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Suppression of T cell activation by hirsutenone, isolated from the bark of *Alnus japonica*, and its therapeutic advantages for atopic dermatitis

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ABSTRACT

The increasing prevalence and severity of atopic dermatitis during recent decades has prompted the development of safe and more highly effective drugs. Although topical corticosteroids have been used for more than 50 years as first line therapy for atopic dermatitis, their potential side effects limits their clinical uses. In light of this, steroid-free topical calcineurin inhibitors were developed and have been used in patients with moderate to severe atopic dermatitis. In the present study, we examined if hirsutenone suppressed the profiles of atopic dermatitis development *in vitro* via mimicry of calcineurin inhibitor actions in mouse splenocytes and RBL-2H3 mast cells. Our results showed that hirsutenone effectively inhibited T cell activation by blocking dephosphorylation of nuclear factor of activated T cells (NFAT). This inhibition was confirmed by inactivation of mitogen-activated protein kinases (MAPKs), which subsequently inhibited production of cytokine mRNAs (interleukin-2, -4, -5, -13 and interferon-γ) after T cell receptor stimulation. We also showed that the early T cell activation marker, CD25, was suppressed in the presence of hirsutenone after T cell receptor stimulation with anti-CD3. Moreover, degranulation of mast cells was remarkably suppressed, comparable to that by cyclosporine A. This indicates that hirsutenone may specifically inhibit calcium-activated processes in both T cells and mast cells. Therefore, our results suggest that hirsutenone may be a new topical drug candidate, which probably acts by mimicking calcineurin inhibitor mechanisms.

1. Introduction

Atopic dermatitis is a common inflammatory skin disease that takes a chronic relapsing course. It is associated with severe pruritus characterized by typically distributed lesions of dry skin, excoriations and lichenification (Spergel and Paller, 2003; Leung et al., 2004). The causes of atopic dermatitis are not completely understood, but a complex inflammatory immune dysregulation and responses to allergens are believed to be involved (Akdis et al., 2002). In atopic dermatitis, immune system dysregulation of the 2 major CD4+ T cell subsets is an important aspect of its pathophysiology (Levy, 2007). Accordingly, atopic dermatitis is characterized by increased inflammatory cell infiltration into the skin and type 2 helper T cell (Th2)-dominated immunity that leads to elevated levels of serum immunoglobulin E (IgE) and peripheral eosinophilia (Leung, 1995).

Helper T cells can be divided into 2 subgroups, Th1 and Th2 cells, based on their cytokine production profiles. Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13. These 2 cytokine groups interact in a counter-regulatory fashion (Leung et al., 2004). In atopic dermatitis, Th2 cells show increased production of IL-4, which stimulates plasma cells to increase IgE production as well as promoting further Th2 cell development (Back et al., 1995).

Much research over the past decades has focused on atopic dermatitis. These studies indicate that the basic cause of this disease is allergic inflammation that is accompanied by a variety of immunological abnormalities (Hussain and Kline, 2004). Thus, the most promising anti-atopic dermatitis drugs may be compounds that are immune-suppressive. These compounds should be able to control the imbalance of Th1/Th2 cells, and also be safe for humans and prevent relapses when used for long-term treatments (Wollenberg et al., 2008).

For the last 50 years, topical corticosteroids have been the primary choice for atopic dermatitis treatment. However, their side effects, such as skin atrophy, striae and perioral dermatitis in sensitive areas (face or skin), has been a major obstacle to their long-term application (Thaci, 2003). Recently, new topical calcineurin inhibitors, including tacrolimus ointment and pimecrolimus cream, have been used for monotherapy treatment of atopic dermatitis when conventional treatments, including corticosteroids, were not possible or were

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unsuccessful. However, topical calcineurin inhibitors are relatively new, and any long-term side effects are not fully known (Reitamo and Remitz, 2006).

Topical calcineurin inhibitors inhibit activation of T cells and mast cells by blocking calcineurin. This suppresses inflammatory cytokines and other mediators of allergic inflammatory reactions (Bornhove et al., 2002). Compared to topical steroids, topical tacrolimus is a potent calcineurin inhibitor that does not exhibit the adverse hormonal reactions associated with classic steroid therapies (Nghiem et al., 2002).

Recently, we isolated several diarylheptanoid compounds from the bark of *Alnus japonica*. These have been used in traditional oriental medicine as remedies for fever, hemorrhage, diarrhea, alcoholism, various skin affections (chronic herpes, eczema and prurigo) and inflammation. Among these investigational candidates, we selected the compound that was likely to suppress T cell activation by the inhibition of calcineurin. In this study, we examined if this selected single compound inhibited atopic dermatitis-associated cytokines from T lymphocytes by mimicking calcineurin inhibitor activity.

2. Materials and methods

2.1. Preparation of hirsutenone

The bark of *A. japonica* was collected at Mt. Chung-gei, Seoul, Korea in November 2002, and a voucher specimen was deposited at the herbarium, College of Pharmacy, Chung-Ang University. The fresh barks were finely cut and extracts were prepared as previously described (Kim et al.,, 2005), with some modification. In brief, 2.5 kg of the barks was incubated for 72 h at room temperature with 80% aqueous acetone. After removing acetone under a vacuum, the aqueous solution was filtered, then concentrated and applied to a Sephadex LH-20 column (25–100 μm, 8×150 cm, Pharmacia, Uppsala, Sweden) and eluted by increasing concentrations of methanol (0–100%). Column chromatography of the target fraction was repeated using MCl-Gel CHP 20P (75–150 μm, 5×80 cm, Mitsubishi Chemical Co., Tokyo, Japan). Elution used an increasing proportional gradient of methanol (40% to 80%). Finally, the eluted solution was applied to a column filled with YMC ODS-gel (s-75 μm, 5×60 cm, YMC Co. Ltd, Kyoto, Japan) and low-pressure liquid column chromatography was performed with increasing proportions of methanol (40% to 80%). The column was connected to a peristaltic pump (Gilson Miniplus 3) with a Gilson 112 UV/VIS detector (254/280 nm), and processed using Gilson 740 ProTech System Controller Software program (Gilson Inc., Middleton, WI, U.S.A.). From these procedures, we obtained optimally purified hirsutenone (0.08 g, 0.0032 w/w %). The lyophilized isolate was dissolved in dimethyl sulfoxide and then further diluted to the desired concentrations from the stock solution (50 mg/ml). The structure of the compound has been characterized by spectral analyses (Fig. 1).

2.2. Cell culture

Mouse spleens were aseptically isolated from Balb/c mice after sacrifice and single primary splenocytes were prepared by mechanical dissociation in cold phosphate buffered saline (PBS) at pH 7.2. Erythrocytes were depleted using a red blood cell lysis buffer (eBioscience, CA, U.S.A.) containing ammonium chloride, which lyses red blood cells with minimal effect on lymphocytes. Splenocytes were cultured in RPMI1640 complete medium with 10% fetal bovine serum (FBS; Hyclone, UT, U.S.A.). The medium also contained an anti-CD3 mAb (eBioscience) and varying concentrations of lyophilized hirsutenone. Splenocytes were cultured at 5×10^6 cells/ml in 96-well microtiter plates or at 1×10^7 cells/ml in 24-well plates and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cell-free supernatants were collected after 24 h of culture and stored at −70 °C until analysis. RBL-2H3 mast cells were grown in RPMI1640 medium (Hyclone) supplemented with 10% FBS, 4 mM/l l-glutamate (Invitrogen, CA, U.S.A.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cultures were maintained under 5% CO_2 at 37 °C in a tissue culture flask. For all experiments, the cells were grown to greater than 90% confluence and subjected to no more than 20 cell passages. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University, Korea.

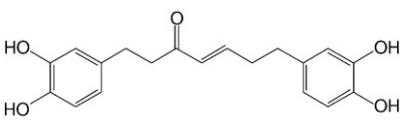

Fig. 1. The chemical structure of hirsutenone (1,7-bis-(3,4-dihydroxyphenyl)-4-heptene-3-one.

2.3. Proliferation assay

The cultures contained either 1 μg/ml T cell mitogen concanavalin A (Sigma. MO, U.S.A.) or 5 μg/ml B cell mitogen lipopolysaccharide (Freund et al., 2000; Sharma et al., 2007). These were incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. Proliferation of splenocyte immune cells was examined by responses to these mitogens after a 24-h culture period using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). This system uses WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. After bioreduction in the presence of the electron carrier 1-methoxy-phenazine methosulfate, this reaction produces a water soluble colored formazan (Kuhn et al., 2003). The reaction plates were measured at 450 nm and the data from triplicate cultures were expressed as the mean ± standard deviation (S.D.).

2.4. Cellular cytotoxicity (lactate dehydrogenase) assay

The cytotoxicity induced by hirsutenone was quantitated by measuring lactate dehydrogenase (LDH) release. LDH content was determined using a commercial non-radioactive LDH assay kit, CytoTox 96® (Promega, WI, U.S.A.), which is based on a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into a red formazan product. The increase in the amount of formazan produced in culture supernatant directly correlates with the increase in the number of lysed cells. The formazan was quantified spectrophotometrically by measuring its absorbance at 490 nm (Spectra Max 340, Molecular Devices, CA, U.S.A.). Cytotoxicity in experimental samples was determined as %LDH release compared with cells treated with 1% Triton X-100.

2.5. Quantitative real-time PCR

Total RNA extracts from mouse splenocytes stimulated by anti-CD3 mAb or RBL-2H3 cells sensitized with 2,4-Dinitrophenyl hapten conjugated to Human Serum Albumin (DNP-HSA; Sigma) were prepared by the Trizol method (Invitrogen). cDNA was synthesized from RNA by the reverse transcription of 1 μg of total RNA using the Improm-II reverse transcription system (Promega, WI, U.S.A.) and oligo dT primers for a total volume of 20 μl. PCR amplification was performed using the primers described in Table 1 (Bioneer, Deajeon, Korea). Quantitative real-time PCR reactions were run on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) using SYBR Green PCR Master Mix (Qiagen, CA, U.S.A.) in 20 μl reaction mixtures. Each real-time-PCR master mix contained 10 μl 2X enzyme mastermix, 7.0 μl RNase free water, 1 μl of each primer (10 pM each) and 1 μl diluted template. PCR was performed with an initial pre-incubation step for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, annealing at 52 °C for 15 s and extension at 72 °C for 10 s. Melting

Table 1
Primer sequences for real-time RT-PCR.

Gene	Primer	Amino acid sequences	Product size (bp)	Accession no.
IL-2	5′ Primer	5'-GCTCTACAGCGGAAGCACAG	235	NM_008366
	3′ Primer	5'-GTCAAATCCAGAACATGCCG		
IFN-γ	5′ Primer	5'-GTTCTGGGCTTCTCCTCCTG	245	NM_008337
	3′ Primer	5'-CTGGCTCTGCAGGATTTTCA		
IL-4	5′ Primer	5'-ATATCCACGGATGCGACAAA	252	NM_021283
	3′ Primer	5'-AAGCCCGAAAGAGTCTCTGC		
IL-5	5′ Primer	5'-GGGGGTACTGTGGAAATGCT	247	NM_010558
	3′ Primer	5'-TTGCACAGTTTTGTGGGGTT		
IL-13	5′ Primer	5'-TGCCATCTACAGGACCCAGA	270	NM_008355
	3′ Primer	5'-CTGAGGCATCTCCCTTCCTC		
PLD_1	5′ Primer	5'-AACACACGGGGAATTCACAT	201	NM_030992
	3′ Primer	5'-TCCTCCTGGATTGCATTTTC		
PLD_2	5′ Primer	5'-TCCCTGTTATAGCCCAGGTG	192	NM_033299
	3′ Primer	5'-CCAGAGGGAGCAGACTCATC		
β-actin	5′ Primer	5'-CTAGGCACCAGGGTGTGATG	291	NM_007393
	3′ Primer	5'-CTACGTACATGGCTGGGGTG		

curve analysis was used to confirm formation of the expected PCR product, and products from all assays were additionally tested by 1.2% agarose gel electrophoresis to confirm the correct lengths. An inter-run calibrator was used, and a standard curve was created for each gene to obtain PCR efficiencies. Relative sample expression levels were calculated using Rotor-Gene 6000 Series Software 1.7, and were expressed relative to β-actin and corrected for between run variability. Data for the experimental samples were expressed as the percentage of the internal control gene.

2.6. Electrophoretic-mobility shift (EMSA) assay

Nuclear extracts for EMSA were prepared from Balb/c mouse splenocytes using Nuclear Extraction Reagents (Pierce, IL, U.S.A.) according to the manufacturer's instructions. EMSA was performed with the Panomics EMSA kit (Panomics, CA, U.S.A.). In brief, nuclear extracts containing equal amounts of proteins for each sample were incubated with poly (dI-dC) (1 μg/μl) for 5 min, followed by the addition of binding buffer (20 mM HEPES pH 7.9, 1 mM DTT, 0.1 mM EDTA, 50 mM KCl, 5% glycerol and 200 μg/ml BSA) and biotinylated oligo (10 ng/μl). To control for specificity of binding for selected samples, a 5-fold excess of unlabeled oligo was added prior to the addition of the biotinylated probe. Binding reaction mixtures were incubated for 30 min at room temperature. Protein-DNA complexes were separated on 6% non-denaturing polyacrylamide gel in 0.5X Tris-borate/EDTA buffer (0.1 M Tris, 0.09 M boric acid containing 1 mM EDTA) at 4 °C. After electrophoresis, gels were transferred to polyvinylidene fluoride (PVDF) membranes. Transferred oligos were immobilized by UV crosslinking for 10 min. For detection of bound oligos, membranes were blocked using blocking buffer (Panomics EMSA Gel-Shift Kit) followed by the addition of Streptavidin-HRPO and blots were developed by ECL according to the manufacturer's instructions (Amersham Bioscience, NJ, U.S.A.).

2.7. Analysis of extracellular CD25 expression using on-chip antibody staining

To analyze an early T cell activation marker, CD25, anti-CD3-treated mouse splenocytes were cultured in 10% FBS RPMI1640 medium for 24 h with hirsutenone or cyclosporine A. Extracellular staining of CD25 was performed using calcein (blue color) and allophycocyanin (APC) (red color) fluorescent dye and analyzed by Agilent 2100 bioanlayzer (Agilent Technologies, CA, U.S.A.). In brief, harvested cells were transferred to a 1.5 ml amber microcentrifuge tube and washed twice with dye loading buffer containing Hanks' balanced saline solution (Invitrogen), 20 mM HEPES and 1% bovine serum albumin (Sigma). Then, cells were stained with 100 μl of 0.5 μM calcein (Molecular Probes, Inc., OR, U.S.A.) for 15 min at 37 °C in the dark and then washed twice with 1 ml of staining buffer containing PBS, 2% BSA and 0.05% NaN3 (Sigma). After staining with calcein, cells were incubated with APC-labeled anti-mouse CD25 (10 μg/ml) in staining buffer for 1 h on ice. The cells were washed twice with staining buffer and resuspended at 2×10^6 cells/ml in cell buffer (cell assay reagents, Agilent Technologies). A cell assay LabChip (Agilent Technologies) was primed with priming solution (Agilent Technologies), after which 10 μl of the cell suspension (20,000 cells) was added to 1 of 6 channels. A focusing dye was applied to another chamber, which acted as a reference for the optical detection system. The chip was then placed in an Agilent Technologies Model 2100 bioanalyzer, which utilizes a vacuum to move cells, single file, through the microfluidic channels past a fluorescent detector. Fluorescent emission from the cells was detected at 510–540 and 635–685 nm. Typically, 500–1000 events/240 s were recorded. Fluorescent events were compared by frequency histogram of anti-CD3-activated splenocytes stained with calcein and APC-labeled CD25. A gate was then set on the calcein-positive cells (live cells), and a software program was used determine the percent positive for the CD markers within the gated calcein-positive cells.

2.8. Western blot analysis

Balb/c mouse splenocytes or RBL-2H3 mast cells were lysed in 1% RIPA buffer containing protease and phosphatase inhibitors (Roche, Mannheim, Germany) and whole cell lysates were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membranes and the membranes were blocked with 5% skim milk in Tris-buffered saline solution with 0.1% Tween-20. Immunoblotting with primary antibodies, anti-NFATc2, anti-phospho-NFATc2, anti-extracelullar signal-regulated protein kinase 1/2 (ERK1/2), anti-phospho-ERK1/2, anti-phospho-p38, anti-p38 antibodies (Santa Cruz Biotechnology, CA, U.S.A.), anti-c-Jun N-terminal kinase (JNK) (Cell Signaling Technology, MA, U.S.A.) was followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Stressgen, CA, U.S.A.). Blots were developed using an ECL solution. Densitometric measurements used Gelquant software (MiniBIS Pro, Jerusalem, Israel).

2.9. β-Hexosaminidase secretion assay

Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase. RBL-2H3 cells were grown on 48-well plates (2.5×10^5 cells per well) and experiments were carried out 3 h after plating. Cells were then treated overnight with 1 μg/ml IgE (Sigma). To remove excess IgE before stimulation, cells were washed 4 times with extracellular buffer (125 mM NaCl, 5 mM KCL, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mmol HEPES, pH 7.3). The cells were then stimulated with 400 ng/ml DNP-HSA (Sigma) suspended in 500 μl extracellular buffer with 0.1% BSA, and incubated at 37 °C for 1 h. Following incubation, 50 μl of the supernatant was incubated with 200 μL of 1 mM *p*-nitrophenyl *N*-acetyl-beta-*D*-glucosamine (Sigma) in 0.05 M citrate buffer (pH 4.5) for 3 h at 37 °C. The enzyme reaction was terminated by the addition of 500 μl of 0.05 M sodium carbonate buffer (pH 10.0) and the OD of each reaction was read at 405 nm (SupraMax340, CA, U.S.A.). The effects of various treatments on β-hexosaminidase release were described as % of IgE control.

2.10. Statistical analysis

Statistical comparisons between the groups used one-way ANOVA with a Dunnet's post-hoc test, which were performed using SPSS software (v. 13). Statistical significance was set a priori at $P<0.05$.

3. Results

3.1. Proliferation and cellular cytotoxicity assay for splenocytes

Cell-mediated immune functions were assessed by measuring the effects of mitogens (concanavalin A for T cells and lipopolysaccharide for B cells) on splenocyte proliferation. The proliferation of both T and B cells was inhibited in the presence of hirsutenone at varying concentrations (0.1 to 50 μM) in a dose-dependent manner (Fig. 2A and B). These results indicate that hirsutenone can suppress an immune response. An anti-proliferative effect was observed at all doses, but the effective thresholds for T cells and B cells were approximately 25 μM hirsutenone. Next, we investigated the effect of hirsutenone on cytotoxicity of splenocytes. Incubation of mouse splenocytes with hirsutenone produced a dose- and time-dependent increase in cell cytotoxicity measured as LDH release. Fig. 1C shows that hirsutenone concentrations from 0.05 to 50 μM did not significantly increase LDH release for exposures up to 24 h, but a higher concentration (500 μM) induced a significant increase in LDH release when incubated for 24 h. The greatest cytotoxic effect (>90%) was observed with 500 μM hirsutenone at 24 h. Because the toxicity with 500 μM hirsutenone was so profound, higher concentrations were not tested.

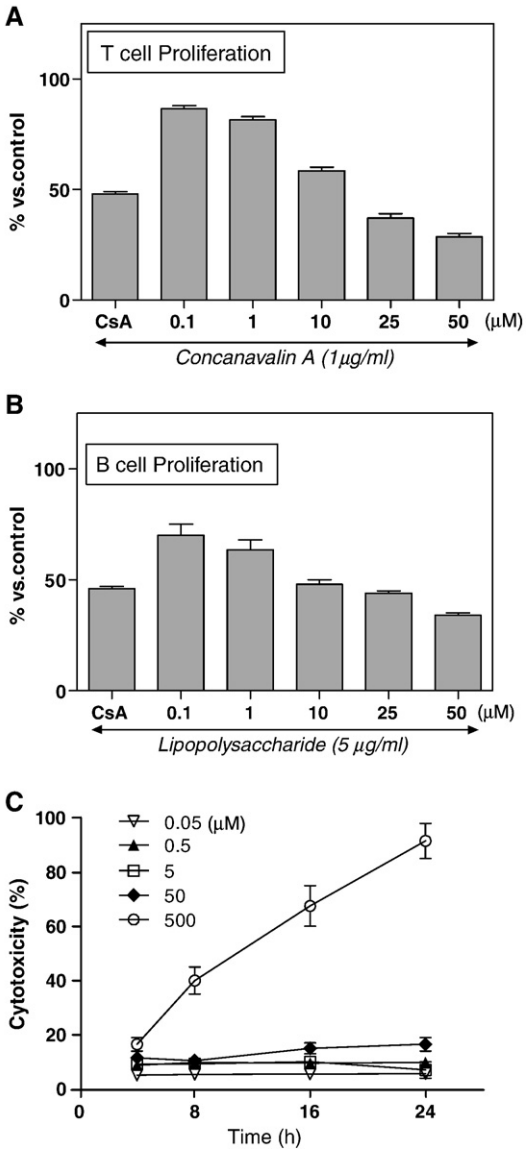

Fig. 2. Effect of hirsutenone on proliferation and cytotoxicity for splenocytes. Spleen cells were prepared from Balb/c mice, seeded in 96-well microplates and cultured with varying concentrations of hirsutenone for 24 h in the presence of either (A) concanavalin A (1 μg/ml) or (B) lipopolysaccharide (5 μg/ml). Cell numbers in triplicate wells were measured as the absorbance (450 nm) of reduced WST-8. Cyclosporine A (0.1 μM) was used as a positive control. Results are representative of 2 separate experiments. (C) Splenocytes were exposed to hirsutenone (0.05, 0.5, 5, 50 and 500 μM) for the indicated times. Concentration dependence and time dependence of cytotoxicity, measured as %LDH released into culture supernatant, was compared with cells treated with 1% Triton X-100. Results are expressed as means ± S.D. from 3 separate experiments.

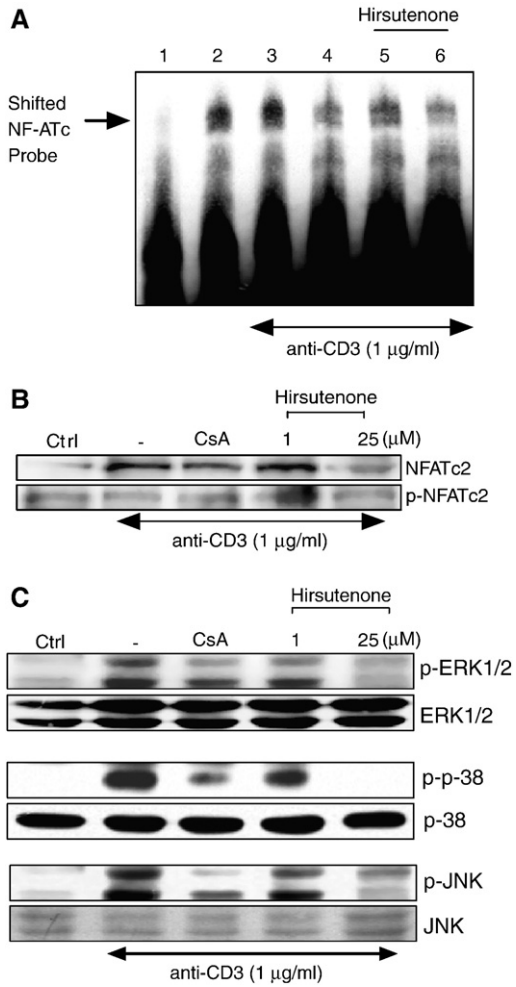

Fig. 3. Electrophoretic-mobility Shift Assay for NFATc and Western blot profiles of NFATc2 and MAP kinase. (A) Anti-CD3-activated splenocytes were treated with hirsutenone or cyclosporine A for 60 min, followed by preparation of nuclear extracts for EMSA as described in Materials and Methods. Lane 1, labeled EMSA probe only with no sample. Lane 2, treated sample with cold and labeled EMSA probe. Lane 3, labeled EMSA probe with anti-CD3-treated sample. Lanes 4 to 6, labeled EMSA probe with 0.1 μM cyclosporine A, 1 μM hirsutenone or 25 μM hirsutenone-treated cells with anti-CD3 mAb activation. (B) T cell receptor-induced dephosphorylation of NFATc2 in cytoplasm was analyzed in the presence or absence of hirsutenone. The blots were stripped and reprobed with specific antibodies as controls for loading. (C) Analysis of ERK, p-38 and JNK phosphorylation in lysates of mouse splenocytes. Cells were activated and cyclosporine A was used as a positive control as in A.

3.2. Inactivation of NFATc2 in anti-CD3-treated splenocytes

To confirm that hirsutenone might be correlated with the inhibition of calcineurin activation, we examined dephosphorylation of NFATc2 in the cytosol and NFATc2 binding to specific DNA sequences. As shown in Fig. 3B, hirsutenone inhibited the dephosphorylation of NFATc2 in the cytosol. This can induce distinct transcriptional programs leading to T cell activation when NFATc2 is

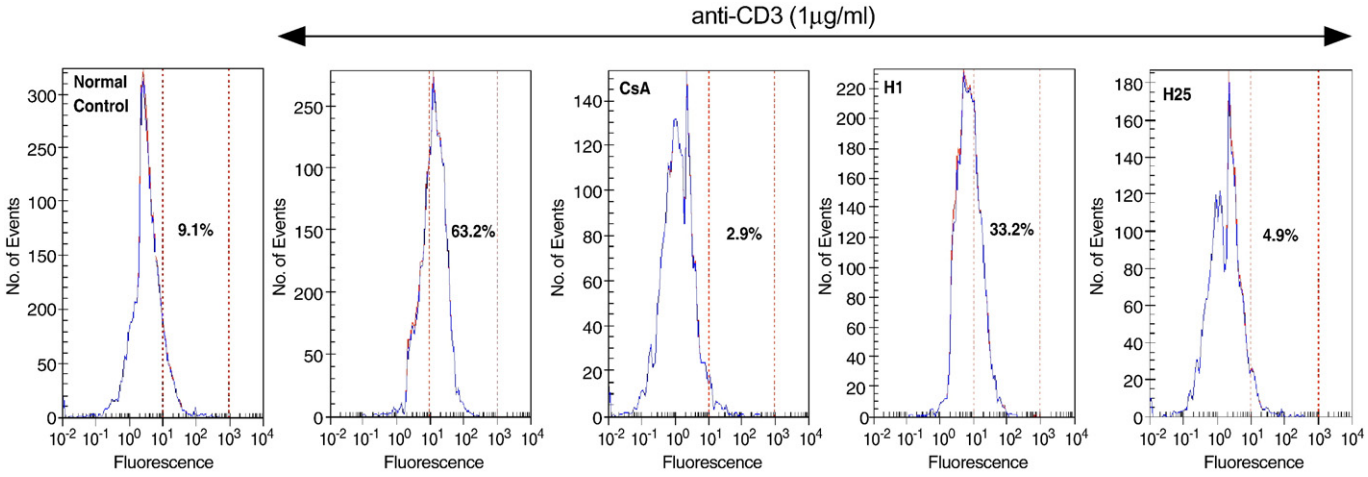

Fig. 4. CD25 expression on anti-CD3 mAb-activated splenocytes. Harvested splenocytes were stained on-chip with anti CD25-APC (1:5.5 dilution) in cell buffer and calcein (1:50 in cell buffer). After incubation for 25 min on the chip, samples were measured as described in Materials and Methods. A gate was set on calcein-positive cells (live cells), and cells positive for CD25 within the gated calcein-positive population were calculated using Agilent 2100 bioanalyzer software program. Positive control treated with cyclosporine A (0.1 μM) was compared with hirsutenone -treated groups. H1; hirsutenone 1 μM, H25; hirsutenone 25 μM.

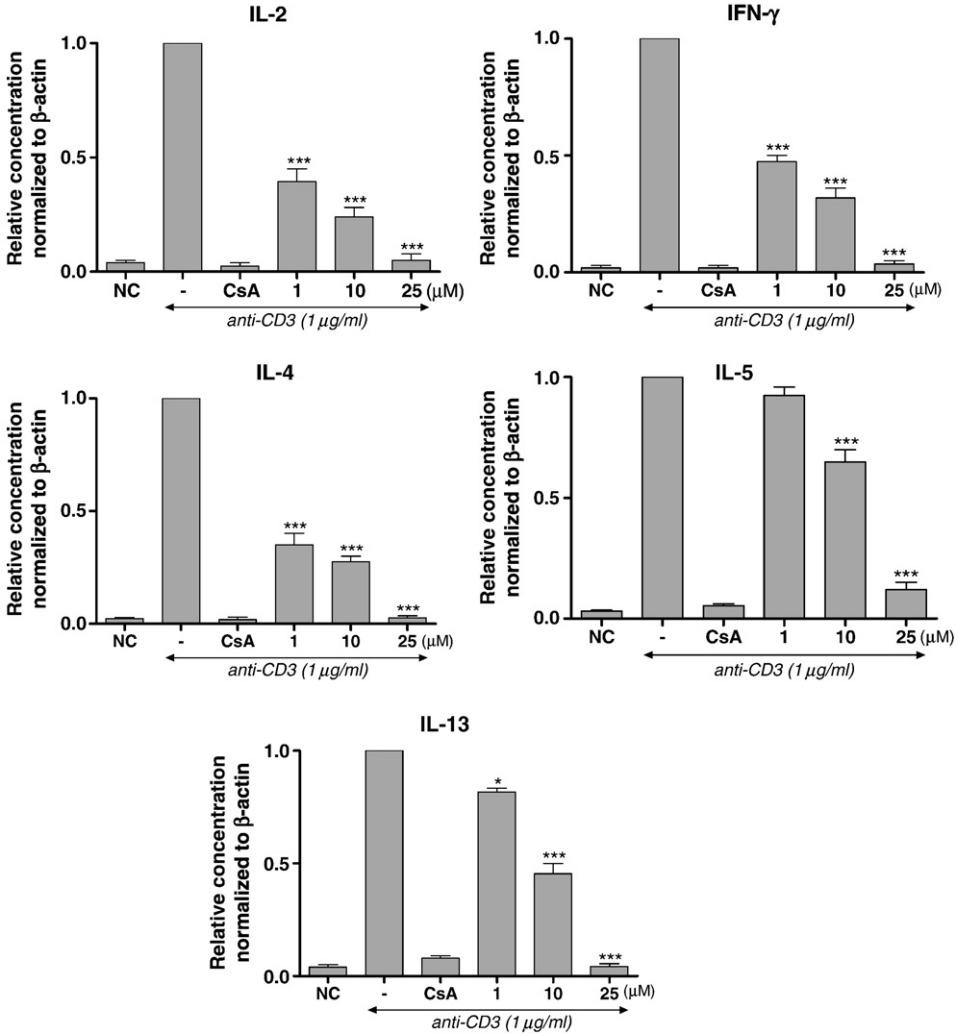

Fig. 5. Th1/Th2 cytokine mRNA expression in splenocytes. Cells were cultured for 24 h as described in Methods and Materials, and cyclosporine A was used as a positive control. Isolated mRNA was analyzed by real-time RT-PCR for IL-2, IFN-γ, IL-4, IL-5, IL-13 and β-actin. Results were internally confirmed by the comparative cycle count (Ct, cycle number threshold) against β-actin as the standard gene. 1 μg/ml Anti-CD3 mAb-activated group was used as a calibrator for a relative comparison for each group. Results are mean ± S.D. ($n = 3$). $^{*}P<0.05$, $^{***}P<0.001$.

translocated into the nucleus, which allows it to bind to specific DNA sequences (Zhou et al., 2002). Using immunoblot analysis of NFATc by EMSA supershift assay, hirsutenone effectively inhibited binding to specific NFATc DNA sequences (Fig. 3A). To check if the observed shifted bands were specific for NFATc, competition tests were included by adding unlabeled ("cold") oligonucleotide to the labeled NFATc probe (Fig. 3A, lane 2). Interestingly, these results suggest that hirsutenone might have as strong a potential to inhibit T cell activation in a dose-dependent manner as is observed with cyclosporine A.

3.3. Inhibition of T cell activation-induced ERK pathway

In T cells, the extracellular signal-regulated kinases (ERK), c-Jun N-terminal Kinase (JNK) and the p38 mitogen-activated protein kinases (MAPK) are known to be involved with T cell immune responses (Dong et al., 2002). To determine if hirsutenone influenced the MAPK pathway initiated after T cell activation, splenocytes were treated with anti-CD3 and cultured for 24 h. The MAPK activities were analyzed by immunoblotting using antibodies against phosphorylated ERK (p-ERK), p-p38 and p-JNK, along with non-phosphorylated ERK, p38 and JNK for experimental validation. As shown in Fig. 3C, a higher concentration of hirsutenone (25 μM) effectively inhibited the T cell activation-induced ERK pathways, which can directly influence the expression of Th1/Th2 immune responses. These experiments demonstrate that hirsutenone might inhibit the ERK pathway in activated T cells.

3.4. Inhibition of T cell activation marker, CD25

An early activation marker for T lymphocytes, CD25, was analyzed by an on-chip antibody staining method. The On-chip evaluation can simultaneously detect live cells and CD25 positive cells via a 2 color (blue and red) detection system. Thus, T cells expressing CD25 on their surfaces can be estimated for the given total events. In this experiment, 100% of the live, antibody-stained cells were CD25 positive and it was therefore expected to always obtain 100% double positive cells. Fig. 4 shows that the % CD25 among gated cells was remarkably decreased by treatment with hirsutenone in a dose-dependent manner (33.2% at 1 μM and 4.9% at 25 μM). Interestingly, the higher dose of hirsutenone (25 μM) showed the same level of CD25 expression as treatment with cyclosporine A (2.9%).

3.5. Profiles of Th1/Th2 cytokine mRNAs in mouse splenocytes

To determine if hirsutenone inhibits T cell receptor-mediated Th1/Th2 cytokines' expressions at the gene level, splenocytes were activated by anti-CD3 mAb. The expressions of IL-2, IL-4, IL-5 and IL-13 mRNAs were examined in the presence of hirsutenone at varying concentrations (1 to 25 μM). Cyclosporine A was included for comparison. mRNA levels for the selected Th1/Th2 cytokines were assessed using quantitative real-time PCR. As shown in Fig. 5, T cells were highly activated by anti-CD3 mAb. However, the mRNA expression levels for IL-2, IFN-γ, IL-4, IL-5 and IL-13 were significantly inhibited after treatment with 25 μM hirsutenone, where the levels of inhibition were similar to that by cyclosporine A treatment. Interestingly, hirsutenone was particularly effective for inhibiting the expressions of IFN-γ, IL-2 and IL-4 mRNAs at a lower concentration (1 μM). Thus, these results clearly demonstrate that hirsutenone can effectively control T cell derived cytokines after stimulation with anti-CD3 mAb.

3.6. Inhibition of β-hexosaminidase release from RBL-2H3

Release of β-hexosaminidase, an indicator of mast cell degranulation, was measured in supernatants after treatment with DNP-HSA (Fig. 6A). Compared to the cyclosporine A positive control, hirsutenone effectively inhibited the release of ß-hexosaminidase from RBL-2H3 at around 10 μM. This inhibitory effect of hirsutenone was optimal at higher concentration (25 μM). Hirsutenone alone at varying concentrations, without antigen stimulation, did not affect β-hexosaminidase secretion (Fig. 6B). Thus, to confirm these results, phospholipase D_2 (PLD_2), which can concomitantly promote degranulation by an increased PLD activity after anti-DNP IgE sensitization, was examined using real-time RT-PCR (Cohen and Brown, 2001). Fig. 6C shows that PLD_2 was remarkably inhibited, corresponding to the inhibition of the release of β-hexosaminidase, while PLD_1 was not responsive to this sensitization.

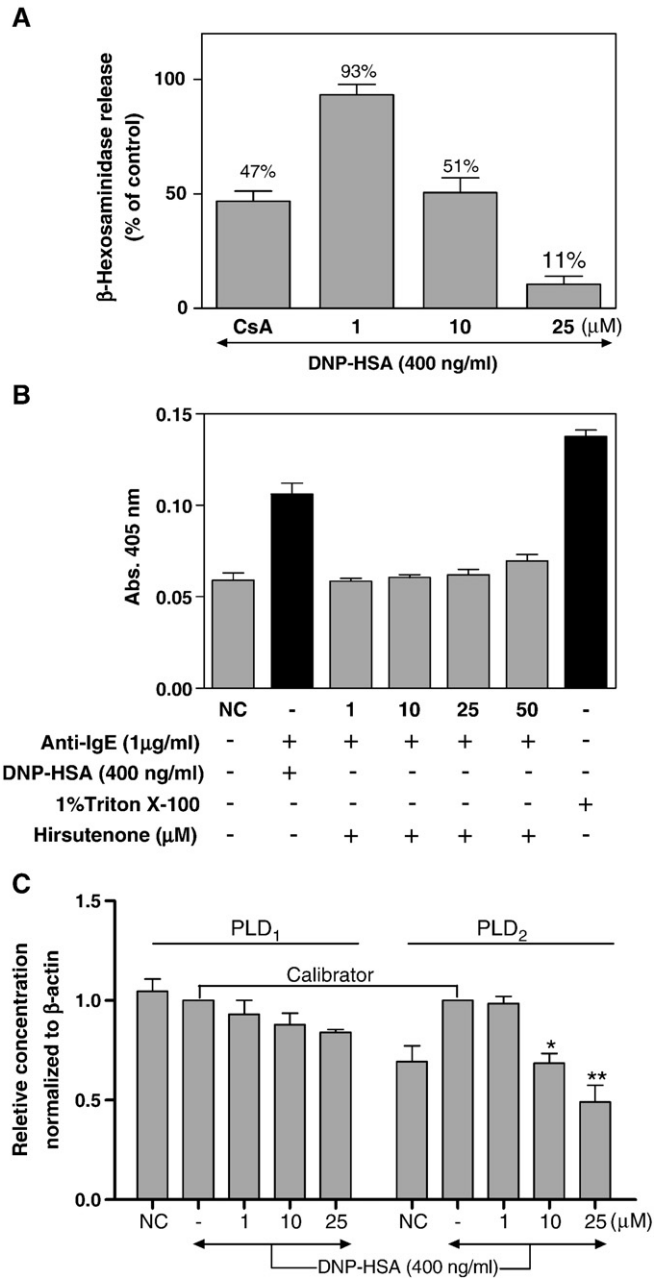

Fig. 6. β-hexosaminidase release and phospholipase D_1/D_2 gene expression. RBL-2H3 cells (2.5×10^5/well) were sensitized with 1 μg/ml of DNP-specific IgE overnight and pretreated with varying doses of hirsutenone (1–25 μM) for 60 min. Cells were stimulated with 400 ng/ml of DNP-HSA for 1 h. (A) β-hexosaminidase release from RBL-2H3 cells was measured using a microplate reader at 405 nm. (B) Control RBL-2H3 cells were treated with or without DNP-HSA after sensitized with DNP-specific IgE overnight, while hirsutenone-treated cell groups (0.1 to 50 μM) were sensitized with DNP-specific IgE alone. 0.1% Triton X-100 (Sigma) was used to lyse whole cells. (C) The mRNA expression levels of PLD_1/PLD_2 were analyzed by real-time PCR. Cyclosporine A (0.1 μM) was used as a positive control. Results are mean ± S.D. ($n=3$). *$P<0.05$, **$P<0.01$.

4. Discussion

In atopic dermatitis, Th1/Th2 T cell subsets provide a framework for understanding the distinction between normal and pathological immune allergic responses. Many studies have suggested that Th1 and Th2 type reactions can reciprocally regulate one another. Therefore, balancing the Th1/Th2 reactions may be a fundamental approach for atopic dermatitis treatment (Heinzel et al., 1989). Despite these mechanistic expectations, there has been no clear resolution for a clinical cure for atopic dermatitis. Thus, the common aims for atopic dermatitis treatment have been to alleviate the signs and symptoms and to reduce the frequency of exacerbations (Alomar et al., 2004).

As a treatment option, topical corticosteroids have been the mainstay of atopic dermatitis treatment for more than 50 years. However, due to their increased potential for side effects, including skin atrophy, striae, telangiectasia, acneiform eruptions, and the risk of systemic effects, such as hypothalamic-pituitary-adrenal axis suppression, topical corticosteroids have not been recommended for maintenance therapy (Ellison et al., 2000; Oikarinen and Autio, 1991; Hardie et al., 1977). Therefore, the development of nonsteroidal topical immunosuppressants has been a goal for researchers.

In this light, topical calcineurin inhibitor has been an historic development in terms of a nonsteroidal topical immunosuppressant with reduced side effects compared to topical corticosteroids (Breuer et al., 2005; Kang et al., 2001). Although cyclosporine A has been used to prevent organ transplant rejection via its actions as a calcineurin inhibition, and is thus included in the topical calcineurin inhibitor family, it is not useful as a topical application, probably due to its large molecular size (M.W. 1202.64) that impedes its ability to penetrate skin (De Rie et al., 1991). By comparison, FDA-approved topical calcineurin inhibitors, tacrolimus (M.W. 822.05) and pimecrolimus (M.W. 810.47), which work by blocking the production and release of pro-inflammatory cytokines after antigen-specific or non-specific activation of T cells and mast cells, are active after topical application and have been shown to be effective for atopic dermatitis treatment (Chapman et al., 2005; Luger et al., 2004). This has prompted the development of smaller molecular weight and highly effective topical calcineurin inhibitor drug candidates, especially for atopic dermatitis.

In the present study, we investigated if hirsutenone had pharmacological immunosuppressive effects comparable to a known calcineurin inhibitor, cyclosporine A. At the molecular level, hirsutenone appreciably blocked the synthesis of Th1/Th2 cytokines by inhibiting the dephosphorylation of NFATc. NFATc can induce the overexpression of atopic dermatitis cytokine genes in the nucleus, thereby initiating the differentiation of Th cells to Th2 cells in atopic dermatitis skin lesions. Interestingly, hirsutenone showed effects on mitogen-activated (concanavalin A & lipopolysaccharide) splenocytes similar to that of cyclosporine A in a dose-dependent manner. These 2 experimental findings suggest that hirsutenone may exert its inhibitory effects on atopic dermatitis -associated T lymphocyte cytokines by mimicking calcineurin inhibitor activity, and also influence the B cell activation that is absolutely required for progression to severe, chronic atopic dermatitis. In addition, CD25, an early T cell activation marker, was also significantly down-regulated at a high dose of hirsutenone.

In conjunction with the blockade of calcineurin function similar to that of cyclosporine A to suppress the production of cytokines during T cell activation, hirsutenone dramatically inhibited the activation of major groups of MAPKs, ERK, JNK and p38. As these protein kinases are activated in T cells via the T cell receptor and are essential for T cell functions (Rincon et al., 2001), interruption of the MAPK pathways may provide mechanistic clues for upstream and downstream pathways of hirsutenone action on T cell inactivation.

In summary, our results demonstrate that hirsutenone may have ideal properties as a new calcineurin inhibitor. It is small in molecular weight and possesses a potential comparable to that of cyclosporine A for the inhibition of allergen-induced T cell and mast cell activation. Moreover, due to its lipophilic properties, it has a high affinity for the skin compartment and a low potential for absorption into the systemic circulation (Meingassner et al., 2005). Thus, hirsutenone is an attractive source for developing a topical drug for T cell-based anti-atopic dermatitis by its actions as a calcineurin inhibitor. Therefore, we conclude that hirsutenone is a promising new topical calcineurin inhibitor candidate derived from a non-chemical natural source that may effectively suppress the overexpression of Th1/Th2 cytokines via the blockade of dephosphorylation of NFATc (calcineurin inhibitor-mimicry), and also suppress mast cell activation, which is a calcium-activated response similar to T cell activation.

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