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ORIGINAL ARTICLE

Theanine is a candidate amino acid for pharmacological stabilization of mast cells

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Abstract The increasing occurrences of allergic disorders may be attributed to exposure to environmental factors that contribute to the pathogenesis of allergy. The health benefits of green tea have been widely reported but are largely unsubstantiated. Theanine is the major amino acid present in green tea. In this study, we investigated the role of theanine in both IgE- and non- IgE-induced allergic response. Theanine inhibited compound 48/80-induced systemic anaphylactic shock and ear swelling responses. IgE-mediated passive cutaneous anaphylaxis was inhibited by the oral administration or pharmaceutical acupuncture of theanine. Histamine release from mast cells was decreased with the treatment of theanine. Theanine also repressed phorbol 12-myristate 13-acetate and calcium ionophore A23187-induced TNF-α, IL-1β, IL-6, and IL-8 secretion by suppressing NF-κB activation. Furthermore, theanine suppressed the activation of caspase-1 and the expression of receptor interacting protein-2. The current study demonstrates for the first time that theanine might possess mast cell-stabilizing capabilities.

Keywords Caspase-1 · Histamine · Nuclear factor-κB · Receptor interacting protein-2 · Theanine

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Introduction

Allergic diseases such as asthma, atopic dermatitis, allergic rhinitis, and food allergy, affect up to 20% of the human population in most countries and are believed to be increasing in prevalence (Eschenauer and Sweet 2006). Allergic disease, defined as a disorder of the immune system, results from exposure to normally harmless environmental substances known as allergens. Mast cells are widely distributed in the connective tissues of mammals and other vertebrates, where they are frequently located in close proximity to blood vessels (Galli et al. 1994). Activated mast cells can produce histamines, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-13, and transforming growth factor (TGF)-β1 (Plaut et al. 1989). The expression of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, is dependent on the activation of a transcription factor, nuclear factor (NF)-κB. When NF-κB binds to a specific consensus DNA element present on the promoter of target genes, it initiates the transcription of various genes (Galien et al. 1996). NF-κB normally resides in the cytoplasm, where it is retained by association with IκB protein (α, β, γ), an endogenous inhibitor (Galien et al. 1996). However, when it is activated, it translocates to the nucleus, binds the DNA, and activates genes. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκB, resulting in the nuclear migration of NF-κB (Galien et al. 1996).

Caspase-1, originally designated IL-1 converting enzyme, is a member of the group of caspases (Lee et al. 2001), and its activation regulates apoptosis and inflammation (Wang et al. 2005). Three specific adaptors have

been reported to regulate the activation of caspase-1. They are receptor interacting protein (RIP)-2 (Lee et al. 2001; Wang et al. 2005; Druilhe et al. 2001), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), a PYRIN-caspase recruitment domain (CARD) protein (Srinivasula et al. 2002; Stehlik et al. 2003; Mariathasan et al. 2004), and Ice-protease activating factor (Ipaf) (Poyet et al. 2001). RIP-2, ASC, and Ipaf are all CARD-containing proteins. Their CARD domains bind to the CARD domain of caspase-1 prodomain via CARD–CARD interactions, induce caspase-1 oligomerization, and promote caspase-1 activation. The activation of caspase-1 induces pro-inflammatory cytokine stimulation (Druilhe et al. 2001; Srinivasula et al. 2002; Stehlik et al. 2003). It has been reported that caspase-1-deficient mice are remarkably resistant to LPS-induced endotoxic shock. Thus, caspase-1 is an attractive target for therapeutic intervention in inflammatory diseases (Humke et al. 2000).

Theanine (L-γ-glutamylethylamide, $C_7H_{14}N_2O_3$), an amino acid found in green tea leaves (*Camellia sinensis*), has a molecular weight of 174.20 Da and is a water-soluble material (Sakato 1949; Borzelleca et al. 2006). Recent studies have reported that theanine has various favorable physiological functions including neuroprotection in transient ischemic neuronal death (Kakuda et al. 2000; Yamada et al. 2005), relaxing effects under resting conditions (Abdou et al. 2006; Lu et al. 2004), hypolipidemic functions (Chiang et al. 2005), regulation of systemic blood pressure (Yokogoshi et al. 1995), suppression of the stimulatory action of caffeine (Nozawa et al. 2000), modulation of neurotransmitter (Yamada et al. 2009), and protection against hepatic toxicity (Sadzuka et al. 2005). Additionally, studies on test rats have shown that repeated, extremely high doses of theanine cause little to no harmful psychological or physical effects (Borzelleca et al. 2006). Therefore, we investigated theanine as part of our continuing search for biologically active anti-allergic agents that may reduce or eliminate allergic reactions induced by environmental allergens.

In the present study, we investigated the effect of theanine on mast cell-mediated anaphylactic and inflammatory reactions. We found that theanine inhibited NF-κB activation and RIP-2/caspase-1 activation pathways.

Materials and methods

Chemical reagents and materials

Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), metrizamide, phorbol 12-myristate 13-acetate (PMA), A23187, *o*-phthaldialdehyde, Avidin-peroxidase, 2′-AZINO-bis (3-ethylbenzithiazoline-sulfonic acid) tablets substrate (ABTS), disodium cromoglycate (DSCG), dexamethasone, and Evans blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The α-minimal essential medium (MEM) was purchased from Flow Laboratories (Irvine, UK). Iscove's Modified Dulbecco's medium (IMDM), ampicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Recombinant human TNF-α, anti-human TNF-α antibody, and biotinylated anti-human TNF-α antibody, and caspase-1 assay kit were purchased from R&D System (Minneapolis, MN, USA). Recombinant human IL-1β, IL-6, and IL-8, anti-human IL-1β, IL-6, and IL-8 antibodies, biotinylated anti-human IL-1β, IL-6, and IL-8 antibodies were purchased from Pharmingen (San Diego, CA, USA). NF-κB, IκBα, RIP-2, and caspase-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The theanine ($_L$-γ-glutamylethylamide) was purchased from TCI (Tokyo kasei kogyo Co., Ltd.).

Animals

Male ICR mice (4-week-old, 20–25 g) and male Sprague–Dawley rats (7-week-old, 200–230 g) were purchased from the Dae-Han Experimental Animal Center (Daejon, Korea), and then maintained at the College of Oriental Medicine, Kyung Hee University. The animals were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1°C and relative humidity of 55 ± 10% throughout the study. All protocols were approved by the Institutional Animal Care and Use Committee of College of Oriental Medicine, Kyung Hee University.

Compound 48/80-induced systemic anaphylactic reaction

Theanine was dissolved in saline and administered orally to mice 1 h before the intraperitoneal injection (8 mg/kg) of the mast cell degranulator, compound 48/80. Mortality was then monitored for 30 min after induction of the anaphylactic reaction. Saline was used for treatments of the control animals in all experiments. Saline did not yield and observable effects in any of the experiments. DSCG and dexamethasone were used a positive control.

Ear swelling response

In these studies, ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. The ear swelling response was determined from any increment in thickness above baseline control values. Compound 48/80 (20 g/l) was freshly dissolved in saline

and injected intradermally (100 μg/site) into the dorsal aspect of the mouse ear using a microsyringe. The ear swelling response was then assessed 40 min later. To assess any protective effects from the theanine, the agent was orally administered to some mice 1 h prior to the compound 48/80.

Passive cutaneous anaphylaxis

IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 μl of the 1:1 mixture of 1 g/l DNP-HSA in PBS and 4% Evans blue via the tail vein. One hour before this injection, theanine was administered orally. To investigate the effect of theanine-pharmaceutical acupuncture on passive cutaneous anaphylaxis (PCA), mice were injected with theanine pharmaceutical acupuncture (1 mg/kg) at the Zusanli acupoint (ST36). ST36 is located 5 mm below and lateral to the anterior tubercle of the tibia. The non-region for acupuncture lateral to ST36 was used as the sham point. The mice were killed 40 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1 M KOH and 4.5 ml of a mixture of acetone and phosphoric acid (with the ratio of 5:13). The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of rat peritoneal mast cells

Rats were anesthetized by diethyl ether, and injected with 20 ml of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, KCl, NaH_2PO_4) containing 0.1% gelatin into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by Pasteur pipette. Then the peritoneal cells were sedimented at $150\times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e. macrophages and small lymphocytes). Peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 mg/l metrizamide (density 1.120 mg/l, Sigma) and centrifuged at room temperature for 15 min at $400\times g$. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Cell culture

HMC-1 cells were maintained in IMDM medium containing 10% FBS, 100 units/ml of penicillin–streptomycin solution, and 1 nM mercaptoethanol at 37°C in 5% CO_2 and 95% humidity. Cells (3×10^5 cells/well) were preincubated with theanine dissolved in distilled water by final concentrations of 0.01, 0.1, and 1 μM for 30 min before challenge with PMA (50 nM) plus calcium ionophore A23187 (1 μM) (PMACI) for 8 h at 37°C.

MTT assay

HMC-1 cell aliquots (3×10^5 cells/ml) were seeded in microplate wells and incubated with 20 μl of an MTT solution (5 mg/ml) for 4 h at 37°C under 5% CO2 and 95% air. Consecutively, 250 μl of DMSO was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

Histamine assay

Purified RPMCs and HMC-1 cells were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. Respective suspensions of RPMCs or HMC-1 cells were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the theanine for 30 min and then incubated for 15 min with compound 48/80 (6 mg/l). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400\times g$ for 5 min at 4°C. The histamine content in the cell-free supernatant was measured by the *o*-phthalaldehyde spectrofluorometric method. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

$$\%\text{inhibition} = (A - B) \times 100/A$$

where A is histamine release without theanine and B is histamine release with theanine.

ELISA

Cytokines were measured by a modified ELISA method. Briefly, 96-well ELISA plates (Nunc, Denmark) were coated with each of the anti-human TNF-α, IL-1β, IL-6, and IL-8 antibodies overnight at 4°C. Before subsequent steps in the assays, coated plates were washed with PBS containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37°C. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium the assay plates were exposed sequentially to each of the biotinylated anti-human TNF-α, IL-1β, IL-6, and IL-8 by using avidin-peroxidase. Optical-density readings were made within 10 min of the addition of the ABTS substrate solution on a Titertk Multiscan (Flow Laboratories) with a 405-nm filter. Appropriate specificity controls were included, and all samples were run in duplicate.

Reverse transcriptase-polymerase chain reaction analysis

We isolated the total RNA from the HMC-1 cells in accordance with the manufacturer's specification using an easy-blue^{TM} RNA extraction kit (Intron Biotech, Korea). According to method by Intron Biotech cDNA synthesis kit, cDNA strand was synthesized for 90 min 37°C and the final volume was 20 μl including 2 μg total RNA. We performed a PCR with the following primers: TNF-α (5′ CAC CAG CTG GTT ATC TCT CAG CTC 3′; 5′ CGG GAC GTG GAG CTG GCC GAG GAG 3′); IL-1β (5′ CCG GAT CCA TGG CAC CTG TAC GAT CA 3′; 5′ GGG GTA CCT TAG GAA GAC ACA AAT TG 3′); IL-6 (5′ GAT GGA TGC TTC CAA TCT GGA T 3′; 5′ AGT TCT CCA TAG AGA ACA ACA TA 3′); IL-8 (5′ CGA TGT CAG TGC ATA AAG ACA 3′; 5′ TGA ATT CTC AGC CCT CTT CAA AAA 3′). To verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions, we also used GAPDH (5′ CAA AAG GGT CAT CAT CTC TG 3′; 5′ CCT GCT TCA CCA CCT TCT TG 3′). The annealing temperature was 56°C for IL-1β and IL-6 or 60°C for TNF-α, IL-8, and GAPDH. We then performed the reverse transcriptase-polymerase chain reaction (RT–PCR) for TNF-α, IL-1β, IL-6, IL-8, and GAPDH with a sample stimulated by PMACI in order to identify the linear response of PCR. The PCR products increased as the concentration of RNA increased. The amplified fragment sizes were 355 bp for TNF-α, 545 bp for IL-1β, 443 bp for IL-6, 200 bp for IL-8, and 446 bp for GAPDH. Finally, the products were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide.

Western blotting

Cells (5×10^6 cells/well) were scraped, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min for 1 h at 4°C and centrifuged at $15{,}000 \times g$ for 5 min at 4°C. Supernatants were assayed. Samples were heated at 95°C for 5 min and briefly cooled on ice. Following centrifugation at $15{,}000 \times g$ for 5 min, 50-μl aliquots were resolved by 12% SDS–PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 0.2 M glycine, and 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 × TBST containing 10% nonfat dry milk and then incubated with respective antibodies (1:500 dilution in blocking solution). After washing in PBS-Tween-20 three times, the blots were incubated with secondary antibodies (1:1000 dilution in blocking solution) for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp., Newark, NJ, USA).

Transient transfection

For the transient transfection, we seeded the HMC-1 cells (1×10^7 cells) in a 100-mm culture dish. We then used Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) to transiently transfect pNF-κB-luciferase (LUC) and pSV40-LUC reporter gene constructs into HMC-1 cells. In brief, we then incubated the cells for 48 h at 37°C in a 5% CO_2 incubator, and the transfected HMC-1 cells were plated and stimulated with PMACI. Theanine was added 1 h before stimulation. Four hours after the stimulation, we harvested the cells and washed them in cold PBS before lysing them in a 500-μl lysis buffer (Dual Luciferase^{R} Reporter Assay System, Promega). After conducting vortex mixing and centrifugation at $12{,}000 \times g$ for 3 min at 4°C, we then stored the supernatant at -70°C until required for the luciferase assay.

Reporter gene assay

Next we mixed 20 μl of cell extract and 100 μl of the luciferase assay reagent at room temperature. To measure the luciferase activity, we used a luminometer (1420 luminescence counter, Perkin Elmer) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ration of firefly luciferase activity to renilla luciferase activity.

Caspase-1 assay

Caspase-1 activity was measured by a caspase colorimetric assay kit (R & D system). Cells were incubated with theanine for 24 h at 37°C. The cells were lysed on ice for 10 min using cell lysis buffer and centrifuged at 10,000 × *g* for 1 min. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amount of total protein was quantified by bicinchoninic acid protein quantification kit (Sigma) in each lysate. Recombinant caspase-1 enzymes are available for use as a positive control.

Statistical analysis

The experiments shown are a summary of the data from at least three experiments and are presented as the mean ± SEM. Statistical significance was compared among each treated group and controlled by a Student's independent *t* test and ANOVA with a Turkey's post hoc test. Results with $p < 0.05$ were considered statistically significant.

Results

Effect of theanine on compound 48/80-induced systemic anaphylactic reaction and ear swelling response

Initially, to examine the contribution of theanine in anaphylactic reactions, we used an in vivo model of systemic anaphylactic reaction. Compound 48/80 (8 mg/kg) was used as a systemic fatal anaphylaxis inducer. After injection with compound 48/80, the mice ($n = 8$/each group) were monitored for 30 min, and then the mortality rate was determined. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the theanine was orally administered at concentrations ranging from 0.01 to 1 mg/kg for 1 h, the mortality from compound 48/80 was dose-dependently reduced. Treatment with theanine (1 mg/kg) alone induced no physiological differences by appearance. DSCG, mast cell stabilizer, also inhibited mortality in a dose-dependent manner. We also tested the effect of dexamethasone (0.5 and 5 mg/kg) used as a positive control in this study. As a result, the anti-allergic effect of theanine 1 mg/kg was higher than that of dexamethasone at 0.5 and 5 mg/kg doses. A previous study showed that compound 48/80 significantly induced an ear swelling response. As shown in Table 2, when mice were pretreated with theanine for 1 h, the ear swelling response derived from compound 48/80 was significantly reduced in a dose-dependent manner ($p < 0.05$).

Table 1 Effect of theanine on compound 48/80-induced systemic anaphylactic reaction in mice

Dose (mg/kg)	Compound 48/80 (8 mg/kg)	Mortality (%)
Saline	+	100.00
DSCG 0.01	+	100.00
DSCG 0.1	+	50.00*
DSCG 1	+	0.00*
Dexa 0.5	+	25.00*
Dexa 5	+	20.00*
Theanine 0.01	+	50.00*
Theanine 0.1	+	33.33*
Theanine 1	+	0.00*
Theanine 1	−	0.00

The groups of mice were orally pretreated with 200 µl of saline, theanine, DSCG, or dexamethasone. Theanine was given at various doses 1 h before compound 48/80 was injected intra-peritoneally ($n = 8$/each group). Mortality (%) is expressed as 'Numbers of dead mice × 100/Total numbers of experimental mice'. Each experiment was carried out at least three times

Dexa dexamethasone

* $p < 0.05$ significantly different from the saline value

Table 2 Effect of theanine on compound 48/80-induced ear-swelling response in mice

Dose (mg/kg)	Thickness (mm)	Inhibition (%)
Saline	0.2674 ± 0.0077	−
Theanine 0.01	0.2072 ± 0.0026*	22.51
Theanine 0.1	0.1912 ± 0.0025*	28.49
Theanine 1	0.1490 ± 0.0018*	44.28

The mice were orally administered with the indicated concentrations of theanine for 1 h prior to compound 48/80 application (100 µg/site). Values are means ± SEM, $n = 3$ for each experiment. Each experiment was carried out at least three times

* $p < 0.05$ significantly different from the saline value

Effect of theanine on PCA

Another way to test anaphylactic reactions is to induce PCA. When theanine was orally administered to mice, the PCA reaction was significantly inhibited in a dose-dependent manner. Theanine pharmaceutical acupuncture also significantly inhibited the PCA reaction (Fig. 1).

Effect of theanine on histamine release

We investigated the inhibitory effects of theanine on compound 48/80-induced histamine release from RPMCs and HMC-1 cells (Table 3). Theanine significantly

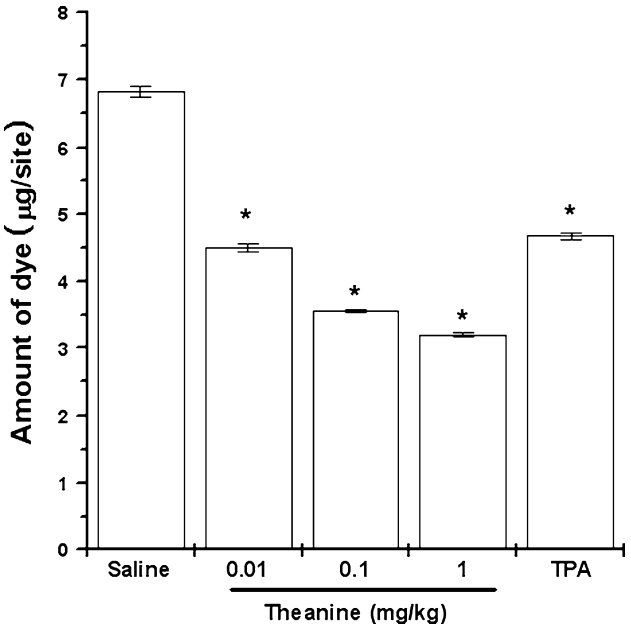

Fig. 1 Effect of theanine on PCA. Theanine (0.01, 0.1, and 1 mg/kg) was administered orally 1 h prior to the challenge with antigen (DNP-HSA).Theanine pharmaceutical acupuncture (1 mg/kg) was also treated prior to the challenge with antigen. Values are means ± SEM of three independent experiments ($n = 9$/each group). *$p < 0.05$ significantly different from the saline value. *TPA* theanine pharmaceutical acupuncture (1 mg/kg)

inhibited compound 48/80-induced histamine release at 1 μM ($p < 0.05$).

Effect of theanine on PMACI-induced inflammatory cytokine secretion and mRNA expression in HMC-1 Cells

To assess the effects of theanine on PMACI-induced TNF-α, IL-1β, IL-6, and IL-8 secretion, HMC-1 cells were pretreated with various concentrations of theanine for 30 min prior to stimulation. The pretreatment of cells with theanine resulted in the inhibition of TNF-α, IL-1β, IL-6, and IL-8 secretion in a dose-dependent manner. The maximal inhibition rates of TNF-α, IL-1β, IL-6, and IL-8 production by theanine (1 μM) were about 54.18, 64.02, 81.36, and 12.72%, respectively (Fig. 2a). Cell cytotoxicity by theanine was not observed up to 1 μM (data not shown). Using the pretreated HMC-1 cells described above, we also performed a RT–PCR analysis for TNF-α, IL-1β, IL-6, and IL-8 to determine whether theanine modulated PMACI-induced cytokine expression. TNF-α, IL-1β, IL-6, and IL-8 mRNA expression was up-regulated by PMACI stimulation, but the up-regulated cytokine mRNA expression was decreased with theanine treatment (Fig. 2b).

Effect of theanine on PMACI-induced NF-κB activation and IκB degradation

The expression of inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 was regulated by the transcription factor, NF-κB. In PMACI-stimulated cells, the expression level of NF-κB in the nucleus was increased. However, as shown in Fig. 3a, the expression level of NF-κB (p65) in the nucleus was decreased with theanine treatment. To examine whether the inhibitory action of theanine was due to its effects on IκB degradation, we investigated the cytoplasmic levels of IκB protein at various concentrations with a Western blot analysis. The expression level of IκB in the cytoplasm was increased with theanine treatment (1 μM). Next, we examined whether theanine could modulate the luciferase expression specifically via NF-κB activation. We transiently transfected pNF-κB-LUC and pSV40-LUC into HMC-1 cells and pretreated with theanine for 1 h before stimulation with PMACI. As shown in Fig. 3b, the PMACI stimulation increased reporter gene activity. This increased activity was significantly decreased with theanine treatment.

Table 3 Effect of theanine on histamine release from RPMCs and HMC-1 cells

Dose (μM)	RPMCs		HMC-1 cells	
	Amount of histamine release (ng/ml)	Inhibition (%)	Amount of histamine release (ng/ml)	Inhibition (%)
Com 48/80				
Saline	337.40 ± 4.21	100.00	59.43 ± 0.41	100.00
Theanine 0.01	335.13 ± 2.75	0.67	54.40 ± 0.80	0.85
Theanine 0.1	314.85 ± 2.44	6.68	49.00 ± 0.15	17.55
Theanine 1	263.12 ± 16.47*	22.02	47.33 ± 0.63*	20.41

Theanine was given at various doses before compound 48/80. The histamine content was measured by the *o*-phthalaldehyde spectrofluorometric procedure, as described. The fluorescent intensity was measured at 440 nm. Values are means ± SEM of three independent experiments

Com 48/80 compound 48/80

* $p < 0.05$ significantly different from the saline value

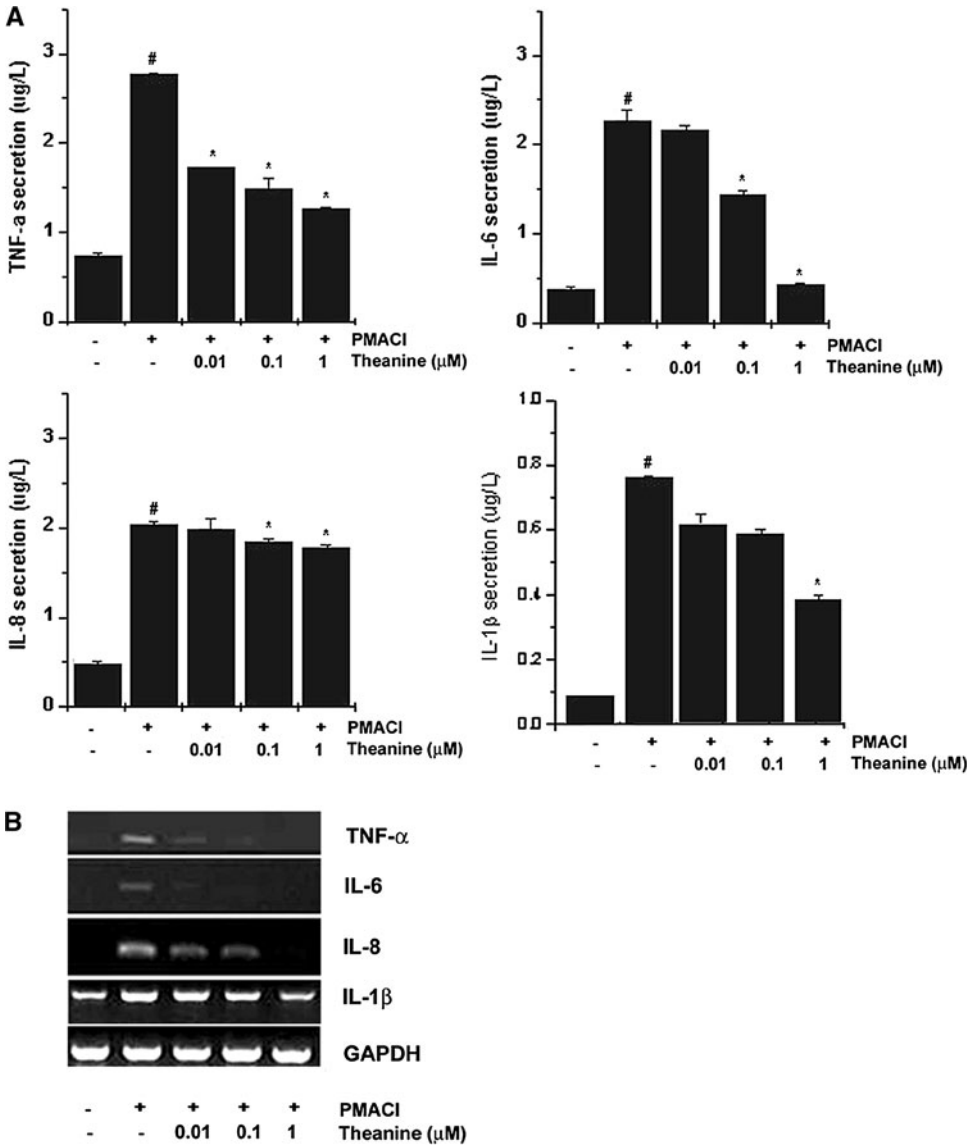

Fig. 2 Effect of theanine on PMACI-induced TNF-α, IL-6, IL-8 and IL-1β secretion (**a**) and mRNA expression (**b**) by HMC-1 cells. HMC-1 cells (3×10^5 cells/well) were grown in IMDM with 10% FBS. Cells were pretreated with various concentrations (0.01, 0.1, and 1 μM) of theanine for 30 min prior to PMACI stimulation. Cytokine levels in supernatant were measured using ELISA method as described. The mRNA was measured with RT–PCR. Values are means ± SEM of three independent experiments. $^{\#}p < 0.05$ significantly different from unstimulated cells. $^{*}p < 0.05$ significantly different from PMACI-stimulated cells

Effect of theanine on RIP-2/Caspase-1 activation in HMC-1

RIP-2 activation induces caspase-1 oligomerization and promotes its activation. Then, caspase-1 induces pro-inflammatory cytokine stimulation. We performed a Western blot analysis to determine the effect of theanine on RIP-2 activation induced by PMACI. The results indicated that theanine (1 μM) suppressed the RIP-2 activation induced by PMACI (Fig. 3c). Next, we investigate the effect of theanine on caspase-1 activation induced by PMACI. Western blot analysis for caspase-1 was performed. Caspase-1 activation was significantly decreased with theanine treatment (Fig. 3c). We also measured the effect of theanine on caspase-1 activation using a caspase-1 assay kit. Once again, this showed that caspase-1 activity was significantly decreased with theanine treatment (Fig. 3d).

Discussion

Recently, increasing occurrences of allergic disorders have been attributed to exposure to environmental factors such as environmental smoke, diesel exhaust, and air pollution that contribute to the pathogenesis of allergy (Baye et al. 2010). In particular, immediate-type hypersensitivity reactions are mediated by various chemicals released from mast cells, which may play a crucial role in many of the physiological changes that occur during immediate allergic responses (Miescher and Vogel 2002). Until now, useful treatments for allergic disease have included

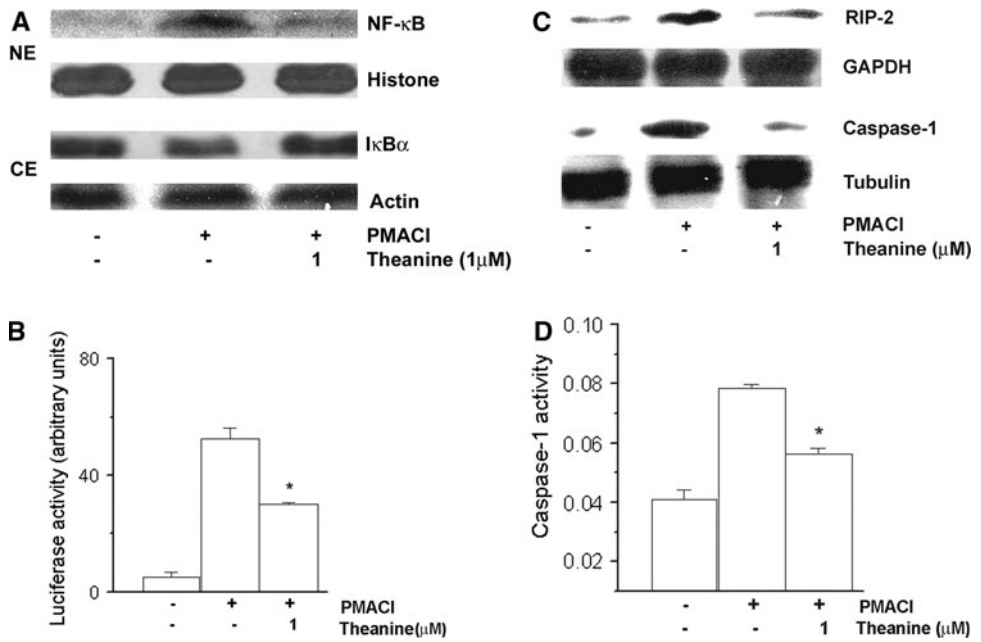

Fig. 3 Inhibitory effect of theanine on NF-κB and caspase-1 activation. HMC-1 cells were treated with 1 μM theanine for 30 min and then stimulated with PMACI for 1 h. NF-κB and IκBα expression was analyzed by western blotting as described in the experimental procedures (**a**). The NF-κB activity was assayed with a luciferase assay (**b**). RIP-2 and caspase-1 expression was analyzed with a Western blotting as described in the experimental procedures (**c**). The enzymatic activity of caspaser-1 was tested with a caspase colorimertic assay (**d**). Values are means ± SEM of three independent experiments. $^{\#}p < 0.05$ significantly different from unstimulated cells. $^{*}p < 0.05$ significantly different from the PMACI-stimulated cells. *NE* nuclear extract, *CE* cytosol extract

antihistamines, which block the activity of the histamine H_1 receptor, leukotriene antagonists, which block the activity of leukotriene mediators, and cromoglicate-based drugs, which block the calcium channel essential for mast cell degranulation (Prussin and Metcalfe 2003). Steroidal anti-inflammatory drugs such as dexamethasone are effective in the treatment of allergic and inflammatory diseases (Okano 2009). Allergen-specific immunotherapy has been also used for 100 years as a desensitizing therapy for allergic diseases and represents the potentially curative and specific method of treatment (Akdis and Akdis 2011). In this study, mice were treated with theanine, which inhibited compound 48/80 or IgE-mediated anaphylactic reaction. Compound 48/80 has been reported to perturb the lipid bilayer membrane and increase its permeability. One report indicated that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells (Tasaka et al. 1986). Hence, it can be hypothesized that theanine might act on the lipid bilayer membrane, preventing perturbation induced by compound 48/80. DSCG prevents the release of type I allergic reaction mediators, acting as a mast cell stabilizer. The drug does not inhibit the binding of IgE to mast cells or the interaction between cell-bound IgE and specific antigens. Instead, DSCG suppresses the release of substances (e.g., histamine, SRS-A) (Cook et al. 2000). Our results suggest that theanine might play a role in stabilizing mast cells.

Activated mast cells release proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8. Elevated levels of circulating TNF-α, IL-1β, IL-4, IL-6, and IL-8 have been reported to cause various pathological states, including sepsis, rheumatoid arthritis, osteoarthritis, asthma, and cerebral infarction (Lin et al. 1994; Hammami et al. 1997; Hougee et al. 2005). Our results showed that theanine treatment inhibited TNF-α, IL-1β, IL-6, and IL-8 release in PMACI-stimulated HMC-1 cells in a dose-dependent manner. Zhang et al. (1992) reported that TNF-α, IL-1β, IL-6, and IL-8 induce allergic inflammation via mast cell activation. Expression of TNF-α, IL-1β, IL-6, and IL-8 genes is dependent on the activation of transcription factor NF-κB/*Rel A* (Jeong et al. 2010). Because suppression of NF-κB activation has been linked with anti-inflammation, we postulated that theanine mediates its effects at least partly through suppression of NF-κB activation. A recent study reported that theanine inhibited neuronal cell death via an inhibition of β-amyloid induced NF-κB activation pathways (Kim et al. 2009). In this study, we also observed that theanine suppresses NF-κB activation induced by PMACI. This suppression is also mediated through inhibition of IκB-α degradation. Therefore, our results indicate that theanine has an anti-inflammatory effect through inhibiting of NF-κB activation.

In particular, Pro-IL-1β can be cleaved by cathepsin G, chymotrypin, elastase, a mast cell chymase, different

matrix metalloproteinsases, and granzyme A, which can be found at sites of neutrophil, lymphocytes, and macrophage infiltration to yield active IL-1β (Irmler et al. 1995). IL-1β is matures in a large caspase1-containing protein complex called the inflammasome (Saleh and Green 2007). Caspase 1 contains an N-terminal caspase recruitment domain shown to be involved in the assembly of protein platforms that promote proteolytic activation of recruited caspases in the context of inflammation and involved in NF-κB signal pathway (Lamkanfi et al. 2004). It was reported that caspase-1–/– mice have a decreased production of IL-6 after stimulation with LPS (Kuida et al. 1995). RIP-2 knockout also reduced the activation of NF-κB (Chin et al. 2002). These studies suggested that therapeutic treatments for inflammatory disease should focus on preventing the activation of RIP2/caspase-1. We postulated that theanine works at least partly by suppressing of RIP2/caspase-1 activation, and we observed that theanine suppressed RIP-2 and caspase-1 activation induced by PMACI. This result suggested that the inhibitory effect of theanine on inflammatory cytokine production might be the result of its regulation of RIP-2 and caspase-1 activation.

In clinical study, Kamath et al. (2003) reported that human Vgamma 2Vdelta 2 T cells were primed to mediate memory and nonmemory antibacterial cytokine responses when they drank 600 ml of brewed black tea containing 2.2 mM L-theanine for 4 weeks. Cystine and theanine supplements (5.8 mM cystine + 1.6 mM theanine daily for 7 days) significantly attenuated the increase in neutrophil count induced by intense endurance exercise in human study (Murakami et al. 2010). Liu et al. (2009) reported that theanine (at the concentrations of 0–125 μM) dose-dependently inhibited A549 cell migration and tumor invasion in vitro. In our study, theanine 1 μM has a potent anti-inflammatory effect. We also found that anti-allergic effect of theanine 1 mg/kg was higher than that of dexamethasone at 5 mg/kg dose. Therefore, we can speculate that the activity found is at a dose that has meaning for actual clinical use.

In conclusion, we showed that theanine inhibited mast cell-mediated anaphylactic reactions. Theanine also inhibited TNF-α, IL-1β, IL-6, and IL-8 secretions and expression by blocking the activation of NF-κB and RIP-2/caspase-1. Our findings suggest that theanine has regulatory effects that may play a beneficial role in the treatment and management of allergic disorders.

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