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Cinnamaldehyde derivatives inhibit degranulation and inflammatory mediator production in rat basophilic leukemia cells

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ABSTRACT

Mast cells play a critical role in allergic diseases. Therefore, development of new therapeutic agents that suppresses the activation of mast cells may help prevent or treat allergic diseases. Here, we investigated the anti-allergic effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in RBL-2H3 cells. β-Hexosaminidase assays revealed that degranulation of RBL-2H3 cells was decreased following treatment with 60 μM 4-chloro-cinnamaldehyde or 4-trifluoro-cinnamaldehyde. Moreover, quantitative real-time reverse transcription polymerase chain reaction showed that the relative expression levels of tumor necrosis factor-α, interleukin-4, and cyclooxygenase-2 mRNAs were decreased in RBL-2H3 cells treated with 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde in a concentration-dependent manner. Additionally, 4-chloro-cinnamaldehyde blocked the phosphorylation of MKKs and MAPKs. These data clearly suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde had inhibitory effects on the inflammatory responses of mast cells and may have potential as novel therapeutic agents for the prevention or treatment of allergic diseases.

1. Introduction

The prevalence of allergic diseases, such as atopic dermatitis and asthma, has become an important health problem worldwide, causing poor quality of life in children and adults [1–4]. Therefore, it is important to identify new therapeutic agents with application in the prevention and treatment of allergic diseases.

Mast cells play a critical role in allergic and other inflammatory responses. Immunoglobulin E (IgE) is an activator of mast cells [5]. The aggregation of IgE receptors (FcεRI) on mast cells triggers complicated biological reactions, such as enhancement of calcium influx, rearrangement of the cytoskeleton, degranulation of prestored granules, and activation of enzymes such as protein kinase C (PKC), cyclooxygenase-2 (COX-2), 5-lipooxygenase (5-LO), and mitogen-activated protein kinases (MAPKs) [6–9]. After the activation of intracellular signaling pathways, mast cells release various inflammatory mediators, such as prestored histamine and proteases, de novo-synthesized cytokines, chemokines, and eicosanoids, such as prostaglandins and leukotrienes [10,11].

Rat basophilic leukemia (RBL)-2H3 cells are the tumor analog of rodent mucosal mast cells that secrete various allergic inflammatory mediators. Therefore, RBL-2H3 cells are suitable for the study of allergic responses [12–18]. Phorbol myristate acetate (PMA), a PKC activator, and A23187, a calcium ionophore that functions to increase Ca^{2+} influx, induce the activation of mast cells [7,19,20]. Additionally, the activation of PKC and increased Ca^{2+} influx, stimulated by PMA and A23187, respectively, are critical factors for allergic responses of mast cells [7,19,21,22]. Thus, PMA and A23187 stimulate mast cells to release inflammatory mediators related to allergic responses [12,13,23].

Cinnamon extract has been reported to have anti-oxidative, antineoplastic, antidiabetic, anti-inflammatory, and anti-allergic effects both in vitro and in vivo [16,24–26]. Cinnamon extracts contain various compounds, including cinnamaldehyde, cinnamic acid, and cinnamate [24,27]. Cinnamaldehyde has been reported to inhibit antigen-induced extracellular calcium influx and degranulation in mouse bone marrow-derived mast cells (mBMMCs), human intestinal mast cells (hiMCs) and RBL-2H3 cells through suppression of phospholipase Cγ1 [17] and extracellular signal-regulated kinase (ERK) phosphorylation [18], whereas cinnamic acid does not have any inhibitory effects on the degranulation of mast cells [17]. Furthermore, while cinnamaldehyde inhibits allergic responses in mast cells, the inhibitory effects of cinnamaldehyde derivatives on production of allergic inflammatory mediators of mast cells have not been investigated.

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In this study, we report the possible anti-allergic activity of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde by analyzing the mechanisms through which these derivatives affect the release of inflammatory mediators in RBL-2H3 cells stimulated with PMA and A23187.

2. Materials and methods

2.1. Materials

Cinnamldehyde derivatives were synthesized and purified as described in [28–30]. Cinnamaldehyde and cinnamaldehyde derivatives were dissolved in dimethyl sulfoxide (DMSO) and diluted in Siraganian buffer (25 mM PIPES, 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 5.6 mM glucose, pH 7.2). The final concentration of DMSO was 0.5%. Phorbol 12-myristate 13-acetate (PMA), A23187 (a calcium ionophore) and U0126 were purchased from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies for total and phosphorylated *c*-Jun. *N*-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), MKK4, MKK3, and MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). *Anti*-β-actin antibodies were purchased from Abcam (Cambridge, UK).

2.2. Cell cultures

RBL-2H3 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin (Hyclone) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Degranulation and cell viability assay

To confirm the effects of cinnamaldehyde and cinnamaldehyde derivatives on degranulation, β-hexosaminidase activity was measured. Cell viability of RBL-2H3 cells treated with cinnamaldehyde and cinnamaldehyde derivatives was determined using MTT assays. The cells were grown in 24-well plates (2×10^5 cells/mL) for 24 h. After treatment with the indicated chemicals for 1 h, the cells were stimulated with PMA (50 nM) and A23187 (1 μM) for 30 min. After stimulation, supernatants were used for β-hexosaminidase assays, and cells were used for MTT assays. Supernatants were incubated with substrate buffer (2 mM 4-*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M sodium citrate buffer, pH 4.5) for 30 min at 37 °C. The enzyme reaction was terminated by stop buffer (0.1 M $NaHCO_3$, pH 10), and the absorbance at 405 nm was measured using an Epoch spectrometer (Biotek, Santa Barbara, CA, USA). After stimulation, the cell medium was replaced with 500 μL MTT solution (5 mg/mL) and incubated for 4 h. The MTT solution was then discarded, and the insoluble formazan products were dissolved in 200 μL DMSO. Absorbance was measured at 550 nm using an Epoch spectrometer (Biotek).

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using X-zol RNA reagent (PhileKorea, Seoul, Republic of Korea), and the RNA concentration was determined with an Epoch spectrometer (Biotek). To determine the relative gene expression levels of tumor necrosis factor-α (*TNF-α*), interleukin-4 (*IL-4*), and cyclooxygenase-2 (*COX-2*) mRNAs, 500 ng of total RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The relative gene expression was determined with THUNDERBIRD SYBR qPCR Mix (TOYOBO), and the house-keeping gene large ribosomal protein (*RPLPO*) was used for normalization. The primer pairs were synthesized by Macrogen (Seoul, Republic of Korea) as follows: *TNF-α* (sense, 5′-TGAACTTCGGGGTGATCG-3′ and antisense, 5′-GGGCTTGTCACTCGAGTTTT-3′), *IL-4* (sense, 5′-TACGGCAACAAGGAACAC-3′ and antisense, 5′-TCTTCAAGCACGGAGGTA-3′), *COX-2* (sense, 5′-TGGTGCCGGGTCTGATGATG-3′ and antisense, 5′-GCAATGCGGTTCTGATACTG-3′), and *RPLPO* (sense, 5′-GTGTTTGACAATGGCAGCAT-3′ and antisense, 5′-ACAGACGCTGGCCACATT-3′). qRT-PCR was performed using a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

2.5. Immunoblotting

RBL-2H3 cells were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (quartett Immunodiagnostika + Biotechnologie Vertriebs Gmbh, Berlin, Germany). Equal amounts of protein (30 μg) in each sample were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to polyvinylidene difluoride membranes (Amersham Hybond-P; GE Healthcare, Buckinghamshire, UK). Blotted membranes were blocked with 1% skim milk in TBS containing 0.05% Tween 20 for 1 h. After three washes in TBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies at 4 °C for 16 h. After three washes in TBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibodies at room temperature for 1 h. After three washes in TBS containing 0.05% Tween 20, the chemiluminescent signals were detected using WesternBright Peroxide chemiluminescent detection reagent (Advansta, Menlo Park, CA, USA). For re-probing the membranes, the antibodies were stripped off by stripping buffer (10% SDS, 0.5 M Tris-Cl and 0.8% β-mercaptoethanol).

2.6. Statistical analysis

Each experiment was performed independently at least three times. Data were expressed as the mean ± standard deviation (SD). Control groups and sample groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS (v.21, for Windows). Differences with P values of <0.05 were considered statistically significant.

3. Results

3.1. 4-Chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited the degranulation of RBL-2H3 cells

To evaluate the effect of cinnamaldehyde derivatives on degranulation of RBL-2H3 cells, β-hexosaminidase assay was performed. RBL-2H3 cells were stimulated with A23187 and PMA. RBL-2H3 cells were treated with cinnamaldehyde and seven cinnamaldehyde derivatives at a concentration of 60 μM for 1 h and stimulated with PMA and A23187 (Fig. 2A). From the seven cinnamaldehyde derivatives, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde significantly reduced the release of β-hexosaminidase in RBL-2H3 cells (Fig. 2A). In addition, the viability of RBL-2H3 cells was not influenced by cinnamaldehyde derivatives when used at a concentration of 60 μM (Fig. 2B). These results suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited degranulation and had no cytotoxic effects on RBL-2H3 cells. We next examined the concentration-dependent effects of 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde on degranulation of RBL-2H3 cells (Fig. 3). At a concentration of 60 μM, 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde dramatically inhibited degranulation of RBL-2H3 cells compared to the stimulated control group. These results suggested that 4-chloro-cinnamaldehyde and 4-trifluoro-cinnamaldehyde inhibited degranulation of RBL-2H3 cells in a concentration-dependent manner.