2/caspase-1/NF-κB in the stimulated HMC-1 cells

Finally, we examined the effect of the regulatory mechanisms of BS on the TSLP production. As can be seen in Figure 7(a) and (b), BS inhibited the expressions of RIP2 and caspase-1 in PMACI-stimulated HMC-1 cells ($P < 0.05$). BS also significantly inhibited the caspase-1

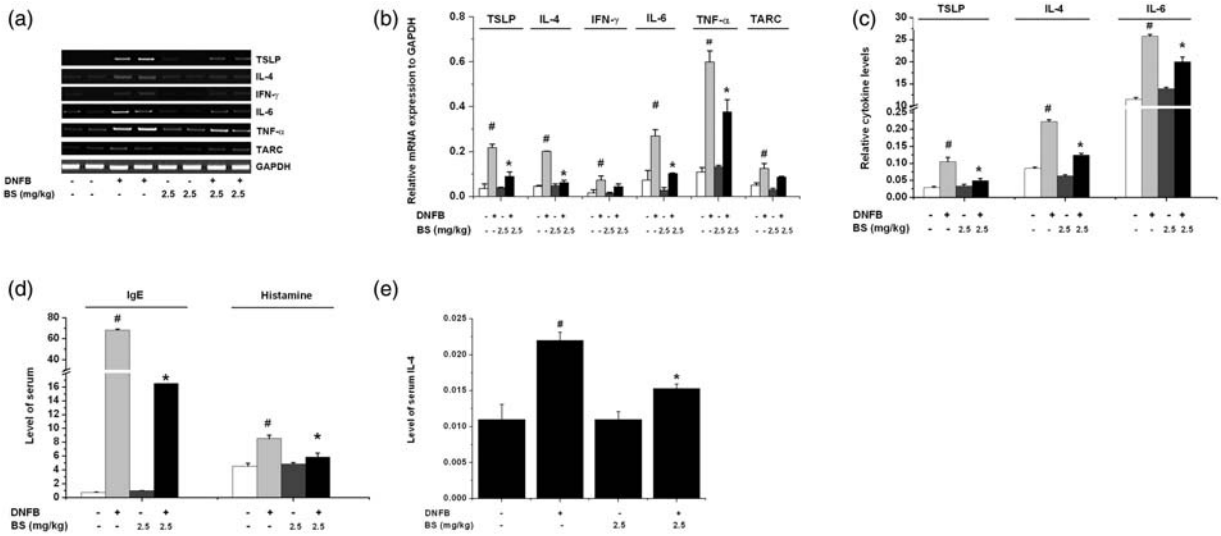

Figure 2 BS reduced the level of TSLP in skin lesions of DNFB-treated NC/Nga mice. (a) The mRNA expressions were analyzed with RT-PCR analysis. (b) The relative mRNA expressions to GAPDH were quantified by densitometry. (c) The protein expressions from the skin lesion homogenate were analyzed with ELISA. (d, e) The intensity of serum histamine was assayed as described in the Materials and methods section. The levels of serum IgE and IL-4 were analyzed with ELISA. $^{\#}P < 0.05$; significantly different from vehicle group. $^{*}P < 0.05$; significantly different from control group (DNFB)

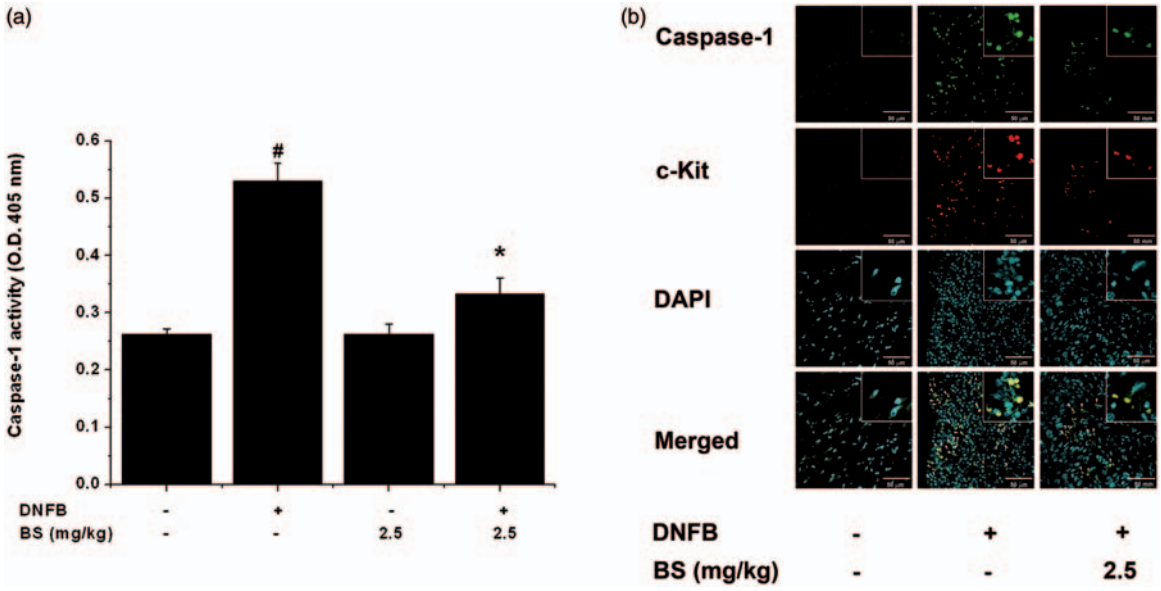

Figure 3 BS reduced the level of caspase-1 in skin lesions of DNFB-treated NC/Nga mice. (a) The caspase-1 activity from the skin lesion homogenate was analyzed with a caspase-1 assay kit. (b) The caspase-1^{+} (FITC) and c-Kit^{+} (PE) cells from DNFB-induced skin lesions were examined with a confocal laser-scanning microscope. Mast cells are identified as c-Kit^{+} cells. The merged image indicated the co-localization of mast cell and caspase-1. The caspase-1^{+}c-Kit^{+} cells were indicated by arrows. Representative photomicrographs were examined at 60 × magnification. (Scale bar = 50 μm). (A color version of this figure is available in the online journal.)

activity in a dose-dependent manner ($P < 0.05$, Figure 7c). A caspase-1 kinetic assay was used to evaluate the binding affinity of BS for the caspase-1 catalytic domain. Recombinant caspase-1 was used to confirm the effect of BS in the kinetic assay. As can be seen in Figure 7(d), caspase-1 activity was increased after treatment with WEHD-pNA (caspase-1 substrate). However, BS significantly inhibited the cleavage of the caspase-specific peptide (tetrapeptide WEHD-*p*NA, substrate of caspase-1) through blocking of the binding reaction between the recombinant caspase-1 and the substrate during the indicated time ($P < 0.05$, Figure 7d). In addition, BS inhibited the NF-κB translocation to the nuclei and the IκBα phosphorylation in cytosol ($P < 0.05$, Figure 7(e) and (f)).

Discussion

The current study provides the first evidence that BS prevented the development of AD and the elevation of IgE and histamine in DNFB-treated NC/Nga mice. The levels of

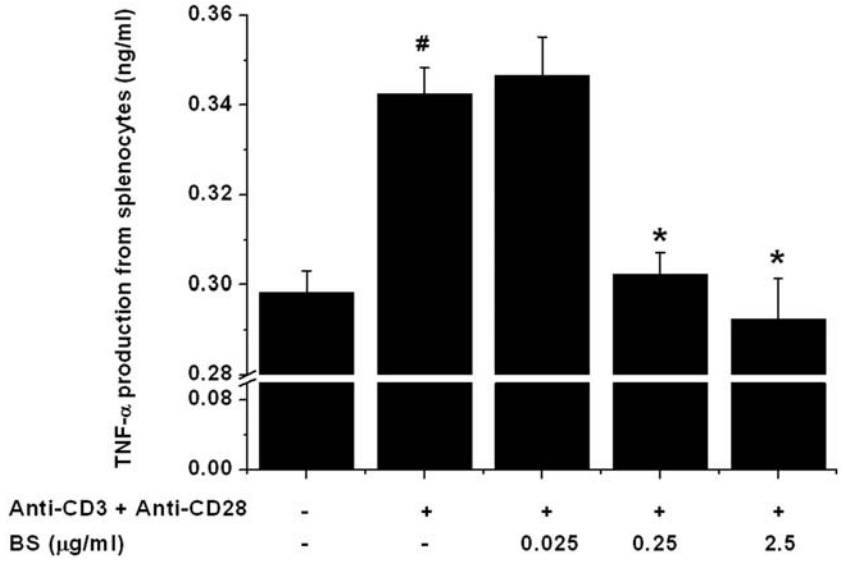

Figure 4 BS reduced the productions of TNF-α from the stimulated splenocytes. Splenocytes (2.5×10^6/mL) were stimulated with immobilized anti-CD3/soluble anti-CD28 antibodies and treated with BS. The productions of TNF-α were analyzed with the ELISA. The datum represents the mean ± SD. of three independent experiments. $^{\#}P < 0.05$, significantly different from unstimulated cells. $^{*}P < 0.05$, significantly different from anti-CD3/anti-CD28-stimulated cells

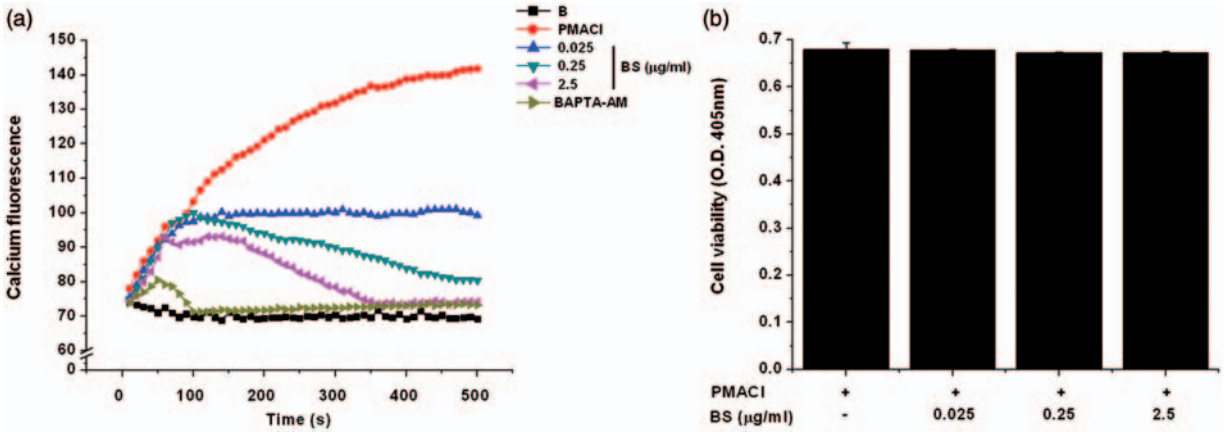

Figure 5 BS reduced the level of intracellular calcium in the PMACI-stimulated HMC-1 cells. (a) HMC-1 cells were pretreated with BS (0.025, 0.25, and 2.5 μg/mL) or BAPTA-AM (10 μM) for 20 min and then stimulated with PMACI. The kinetics of intracellular calcium was measured every 10 s for 500 s. B: unstimulated cells; PMACI: PMACI-stimulated cells; BAPTA-AM: BAPTA-AM and PMACI-stimulated cells. (b) Cell viability was analyzed with an MTT assay. Each datum represents the mean ± SD of three independent experiments. $^{*}P < 0.05$, significantly different from PMACI-stimulated cells. (A color version of this figure is available in the online journal.)

inflammatory cytokines and chemokine in the AD mice were also significantly reduced by BS administration. BS is responsible for the inhibition of TNF-α production from the stimulated splenocytes. In addition, BS reduced the level of TSLP through a blockade of intracellular calcium/RIP2/caspase-1/NF-κB pathways in human mast cells.

AD is induced by initial sensitization to environmental antigens, infections, and stress through skin with altered epidermal barrier function.[32] Increased incursion of microbial products through deficient-skin barriers during AD activates innate receptors and induces proinflammatory signals contributing to sensitization.[33] Recently, it has been proposed that TSLP acts as an important switch factor in allergic responses based on its capacity to differentiate proinflammatory TH2 cells from naive CD4 T-cell precursors in human subjects.[34] The proinflammatory TH2 cells produce high levels of IL-4, IL-5, and IL-13 in conjunction with TNF-α but only low levels of IFN-γ and IL-10.[35,36] In addition, the cytokine balance skews from TH1 to TH2, thereby increasing symptoms of allergic disease. In mice lacking the TSLP gene, allergic reactions were attenuated.[37] Local infiltrations by eosinophils, Th2 cytokine production, and serum IgE levels were dramatically reduced in TSLP receptor-deficient mice.[33] Conversely, mice expressing an inducible TSLP transgene in the skin develop eczematous lesions.[38] TSLP is highly expressed in the skin lesions of patients with AD.[35,36,39] The interactions of human DCs with TSLP-expressing epithelial cells within

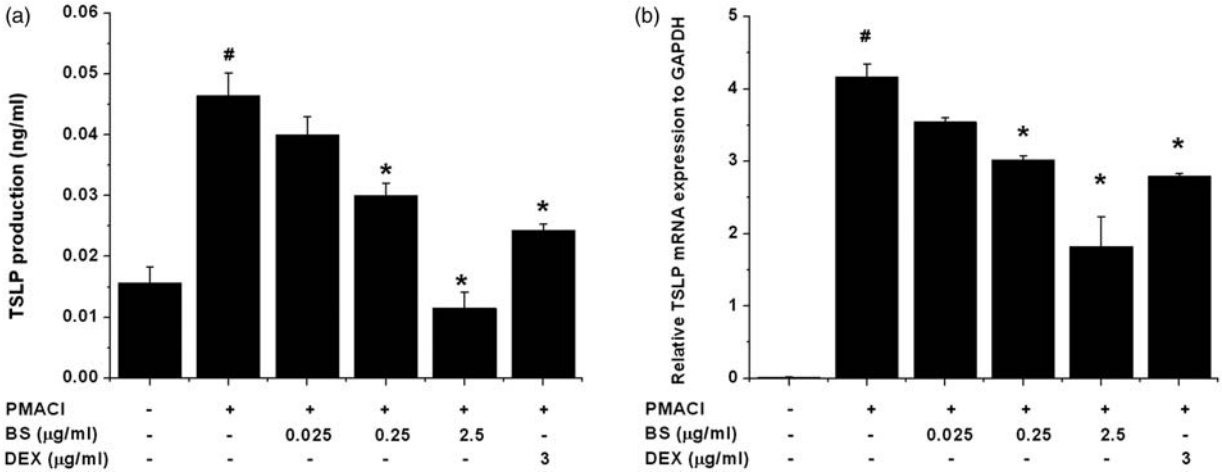

Figure 6 BS reduced the level of TSLP from the PMACI-stimulated HMC-1 cells. (a) HMC-1 cells (4×10^5) were pretreated with BS and DEX for 2 h and stimulated with PMACI for 7 h. The production of TSLP was analyzed with the ELISA. (b) HMC-1 cells (1×10^6) were pretreated with BS and DEX for 2 h and stimulated with PMACI for 5 h. And mRNA expression of TSLP was analyzed with the real time-PCR analysis. Each datum represents the mean ± S.D. of three independent experiments. $^{\#}P < 0.05$, significantly different from unstimulated cells. $^{*}P < 0.05$, significantly different from PMACI-stimulated cells

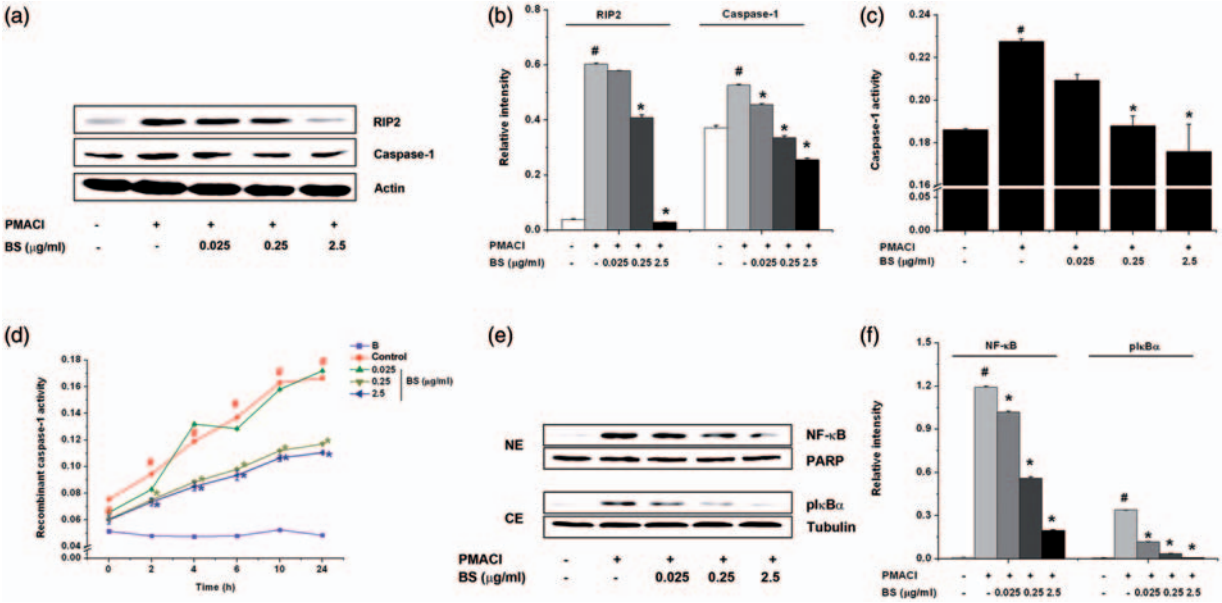

Figure 7 BS reduced the activation of RIP2/caspase-1/NF-κB in the PMACI-stimulated HMC-1 cells. (a) HMC-1 cells (5×10^6) were pretreated with BS (0.025, 0.25, and 2.5 μg/mL) for 2 h and stimulated with PMACI for 1 h. The levels of RIP2 and caspase-1 were analyzed with Western blot analysis. (b) The relative intensities to actin were quantified by densitometry. (c) The caspase-1 activity was analyzed with a caspase-1 assay kit. (d) Catalytic activity of recombinant caspase-1 (0.5 units) was analyzed by tetrapeptide WEHD-*p*NA (substrate) for indicated times. B: no substrate; Control: substrate-treated group; BS: substrate and BS-treated group. Each datum represents the mean ± SD of three independent experiments. $^{\#}P < 0.05$, significantly different from no substrate. $^{*}P < 0.05$, significantly different from substrate-treated group. (e) HMC-1 cells (5×10^6) were pretreated with BS for 2 h and stimulated with PMACI for 2 h. The activation of NF-κB and phosphorylation of IκBα were analyzed with Western blot analysis. NE: nuclear extract; CE: cytoplasmic extract. (f) The relative intensities to PARP or tubulin were quantified by densitometry. $^{\#}P < 0.05$, significantly different from unstimulated cells. $^{*}P < 0.05$, significantly different from PMACI-stimulated cells. (A color version of this figure is available in the online journal.)

the respiratory tract or skin are likely important to the generation and maintenance of the TH2 responses that orchestrate allergic disease. The TSLP-mediated amplification of TH2 responses triggered FcεRI pathways and increased levels of the TH2-attracting chemokine TARC/CCL17.[34] Recently, we demonstrated that TSLP was expressed and produced through intracellular calcium, caspase-1, and NF-κB in mast cells.[13] Furthermore, an initial step in allergy immunotherapy is desensitization of FcεRI-bearing mast cells. Therefore, therapies targeting mast cells may prove beneficial for treatment of AD. In this study, BS reduced the TSLP levels in DNFB-induced AD mice and stimulated

HMC-1 cells. So, we postulate that the protective effect of BS on AD might be derived from the down-regulation of TSLP. We can also deduce that BS has an anti-AD effect.

Rapidly emerging studies implicate IL-25 and IL-33, as well as TSLP, as critical regulators of innate and adaptive immune responses associated with TH2 cytokine-mediated inflammation.[40] IL-25 was observed in tissues from patients with chronic asthma and AD.[41] The expression of IL-33 in the skin activated an immune response and this process played a critical role in the pathogenesis of allergic inflammation that is characteristic of AD.[42] Thus, further study is necessary to clarify the regulatory effect of BS on the level of IL-25 and IL-33.

The caspase-1 is a key regulator of pathogen recognition and inflammation. It leads to the processing and release of the proinflammatory cytokines IL-1β, IL-18, IL-33, and TSLP.[13,14,43] Caspase-1 transgenic mice overexpressing caspase-1 spontaneously suffered from chronic dermatitis and significantly increased serum levels of histamine and IgE.[44,45] Caspase-1 and RIP2 lead to activation of the inflammatory cytokine transcription factor, NF-κB[14,46] In this study, histologic study revealed that there were few mast cells-derived caspase-1 in the dermis of mice that were given BS. Both the relative intensity and activity of caspase-1 were also reduced by BS in a dose-dependent manner. Furthermore, BS suppressed the binding reaction between recombinant caspase-1 and the substrate. However, the recombinant caspase-1 activity was the same level in the condition of BS doses of 0.25 and 2.5 μg/mL. This would result from the saturation of recombinant caspase-1 by BS (0.25 μg/mL). Thus, we can presume that caspase-1 is the signaling molecule that is specifically targeted by BS. We contend that the clinical efficacy of BS against AD might be derived from the regulation of caspase-1 signaling pathways.

Cytokines produced from the spleen mediate various allergic diseases, such as AD, asthma, and ear swelling.[47–49]Moreover, mixed Th1 and Th2 type cytokines were produced from splenocytes of skin-sensitized mice.[49] The release of TNF-α is of major importance in the initiation and development of many inflammatory skin disorders.[50] Histamine did not affect the phosphorylation of ERK1/2, which is involved in allergic inflammation via H1 receptors in splenocytes from TNF-α knockout mice.[51] In this study, although the differences of TNF-α from splenocytes in individual condition were very small, BS inhibited the production of TNF-α in a dose-dependent manner. Ding *et al.* and Rizvi *et al.* have reported that immune responses were induced, although the amounts of TNF-α released from splenocytes were very small.[52,53] Thus, these data suggests that the anti-inflammatory effects of BS could be mediated at least partially through an inhibitive action on splenocytes.

Recent treatment strategies for allergies have focused on immune intervention. Topical steroids, emollients, and oral anti-histamines are used as the first-line therapy for AD. However, allergy immunotherapy remains a niche treatment secondary to diagnostic drugs because of its cost, long duration of treatment, and concerns regarding safety and effectiveness.[54] Natural product-based anti-inflammatory drugs with good efficacy and lower risk of adverse effects offer promising treatment and prevention of AD for patients.[55] In the present study, we showed that BS has an anti-AD effect in *in vivo*, *ex vivo* and *in vitro* models. Our results suggest that BS possesses preventive and curative effects against AD. However, further work should address the possibility that BS may also be active in human models.

Conclusion

BS has not been elucidated for its inhibitory effects and mechanism against AD. We for the first time observed that BS suppressed the levels of inflammatory cytokines, chemokine, IgE, and histamine in DNFB-treated NC/Nga mice. BS reduced the level of TSLP through a blockade of the caspase-1 pathway in DNFB-treated NC/Nga mice and stimulated HMC-1 cells. Therefore, we hypothesize that BS can be helpful in treating allergic inflammatory diseases including AD.

Author contributions: NR Han performed the research and wrote the paper, HM Kim and HJ Jeong designed the study and analyzed the data.

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