



Tryptanthrin inhibits Th2 development, and IgE-mediated degranulation and IL-4 production by rat basophilic leukemia RBL-2H3 cells

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ABSTRACT

Ethnopharmacological relevance: Tryptanthrin is a compound isolated from *Polygonum tinctorium*, which is a known folk medicine with various biological activities.

Aim of the study: Allergic diseases are initiated by the development of allergen-specific T helper type 2 (Th2) cells and amplified by the degranulation of and cytokine release from basophils and mast cells during an effector phase. We found that Tryptanthrin could down-regulate IL-4 production by Th2 cells, while IFN- γ production by Th1 cells was not affected. Since IL-4 produced by basophils and effector Th2 cells has been shown to play important roles in the development and amplification of Th2-dominated allergic responses, we examined the effects of Tryptanthrin on the initiation and effector phase responses of Type I allergy *in vitro*.

Materials and methods: To determine the mechanisms of Tryptanthrin-induced down-regulation of IL-4 production, the expression of Th2-specific transcription factors, c-Maf and GATA-3, was analyzed by RT-PCR. The effects of Tryptanthrin on Th cell differentiation were evaluated using CD4⁺ T cells purified from spleen cells of Sugi basic protein (SBP)-immunized BALB/c mice. In primary cultures, cells were stimulated with SBP and antigen-presenting cells under neutral or Th2-skewing conditions in the presence or absence of Tryptanthrin. Cytokines produced by differentiated Th cells in secondary cultures were analyzed by ELISA. The effects of Tryptanthrin on IgE-mediated degranulation and IL-4 production were determined using rat basophilic leukemia (RBL-2H3) cells. Phosphorylation of ERK1/2 and Akt in Tryptanthrin-treated RBL-2H3 cells was analyzed to determine the mechanism of Tryptanthrin actions.

Results: Tryptanthrin suppressed c-Maf mRNA expression in Th2 clone cells, and even under Th2-skewing conditions, Tryptanthrin inhibited differentiation toward the Th2 phenotype, which is an essential event for the initiation phase of allergic diseases. Tryptanthrin also inhibited the IgE-mediated degranulation of and IL-4 production by RBL-2H3 cells, probably due to inhibiting IgE-mediated signaling pathways, including the phosphorylation of ERK1/2 and Akt.

Conclusion: These findings suggest that Tryptanthrin effectively inhibits the effector and exacerbation responses, as well as the initiator responses, of Type I allergy. Thus, Tryptanthrin may have beneficial effects for immediate-type allergic responses.

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1. Introduction

The prevalence of Type I allergy, such as pollinosis, allergic rhinitis, food allergy, and allergic asthma, have gradually increased in recent decades, particularly in developed countries. For example, the prevalence of Japanese cedar pollinosis in the general population in Japan was estimated to be 24.5% in 2004 (Kaneko et al., 2005). Due to the soaring healthcare costs and the physical suffer-

ing it causes, Japanese cedar pollinosis is becoming a major problem in Japan.

Allergic diseases are initiated by the development of allergen-specific T helper type 2 (Th2) cells. Recently, it has become recognized that IL-4 produced by basophils and mast cells plays a crucial role in the differentiation of naïve CD4⁺ T cells into Th2 cells and the development of allergic reactions (Oh et al., 2007; Sokol et al., 2008). Th2 cells produce IL-4, IL-5, IL-9, and IL-13, and regulate B lymphocyte class switching to IgE via their production of IL-4 (Constant and Bottomly, 1997). These allergen-specific IgE antibodies, in turn, activate basophils and mast cells by cross-linking of high affinity IgE Fc receptors (Fc ϵ RI) on these cells' surfaces (Yamaguchi

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Table 1

Sequences of primers for the real-time polymerase chain reaction analysis.

Gene name	Primer sequence (5'–3')		GenBank accession no.
	Forward	Reverse	
GAPDH ^a	ACCATCTTC CAGGAGCGAG	AGTGATGGCATGGACTGTGG	NM.008084
GATA-3 ^b	TCTGGAGGAGGAACGCTAATGG	GAAGCTCTTCGCACACTTGGAGACT	NM.008091
c-Maf ^c	AGCAGTTGGTGACCATGTCC	TGGAGATCTCTGCTTGAGG	NM.001025577

^a GAPDH: glyceraldehyde-3-phosphate dehydrogenase.^b GATA-3: GATA binding protein 3.^c c-Maf: Maf avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog.

et al., 1997). During an effector phase, activated basophils and mast cells secrete a range of mediators upon rechallenge with antigen, including cytokines, chemokines, histamine, and arachidonic derivatives, which results in smooth muscle constriction, vascular hyperpermeability, and inflammatory cell recruitment (Marshall, 2004; Theoharides and Kalogeromitros, 2006).

Current drugs that are used to treat Type I allergy, such as chemical mediator release inhibitors, antihistamines, phosphodiesterase E4 inhibitors, leukotrienes antagonists and even anti-IgE antibodies, are aimed to intervene in or suppress effector phase responses. These drugs can suppress certain symptoms or part of the allergic inflammatory process, but fail to regulate the complete allergic cascade (Beier et al., 2007). To date, very few drugs have become available that can inhibit both the initiation phase of Th2 development and the effector phase responses of allergic reactions.

Tryptanthrin, 6,12-dihydro-6,12-dioxindolo-(2,1-b)-quinazoline, is a compound extracted from *Polygonum tinctorium*. It is a known folk medicine that is used in Japan for its anti-inflammatory, anti-pyretic, and detoxification effects. Tryptanthrin has a range of biological activities, such as anti-microbial (Kataoka et al., 2001), anti-tumor (Kimoto et al., 2001; Koya-Miyata et al., 2001), and anti-inflammatory activities (Ishihara et al., 2000). In our previous reports, we found that Tryptanthrin suppressed dextran sodium sulfate-induced colitis in mice (Micallef et al., 2002) and inhibited IFN- γ production by Peyer's Patch lymphocytes derived from mice that had been orally administered Staphylococcal enterotoxin (Takei et al., 2003). However, to our knowledge, possible immunoregulatory effects of Tryptanthrin on Th cell activation have not been studied.

In this study, we found that Tryptanthrin down-regulated IL-4 production by Th2 cells, while IFN- γ production by Th1 cells was not affected. It is well recognized that IL-4 produced by basophils and effector Th2 cells plays important roles in the development and amplification of Th2-dominated allergic responses. To identify possible anti-allergic actions of Tryptanthrin, we examined the effects of Tryptanthrin on allergic reactions by focusing on Th2 cell development (initiation phase response) and on the degranulation of basophils (effector phase response) *in vitro*.

2. Materials and methods

2.1. Mice

BALB/c female mice, aged 7–8 weeks, were purchased from Charles River Japan (Kanagawa, Japan). Mice were fed rodent chow (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. All animal experiments were conducted according to the guidelines of the Animal Experiments Review Board at the Hayashibara Biochemical Laboratories, Inc.

2.2. Antigens

Sugi basic protein (SBP), a major allergen from Japanese cedar (*Cryptomeria japonica*) pollen, was prepared according to the pro-

cedure described by Taniguchi et al. (1989). OVA was purchased from Sigma Chemical (St. Louis, MO).

2.3. T cell clones

OVA-specific Th1 clone #4 was established from BALB/c mice in our laboratory (Kohno et al., 1997), and were maintained by repeated stimulation with specific Ag and subsequent culture in medium containing 10 U/ml human IL-2. A conalbumin-specific Th2 clone, D10.G4.1, was maintained by repeated stimulation with mitomycin C (MMC)-treated C57BL/6 spleen cells, as described (Kohno et al., 1997). All cell cultures used complete medium comprised of RPMI 1640 supplemented with 10% FCS (Life Technologies, Grand Island, NY), 50 μ M 2-ME, 60 μ g/ml penicillin, and 50 μ g/ml streptomycin. These cell clones were used for experiments after culture for at least 2 weeks following the last Ag stimulation.

2.4. Stimulation of T cell clones for cytokine production and proliferation

Th1 clone #4 and Th2 D10.G4.1 cells (2.5×10^4 cells/well) were stimulated with immobilized anti-CD3 mAb (145-2C11; Cedarlane Laboratories Limited, Ontario, Canada) in the presence or absence of varying concentrations of Tryptanthrin (Wako Pure Chemical Industries, Osaka, Japan) in 96-well plates. Immobilized anti-CD3 mAb was prepared by incubating plates with 8 μ g/ml of anti-CD3 mAb in PBS (50 μ l/well) for 3 h at 37 °C followed by washing the plates 3 times with cold PBS. For IFN- γ and IL-4 assays, 50 μ l/well of culture supernatants were collected after 48 h. To measure cell proliferation, 20 μ l alamarBlue dye (Trek Diagnostic Systems, OH), a redox indicator, was added to each microplate well for the last 2–3 h of the incubation period (Ahmed et al., 1994). Fluorescence intensity (FI) was measured using a 544 nm excitation wavelength and a 590 nm emission wavelength.

2.5. RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Briefly, D10 G4.1 cells (2×10^6 cells/well) were dispensed into anti-CD3 mAb coated 6-well plates, with or without varying concentrations of Tryptanthrin, and cultured for 48 h at 37 °C in 5% CO₂. Total RNA was extracted from D10.G4.1 cells using an RNeasy Mini kit (QIAGEN, Tokyo, Japan) and DNase (QIAGEN) according to the manufacturer's instructions. Subsequently, first-strand cDNA was synthesized using Superscript reverse transcriptase III (Invitrogen, Carlsbad, CA) and Oligo (dT) (Invitrogen). Specific primers for PCR were identical to those described previously (Srivastava et al., 2004; Tanaka et al., 2005). All PCR primer sequences are shown in Table 1. Synthesized cDNA was mixed with SYBR Green Master Mix (Roche, Mannheim, Germany) and different sets of gene-specific primers. Real-time PCR was performed using a LightCycler 480 system (Roche). For quantitative purposes, the expression of each gene was normalized to a house keeping gene, GAPDH.

2.6. Purification of CD4⁺ T cells and cell culture for T cell differentiation

BALB/c mice were immunized 3 times by subcutaneous (s.c.) and intraperitoneal (i.p.) injections with 10 µg SBP absorbed onto 2 mg alum adjuvant every 2 weeks. CD4⁺ T cells were purified from spleen cells of SBP-immunized BALB/c mice, as described previously (Kohno et al., 2008). Briefly, spleen cells were incubated in FCS-coated plastic plates for 1.5 h at 37 °C. After incubation, non-adherent cells were seeded onto plates coated with goat anti-mouse Ig (DAKO, Glostrup, Denmark) for 1 h at 4 °C to remove B cells. The recovered non-adherent cells were incubated with ascites fluid containing rat anti-mouse CD4 mAb from the GK1.5 hybridoma (a gift from Dr. E. Nakayama, Okayama University Medical School, Okayama, Japan) for 30 min on ice, and then washed twice with medium. The harvested cells were incubated in plates coated with goat anti-rat IgG (ICN Pharmaceuticals, Aurora, OH) for 1 h at 4 °C to recover CD4⁺ T cells. Adherent cells were primarily CD4⁺ T cells with a purity of >90% as verified by flow cytometric analysis.

Purified CD4⁺ T cells (2×10^6 cells/ml) were stimulated in 24-well plates with 10 µg/ml SBP and MMC-treated BALB/c mouse spleen cells (4.8×10^6 cells/ml) in the presence or absence of varying concentrations of Tryptanthrin (primary culture, neutral conditions). For some experiments, 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN) and 10 µg/ml of anti-IL-12 mAb (BD PharMingen, San Diego, CA) were added to the primary culture (primary culture, Th2 conditions). After 7 d, T cells were harvested, washed, and restimulated at 4×10^5 cells/ml with 10 µg/ml SBP and MMC-treated BALB/c mouse spleen cells (secondary culture). After 48 h incubation, culture supernatants were collected for cytokine assays.

2.7. Degranulation assay for RBL-2H3 cells

The inhibitory effect of Tryptanthrin on the release of β-hexosaminidase from RBL-2H3 cells during degranulation was evaluated using a previously reported method (Morikawa et al., 2002) with minor modifications. Briefly, RBL-2H3 cells (2×10^5 cells/well) were cultured in 24-well plates with Eagle's minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 10% FCS, 0.3% glutamine, and 60 ng/ml of monoclonal anti-dinitrophenyl IgE antibody (anti-DNP IgE) (Sigma–Aldrich, St. Louis, MO). The cells were incubated overnight at 37 °C in 5% CO₂ to sensitize the cells. Then, the cells were washed twice with 500 µl of Siraganian buffer containing 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), and 40 mM NaOH at pH 7.2, and incubated in 160 µl of Siraganian buffer supplemented with 5.6 mM glucose, 1 mM CaCl₂ and 0.1% bovine serum albumin (BSA) for 15 min at 37 °C. In addition, 20 µl of varying concentrations of Tryptanthrin or Oxatomide (Wako Pure Chemical Industries) were added to each well and incubated for 15 min, followed by the addition of 20 µl of dinitrophenylated albumin (DNP-OVA) (Sigma–Aldrich) at a final concentration of 5 µg/ml at 37 °C for 15 min to induce an allergic-type degranulation reaction. The reaction was stopped by cooling the plate on ice for 10 min. Supernatants (100 µl) were transferred to a 96-well microplate and incubated with 50 µl of 1 mM substrate (4-nitrophenyl N-acetyl-β-D-glucosaminide) (Sigma–Aldrich) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 50 µl of stop solution (0.1 M sodium carbonate buffer, pH 10.0). The absorbance was measured using a microplate reader at 405 nm. Tryptanthrin or Oxatomide was dissolved in dimethylsulfoxide (DMSO), and the solution was diluted with Siraganian buffer. We confirmed that DMSO that was contained in the high concentrations of Tryptanthrin or Oxatomide did not affect the results of this degranulation assay.

The percentage degranulation of IgE-sensitized RBL-2H3 cells by the test material was calculated using the following equation:

$$\text{Percent degranulation (\%)} = 100 \times \frac{(T - N)}{(C - N)},$$

where C is the absorbance measured using an extract of non-stimulated RBL-2H3 cells prepared by freeze-thawing 3 times, T is the absorbance measured using a culture supernatant of IgE-sensitized RBL-2H3 cells followed by induction with DNP-OVA and test samples, and N is the absorbance measured using a culture supernatant of IgE-non-sensitized RBL-2H3 cells followed by induction with DNP-OVA.

To determine the growth inhibitory effects of test samples in this degranulation assay, RBL-2H3 cells (4×10^4 cells/well) were cultured in 96-well plates and treated as described above. After 15 min of induction, 20 µl of alamarBlue dye was added to the culture and the plates were incubated for an additional 2–3 h at 37 °C.

2.8. IL-4 production by RBL-2H3 cells

RBL-2H3 cells were dispensed into 24-well plates as described above and incubated for 6 h at 37 °C in 5% CO₂. Then, cells were stimulated for measurements of IL-4 production with 60 ng/ml anti-DNP-IgE and 2 µg/ml DNP-OVA in the presence or absence of varying concentrations of Tryptanthrin or Oxatomide and incubated overnight.

Proliferation of RBL-2H3 cells was assessed by adding 20 µl of alamarBlue dye to the culture, as described above.

2.9. Cytokine assays

Cytokines in the culture supernatants (mouse IL-4 and IFN-γ) were measured by two-site sandwich ELISA. The Abs for coating the plates and the biotinylated secondary mAbs were as follows: for IL-4, rat mAb anti-mouse IL-4 (BVD4-1D11, BD PharMingen) and biotinylated mAb anti-mouse IL-4 (BVD6-24G2, BD PharMingen); for IFN-γ, polyclonal rabbit anti-mouse IFN-γ Ab (prepared in our laboratory) and biotinylated rat mAb anti-mouse IFN-γ (XMG1.2, BD PharMingen). HRP-conjugated Streptavidin was purchased from Zymed (San Francisco, CA).

Levels of rat IL-4 secreted by RBL-2H3 cells were determined with an ELISA kit (R&D Systems, Minneapolis, MN).

2.10. Western immunoblotting analysis of cell signaling molecules

Western blotting used whole cell extracts from RBL-2H3 cells treated with anti-DNP IgE and DNP-OVA in the presence or absence of varying concentrations of Tryptanthrin, as described previously (Tsuji-Takayama et al., 2006). Membranes were probed with anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit mAb (D13.14.4E; Cell Signaling Technology, Danvers, MA) and anti-phospho-Akt (Ser473) rabbit mAb (193H2; Cell Signaling Technology). Specific bands were detected using a chemiluminescent system (Immobilon Western Chemiluminescent HRP substrate; Millipore, Bedford, MA). After treatment with a reprobing solution (Restore Western Blot Stripping Buffer; Pierce Biotechnology, Rockford, IL) for 15 min at room temperature, the membrane was used for secondary detection with anti-ERK1 rabbit pAb (C-16; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt mouse mAb (2H10; Cell Signaling Technology), and anti-actin mouse mAb (MAB1501; Chemicon, Temecula, CA). Band density was measured using an Image Master densitometer (Pharmacia Biotech, Uppsala, Sweden).

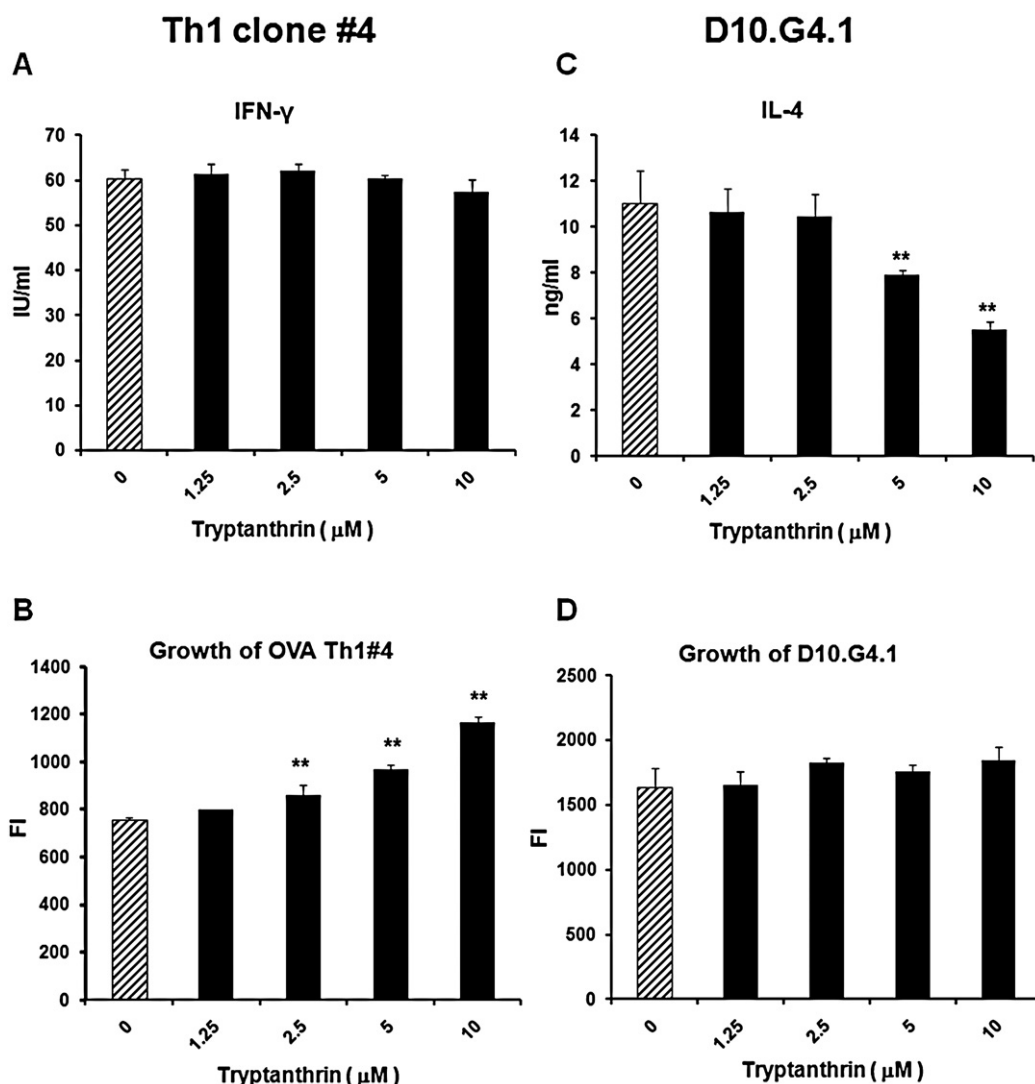


Fig. 1. The effect of Tryptanthrin on cytokine production by anti-CD3-stimulated Th1 and Th2 clone cells. OVA-specific Th1 clone #4 and conalbumin-specific Th2 clone D10.G4.1 cells (2.5×10^4 cells) were stimulated with immobilized anti-CD3 mAb ($8 \mu\text{g/ml}$) in the presence or absence (control) of varying concentrations of Tryptanthrin at 37°C for 48 h. Concentrations of IFN- γ (A) and IL-4 (C) in culture supernatants were measured by ELISA. Growth of Th1 clone #4 (B) and D10.G4.1 (D) cells was assessed by alamarBlue dye for the last 2–3 h of the incubation period. Results are given in units of fluorescence intensity. Results are the means $\pm$ S.D. of triplicate cultures. Results are representative of two independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, compared with control cultures.

2.11. Statistical analysis

Data were analyzed by Dunnett's multiple comparisons test using a JMP 6 software (SAS Institute, Cary, NC). p -Values < 0.05 were considered statistically significant.

3. Results

3.1. Tryptanthrin suppresses the production of IL-4 by anti-CD3-stimulated Th2 clone D10.G4.1 cells

First, we examined whether Tryptanthrin could regulate either cellular or humoral immune responses via directly affecting effector Th cell activation. For this purpose, OVA-specific Th1 clone #4 cells and conalbumin-specific Th2 clone D10.G4.1 cells were stimulated with immobilized anti-CD3 mAb in the presence or absence of varying concentrations of Tryptanthrin for 48 h. Tryptanthrin significantly and dose-dependently suppressed IL-4 production by D10.G4.1 cells (Fig. 1C). At 5 and $10 \mu\text{M}$ Tryptanthrin, IL-4 production was significantly reduced by 28% ($p < 0.01$) and 50% ($p < 0.01$) that of control culture, respectively. Since the growth of D10.G4.1

cells was not affected by Tryptanthrin (Fig. 1D), the reductions in IL-4 production by Tryptanthrin were not due to the inhibition of cell growth of D10.G4.1 cells. In contrast, Tryptanthrin had no effect on IFN- γ production by Th1 clone #4 cells (Fig. 1A). However, cell growth was significantly enhanced at concentrations above $2.5 \mu\text{M}$ Tryptanthrin (Fig. 1B), although the reasons for this remain to be determined. These results suggest that Tryptanthrin directly suppressed the activation of Th2 cells, but not Th1 cells.

3.2. Tryptanthrin suppresses the expression of Th2-specific transcription factor *c-Maf*, but not *GATA-3*

Because Tryptanthrin directly suppressed IL-4 production by anti-CD3 mAb-stimulated D10.G4.1 cells, we speculated that Tryptanthrin would suppress the expression of Th2-specific transcription factors. It is well established that *GATA-3* and *c-Maf* are critical Th2-specific transcription factors (Kurata et al., 1999). Thus, we examined the effects of Tryptanthrin on *GATA-3* and *c-Maf* expressions by immobilized anti-CD3 mAb-stimulated D10.G4.1 cells using real-time PCR. As shown in Fig. 2, Tryptanthrin had no

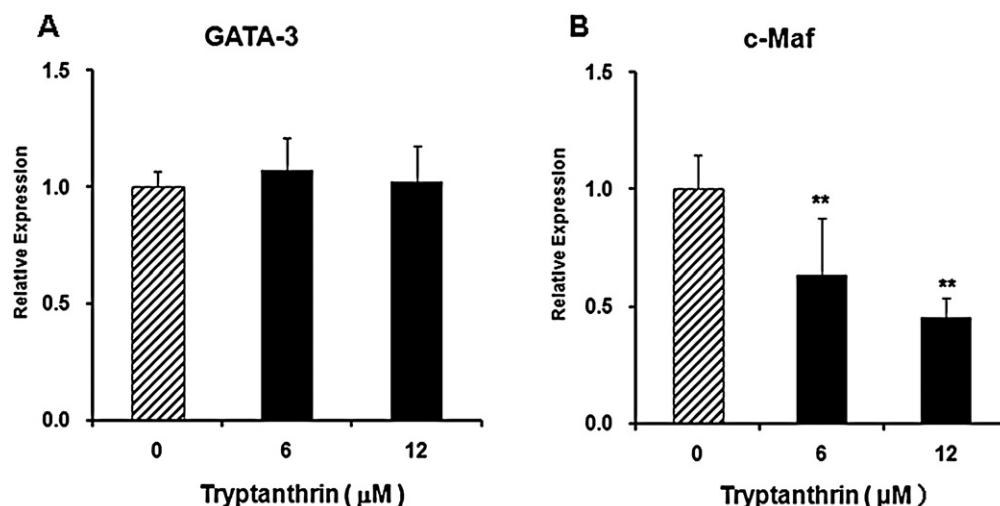


Fig. 2. The effect of Tryptanthrin on GATA-3 and c-Maf expressions in anti-CD3-stimulated D10.G4.1 cells. Total RNA was extracted from D10.G4.1 cells (2×10^6 cells) stimulated with immobilized anti-CD3 mAb ($8 \mu\text{g/ml}$) in the presence or absence (control) of varying concentrations of Tryptanthrin at 37°C for 48 h. First-strand cDNA was synthesized using Superscript III reverse transcriptase, as described in Section 2. Expression levels of genes associated with Th2 specific transcription factors, GATA-3 (A) and c-Maf (B), were analyzed by real-time PCR. GAPDH mRNA expression levels were used for normalization. Results are given as changes in gene expression relative to the mean values of control. Results are the means $\pm$ S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control cultures.

effect on GATA-3 mRNA expression (Fig. 2A), whereas c-Maf mRNA was significantly and dose-dependently suppressed by the addition of Tryptanthrin ($p < 0.01$ for both 6 and $12 \mu\text{M}$ Tryptanthrin) (Fig. 2B). Because c-Maf binds to the IL-4 proximal promoter and regulates IL-4 production (Ho et al., 1996; Kim et al., 1999), these results indicate that Tryptanthrin down-regulates c-Maf mRNA expression, which results in reduced IL-4 production by D10.G4.1 cells.

3.3. Tryptanthrin inhibits Th2 differentiation under Th2-skewing conditions

Since it has been shown that c-Maf functions during the differentiation of normal primary T cells into Th2 subset (Ho et al., 1998), we investigated the effect of Tryptanthrin on Th cell differentiation. For this purpose, we purified CD4^+ T cells from spleen cells of SBP-immunized BALB/c mice. Purified CD4^+ T cells were stimulated with SBP and MMC-treated spleen cells from normal BALB/c mice as antigen-presenting cells (APCs) in the presence or absence of varying concentrations of Tryptanthrin with (Th2 conditions) or without (neutral conditions) IL-4 and anti-IL-12 mAb (primary culture). After 7 d, T cells were harvested, re-stimulated with SBP and MMC-treated spleen cells, and evaluated for cytokine production after 48 h (secondary culture). Under neutral conditions, the Th2 cytokine IL-4 secretion was significantly suppressed by the addition of Tryptanthrin to the primary cultures in a concentration-dependent manner ($p < 0.01$ for both 4 and $8 \mu\text{M}$ Tryptanthrin) (Fig. 3B). In contrast, comparable amounts of the Th1 cytokine IFN- γ were detected at $4 \mu\text{M}$ Tryptanthrin, but IFN- γ was significantly ($p < 0.01$) and markedly reduced at $8 \mu\text{M}$ Tryptanthrin (Fig. 3A). Under Th2 conditions, IL-4 secretion was significantly and dose-dependently suppressed by the addition of Tryptanthrin to the primary cultures ($p < 0.05$ for $4 \mu\text{M}$ Tryptanthrin; $p < 0.01$ for $8 \mu\text{M}$ Tryptanthrin) (Fig. 3D), whereas IFN- γ secretion was significantly enhanced ($p < 0.01$ for both 4 and $8 \mu\text{M}$ Tryptanthrin) (Fig. 3C). The secretion of other Th2 cytokines, IL-5 and IL-10, was also suppressed in a manner similar to that of IL-4 (data not shown). These results suggest that Tryptanthrin inhibits Th cell differentiation toward Th2 cells, even under Th2-skewing conditions.

3.4. Tryptanthrin inhibits degranulation of rat basophilic leukemia (RBL-2H3) cells

Next, we examined the effects of Tryptanthrin on IgE-mediated degranulation, an effector phase response of allergic hypersensitivity. Basophils and mast cells are important effector cells in allergic hypersensitivity that release a number of chemical mediators after Fc ϵ RI receptors on their surfaces are cross-linked. RBL-2H3 is an established cell line from rats (Seldin et al., 1985) that has been used to screen for inhibitors of degranulation and cytokine release (Morikawa et al., 2002; Lee et al., 2008). We then examined the effect of Tryptanthrin on degranulation by the release of β -hexosaminidase from RBL-2H3 cells. Tryptanthrin significantly and dose-dependently inhibited the degranulation of RBL-2H3 cells ($p < 0.05$ for $1.25 \mu\text{M}$ Tryptanthrin; $p < 0.01$ for both 2.5 and $5 \mu\text{M}$ Tryptanthrin) (Fig. 4A). At $5 \mu\text{M}$ Tryptanthrin, 81% inhibition was observed. The growth of RBL-2H3 cells was not affected by addition of Tryptanthrin at concentrations up to $5 \mu\text{M}$ (Fig. 4B), suggesting that the inhibition of degranulation by Tryptanthrin was not due to its growth inhibitory effects. We then compared the inhibitory effect of Tryptanthrin with that of Oxatomide, an anti-allergic agent that inhibits the release of both chemical mediators and peptide leukotrienes (LTs) (Kosaka et al., 1987; Paulussen et al., 1996). Oxatomide also significantly inhibited degranulation, but, on a molar basis, the inhibitory effects were relatively weak compared to Tryptanthrin (Fig. 4A). These findings suggest that Tryptanthrin may have therapeutic effects for IgE-mediated hypersensitivity.

3.5. Tryptanthrin suppresses IL-4 secretion by RBL-2H3 cells

Because basophils and mast cells can secrete a number of cytokines, such as IL-4, IL-6, TNF- α , and IL-13, upon cross-linking of their Fc ϵ RI receptors (Turner and Kinet, 1999), we investigated whether RBL-2H3 cells secrete IL-4 after cross-linking their Fc ϵ RI receptors with DNP-OVA and DNP-specific IgE antibodies. As shown in Fig. 5A, RBL-2H3 cells secreted substantial amounts of IL-4 upon Fc ϵ RI receptor cross-linking. Since Tryptanthrin inhibited IgE-mediated degranulation of RBL-2H3 cells, we examined the effects of Tryptanthrin on IgE-mediated IL-4 secretion. For this purpose, we stimulated RBL-2H3 cells with anti-DNP IgE and DNP-OVA in the presence or absence of varying concentrations of Tryptanthrin or

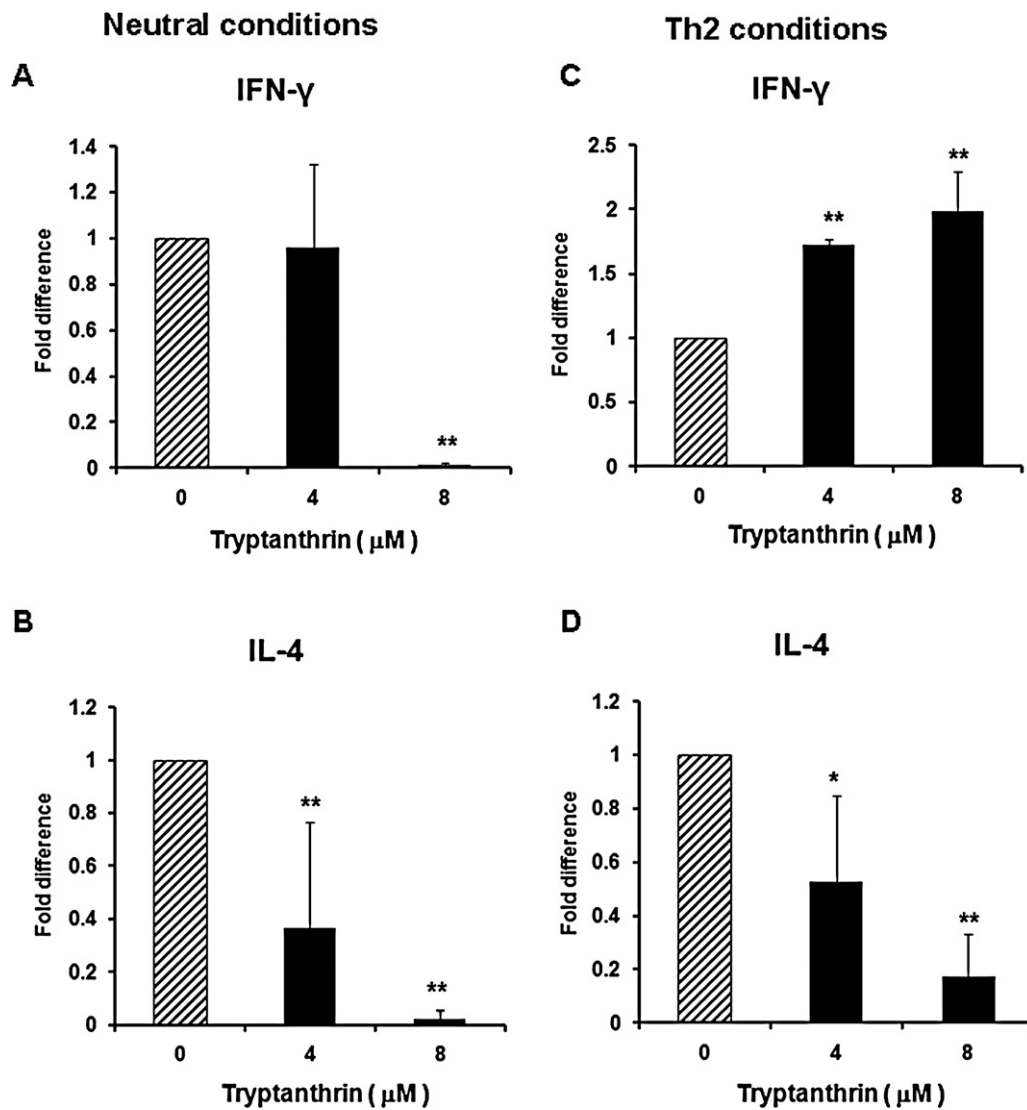


Fig. 3. The effect of Tryptanthrin on the differentiation of CD4⁺ T cells purified from SBP/Alum-immunized mice. CD4⁺ T cells purified from spleen cells of SBP/Alum-immunized mice were stimulated with SBP and APC in the presence or absence (control) of varying concentrations of Tryptanthrin with (Th2 conditions) or without (neutral conditions) IL-4 (10 ng/ml) and anti-IL-12 mAb (10 μg/ml) at 37 °C for 7 d (primary culture). After the primary culture, T cells were recovered, washed, and re-stimulated with SBP and APCs at 37 °C for 48 h (secondary culture). Concentrations of IFN-γ (A and C) and IL-4 (B and D) in the secondary cultures under neutral conditions (A and B) and Th2 conditions (C and D) were measured by ELISA. Results are given as fold differences in relation to the Tryptanthrin-untreated value of either neutral or Th2 conditions from four independent cultures of two separate experiments. **p* < 0.05, ***p* < 0.01, compared with control cultures.

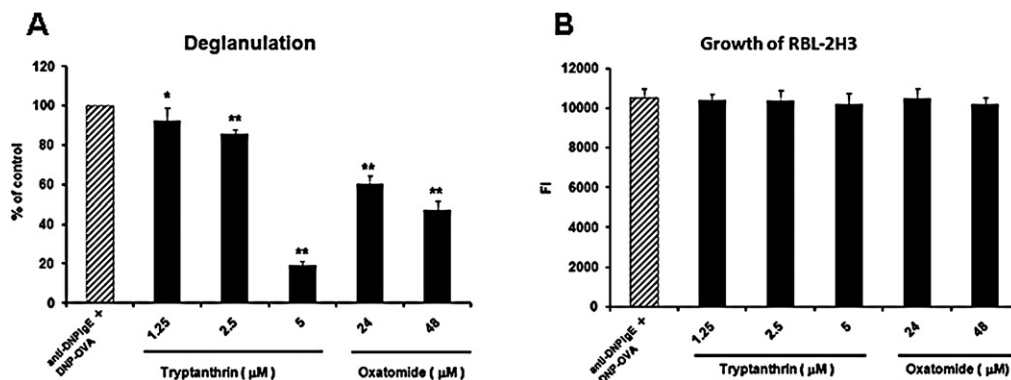


Fig. 4. The effect of Tryptanthrin on the IgE-mediated degranulation of RBL-2H3 cells. RBL-2H3 cells (2×10^5 cells/well) were incubated overnight with anti-DNP IgE (60 ng/ml) in 24-well plates. After the incubation period, cells were washed and treated with or without (control) varying concentrations of Tryptanthrin or Oxatamide for 15 min. Subsequently, cells were stimulated with DNP-OVA (5 μg/ml) for 15 min to induce β-hexosaminidase release (A). Percentages of degranulation were calculated as described in Section 2. Effect of Tryptanthrin on the growth of RBL-2H3 cells treated as described above was assessed by alamarBlue (B). Results are the means ± S.D. of triplicate cultures. Results are representative of two independent experiments with similar results. **p* < 0.05, ***p* < 0.01, compared with control cultures.

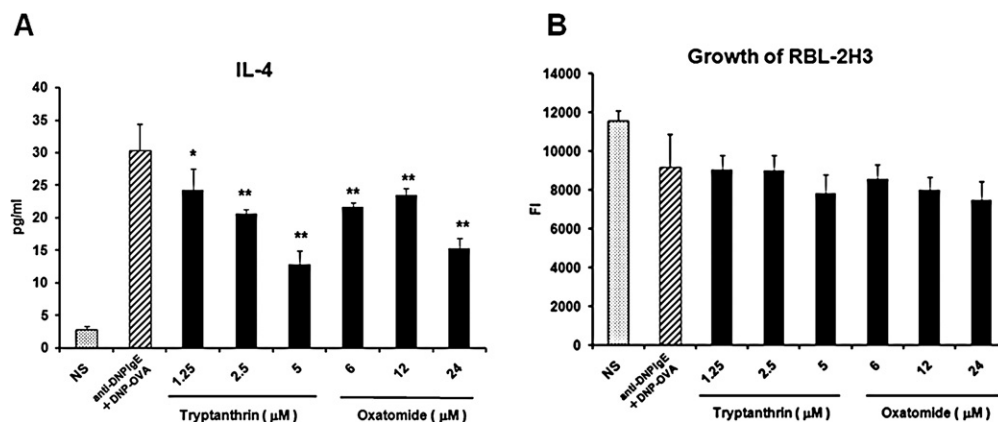


Fig. 5. Effect of Tryptanthrin on IgE-mediated IL-4 secretion from RBL-2H3 cells. RBL-2H3 cells (2×10^5 cells/well) were incubated with anti-DNP IgE (60 ng/ml) and DNP-OVA (2 μ g/ml) in the presence or absence of varying concentrations of Tryptanthrin or Oxatomide for 24 h in 24-well plates. Non-stimulated control cells (NS) were incubated without anti-DNP IgE and DNP-OVA. Concentrations of IL-4 in culture supernatants were measured by ELISA (A). Growth of RBL-2H3 cells (4×10^4 cells/well) was assessed by alamarBlue (B). Results are the means $\pm$ S.D. of triplicate cultures. Results are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, compared with control cultures.

Oxatomide for 24 h. As shown in Fig. 5A, IgE-mediated IL-4 secretion was significantly and dose-dependently inhibited by adding Tryptanthrin ($p < 0.05$ for 1.25 μ M Tryptanthrin; $p < 0.01$ for both 2.5 and 5 μ M Tryptanthrin). At 5 μ M Tryptanthrin, 58% inhibition was observed. Oxatomide also inhibited IL-4 secretion by RBL-2H3 cells, although to a lesser extent. Since the growth of RBL-2H3 cells was not significantly inhibited by the addition of Tryptanthrin or Oxatomide at concentrations used in this experiment (Fig. 5B), the inhibition of IL-4 production by Tryptanthrin or Oxatomide was not due to growth inhibition of RBL-2H3 cells. These results suggest that Tryptanthrin inhibits IL-4 secretion, as well as degranulation, upon cross-linking of Fc ϵ RI receptors on basophils.

3.6. Tryptanthrin inhibits IgE-mediated signaling pathways

To investigate the mechanism by which Tryptanthrin inhibits IL-4 production by RBL-2H3 cells, we examined whether Tryptanthrin influences IgE-mediated signaling pathways, including the phosphorylation of ERK1/2 (MAP kinases) and Akt, a downstream effector of phosphatidylinositol 3-kinase (PI3K) (Fig. 6). For mast cells and basophils, phosphorylation of ERK1/2 and Akt leads to the production of cytokines, including TNF- α and IL-4 (Huang et al., 2008).

RBL-2H3 cells were stimulated with anti-DNP IgE and DNP-OVA in the presence or absence of varying concentrations of Tryptanthrin for 15 min or 30 min. As shown in Fig. 6A–C, phosphorylation of ERK1/2 and Akt in cells stimulated with anti-DNP IgE and DNP-OVA was increased compared with that in non-stimulated RBL-2H3 cells. This phosphorylation was suppressed by the addition of Tryptanthrin in a concentration-dependent manner, while Tryptanthrin had no effect on total protein concentration. We also confirmed that Tryptanthrin did not interfere with the binding of anti-DNP IgE antibodies to RBL-2H3 cells as determined by cell ELISA (data not shown). These results suggest that Tryptanthrin inhibited IL-4 production via inhibiting the phosphorylation of ERK1/2 and Akt in antigen- and IgE-stimulated RBL-2H3 cells.

4. Discussion and conclusions

In this study, we have shown that Tryptanthrin has anti-allergic properties *in vitro*. First, Tryptanthrin directly suppressed IL-4 production by D10.G4.1 cells, a Th2 clone, and even under Th2-skewing conditions, Tryptanthrin inhibited differentiation toward the Th2 phenotype, which is an essential event for the initiation phase

of allergic disease. Second, Tryptanthrin inhibited IgE-mediated degranulation and IL-4 production by RBL-2H3 cells, which are essential events for the effector phase of hypersensitivity reactions. In accordance with our results, Xu et al. (2009) recently reported that Tryptanthrin inhibits the IgE-mediated degranulation of RBL-2H3 cells, although the mechanism(s) of action has not been determined (Xu et al., 2009). We have shown here that the Tryptanthrin-induced suppression of IL-4 production by RBL-2H3 cells is due to the inhibition of IgE-mediated signaling pathways, including the phosphorylation of ERK1/2 and Akt.

Th2 cells, basophils, and mast cells are important players in allergic responses. Th2 cells produce numerous cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and regulate antibody production for defense against extracellular pathogens (Mosmann and Coffman, 1989; Seder and Paul, 1994). In particular, IL-4 is a determining factor for Th2 differentiation and induces IgE antibody production by B cells, and thereby contributes to the development of allergic responses (Constant and Bottomly, 1997).

Here, we demonstrated that Tryptanthrin directly suppressed IL-4 production by anti-CD3-stimulated D10.G4.1 cells. However, IFN- γ production by Th1 clone #4 cells was not affected. This suppression was due to decreased expression of the Th2-specific transcription factor c-Maf, but not GATA-3. c-Maf was identified as the first Th2-specific transcription factor that bound to the IL-4 proximal promoter and regulated IL-4 production (Ho et al., 1996; Kim et al., 1999).

Recently, Saraiva et al. (2009) showed that ERK1/2 in Th cells possibly regulates the expression of c-Maf, but not T-bet, a critical regulator of Th1 development and IFN- γ production (Saraiva et al., 2009). As shown in Fig. 6, Tryptanthrin inhibited the phosphorylation of ERK1/2 in RBL-2H3 cells. Although the possible effects of Tryptanthrin on inhibiting ERK1/2 expression were not examined in our clones of Th1 or Th2 cells, these findings lead us to suggest that Tryptanthrin suppresses c-Maf expression in D10.G4.1 cells via inhibiting the activation of ERK1/2, resulting in reduced IL-4 production.

Tryptanthrin inhibited the development of IL-4-producing Th2 cells not only under neutral conditions, but also under Th2-skewing conditions. Under neutral conditions, adding Tryptanthrin to primary cultures resulted in significant and dose-dependent reduction of IL-4 production in secondary cultures. Because IL-4 is a key cytokine for Th2 differentiation, suppressing IL-4 production undoubtedly resulted in reduced Th2 differentiation. It would seem likely that the reduced c-Maf expression was involved in the inhibition of IL-4 production.

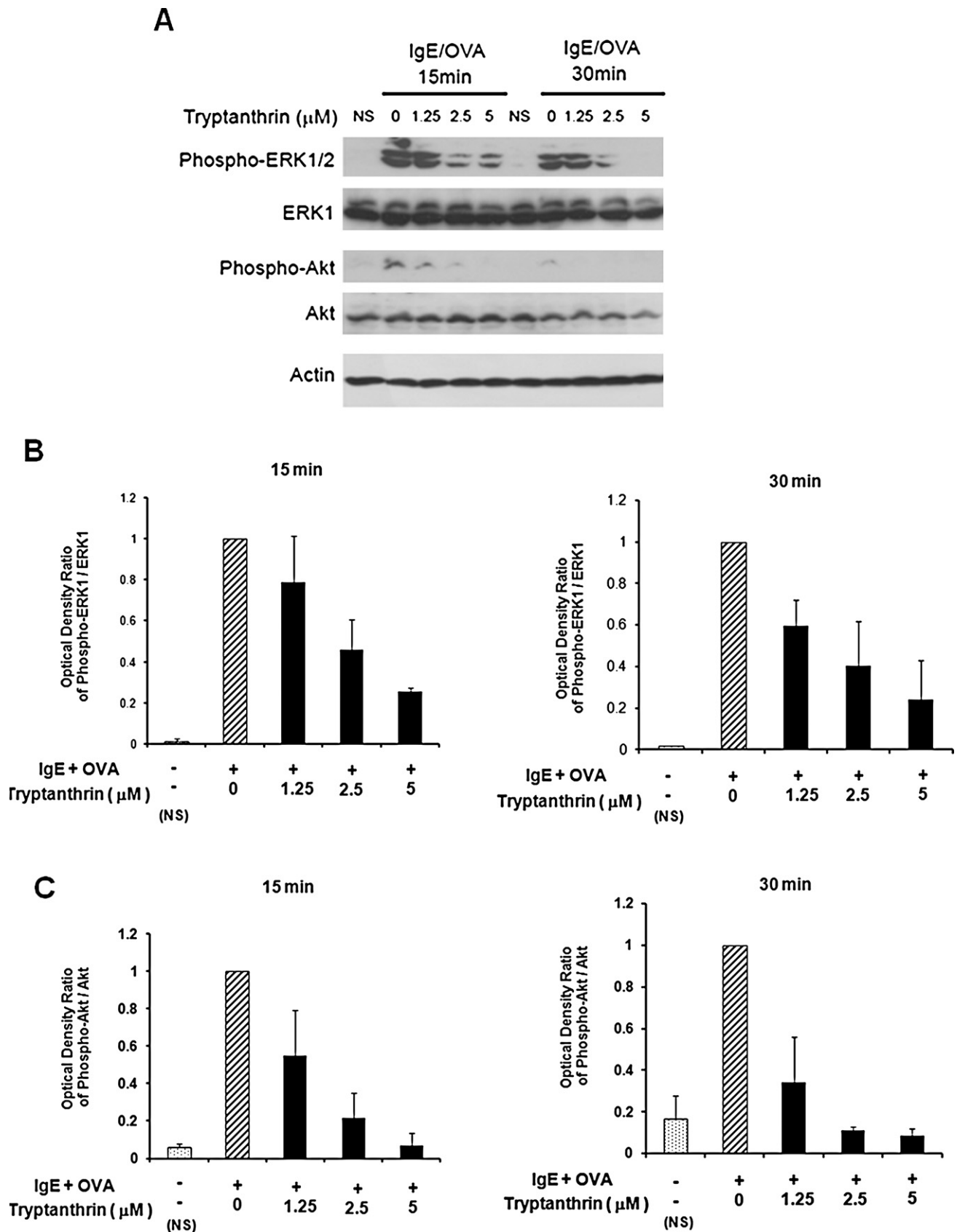


Fig. 6. Effect of Tryptanthrin on IgE-mediated phosphorylation of ERK1/2 and Akt. RBL-2H3 cells (1×10^6 cells/well) were incubated with anti-DNP IgE (60 ng/ml) and DNP-OVA (2 $\mu\text{g}/\text{ml}$) in the presence or absence of varying concentrations of Tryptanthrin for 15 min and 30 min in 6-well plates. Phosphorylation of ERK1/2 and Akt was determined by immunoblotting of whole-cell lysates using specific antibodies against the phosphorylated or total proteins (A). The optical density ratio of phospho-ERK1 to total ERK1 (B) and of phospho-Akt to total Akt (C) was also shown. Results are expressed as the means $\pm$ data range of two independent experiments. NS: non-stimulated.

In this experiment, in addition to the reduced IL-4 production, IFN- γ production was also markedly inhibited at 8 μ M Tryptanthrin. We consider that these marked reductions in IFN- γ and IL-4 production were not due to the growth inhibition of Th cells for the following reasons: (1) Tryptanthrin at or below 10 μ M did not directly inhibit the growth of anti-CD3 mAb-stimulated Th1 and Th2 cells (Fig. 1B and D). (2) Tryptanthrin at or below 20 μ M did not inhibit but rather increased the Con A-induced proliferative response of spleen cells from normal BALB/c mice, although IFN- γ production by Con A-stimulated spleen cells was dose-dependently and significantly inhibited (data not shown). (3) We previously found that Tryptanthrin at or below 10 μ M had little if any effect on the SBP-specific proliferative response of spleen cells from SBP-immunized mice (spleen cell proliferation determined by alamarBlue dye on day 3: 2781 ± 59 FI for control culture, 2826 ± 149 FI for 5 μ M Tryptanthrin, 2740 ± 61 FI for 10 μ M Tryptanthrin; means $\pm$ S.D., $n = 3$). In this experiment, production of both IFN- γ and IL-4 was significantly inhibited at 10 μ M Tryptanthrin (IFN- γ production: 0.29 ± 0.06 IU/ml for control culture, 0.31 ± 0.02 IU/ml for 5 μ M Tryptanthrin, 0.12 ± 0.06 IU/ml for 10 μ M Tryptanthrin, $p < 0.05$ for 10 μ M Tryptanthrin; IL-4 production: 64.8 ± 13.7 pg/ml for control culture, 41.5 ± 11.2 pg/ml for 5 μ M Tryptanthrin, 23.8 ± 2.6 pg/ml for 10 μ M Tryptanthrin, $p = 0.057$ for 5 μ M Tryptanthrin, $p < 0.01$ for 10 μ M Tryptanthrin; means $\pm$ S.D., $n = 3$). These results suggest that Tryptanthrin exerts its immunoregulatory effect on primary T cells without affecting cell growth at least until concentrations of 10 μ M.

It has been reported that endogenous IL-4 is required for the development of protective CD4⁺ Th1 responses against *Candida albicans* infections (Mencacci et al., 1998). Thus, the dramatic reduction of endogenous IL-4 production by the inhibition of c-Maf expression possibly resulted in inhibiting the development of IFN- γ -producing Th1 cells.

Under Th2-skewing conditions, Tryptanthrin dose-dependently inhibited the secretion of Th2 cytokines, including IL-4, IL-5, and IL-10, in the secondary cultures, indicating the inhibition of Th2 cell development. Conversely, IFN- γ secretion was promoted. These results cannot be explained by the reduced expression of c-Maf due to Tryptanthrin, as sufficient exogenous IL-4 to induce Th2 cell development was added to the cultures. Therefore, it seems likely that Tryptanthrin inhibited Th2 cell development via a mechanism(s) other than the suppression of c-Maf expression.

Other possible mechanisms may involve IL-4 receptor signals that are involved with Th2 development via Janus kinase (JAK) and signal transducer and activator of transcription 6 (STAT6) (Kubo et al., 1997). Upon activation, STAT6 translocates into the nucleus and rapidly induces the expression of GATA-3 (Kurata et al., 1999; Ouyang et al., 1998). Yamashita et al. (2005) reported that the T cell receptor-mediated Ras-ERK MAPK cascade is required for the stable expression of GATA-3 protein in developing Th2 cells. A MEK/ERK MAPKK-specific inhibitor, PD98059, inhibited the development of Th2 cells under Th2-skewing conditions (Yamashita et al., 2005). In our study, Tryptanthrin inhibited the phosphorylation of ERK in RBL-2H3 cells that were stimulated by Fc ϵ RI cross-linking. These results suggest the possibility that Tryptanthrin inhibits Th2 cell development under Th2-skewing conditions via reduced GATA-3 expression by suppressing T cell receptor-mediated activation of ERK. Because GATA-3 counter-regulates T-bet (Yin et al., 2002), it seems likely that increased production of IFN- γ in the secondary cultures observed after adding Tryptanthrin to the primary cultures under the Th2-skewing conditions may have been caused by the up-regulation of T-bet. Additional studies will be necessary to confirm these possibilities.

In our study, IL-4 production during the effector phase was also inhibited by Tryptanthrin. Several studies have reported molecular mechanisms for regulating cytokine production by basophils.

Basophils can rapidly produce large amounts of IL-4 in response to several stimuli, including IL-3, parasite components, and dietary allergens, and play a role as APCs that preferentially induce Th2 cells in response to complexes of antigen plus antigen-specific IgE *in vitro* and *in vivo* (Yoshimoto, 2010). Thus, basophils are possibly a source of early IL-4 production that is critical during the initiation of Th2 cell differentiation and play a critical role in allergic responses.

In basophils and mast cells, cross-linking of IgE antibodies by antigens first activates the Src family kinases, such as Lyn and Fyn (Parravicini et al., 2002; Gilfillan and Tkaczky, 2006). The tyrosine kinase Lyn leads to the phosphorylation of FcR γ , and induces the recruitment of Syk to FcR γ . Syk plays a key role in the activation of mast cells and as an upstream kinase for the activation of LAT and SLP-76 (Siraganian et al., 2002). ERK1/2 is involved downstream of LAT and SLP-76 and regulates the production of several cytokines, including TNF- α and IL-13 (Zhang et al., 1997). In contrast, the tyrosine kinase Fyn phosphorylates a linker protein, Gab2, which leads to the activation of PI3K (Gu et al., 2001; Parravicini et al., 2002). Akt, which is subsequently activated downstream of PI3K, regulates IL-4 production (Frossi et al., 2007; Huang et al., 2008). Although we have shown here that Tryptanthrin inhibits the phosphorylation of ERK1/2 and Akt, it would be interesting to study whether Tryptanthrin can inhibit molecules upstream of ERK1/2 and Akt.

In summary, our findings indicate that Tryptanthrin inhibits Th2 differentiation and IL-4 production by established Th2 clone cells. Tryptanthrin also inhibited the degranulation of and IL-4 production by RBL-2H3 cells. These findings suggest that Tryptanthrin may effectively inhibit the effector and exacerbation responses, as well as the initiating events, of allergic reactions and have beneficial effects for allergic diseases. It has been recently shown that Tryptanthrin exhibits limited anti-inflammatory activities *in vivo* because of its low solubility and bioavailability (Recio et al., 2006). Thus, to address this issue, increasing the solubility of Tryptanthrin would be required in order to fully exploit the anti-allergic effects of Tryptanthrin *in vivo*.

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