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Galangin attenuates mast cell-mediated allergic inflammation

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ABSTRACT

A great number of people are suffering from allergic inflammatory disease such as asthma, atopic dermatitis, and sinusitis. Therefore discovery of drugs for the treatment of these diseases is an important subject in human health. In this study, we investigated anti-allergic inflammatory effect of galangin and underlying mechanisms of action using *in vitro* and *in vivo* models. Galangin inhibited histamine release by the reduction of intracellular calcium in phorbol 12-mystate 13-acetate plus calcium ionophore A23187-stimulated human mast cells (HMC-1). Galangin decreased expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-8. The inhibitory effect of galangin on theses pro-inflammatory cytokines was related with c-Jun N-terminal kinases, and p38 mitogen-activated protein kinase, nuclear factor-κB, and caspase-1. Furthermore, galangin attenuated IgE-mediated passive cutaneous anaphylaxis and the expression of histamine receptor 1 at the inflamed tissue. The inhibitory effects of galangin were more potent than cromolyn, a known anti-allergic drug. Our results showed that galangin down-regulates mast cell-derived allergic inflammatory reactions by blocking histamine release and expression of pro-inflammatory cytokines. In light of *in vitro* and *in vivo* anti-allergic inflammatory effects, galangin could be a beneficial anti-allergic inflammatory agent.

1. Introduction

Mast cells are effector cells in the initiation of inflammatory reactions associated with allergic disorders such as asthma, atopic dermatitis, and sinusitis. In vertebrates, mast cells are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment, including the skin, airways, conjuctiva and gastrointestinal tract (Grimbaldeston et al., 2006).

Upon exposure of an allergen, which is recognized by immunoglubulin (Ig)E antibodies bound to the high affinity IgE receptor (FcεRI) expressed on mast cell surface, aggregation of FcεRI triggers a intracellular signaling process (Rivera et al., 2008). Phosphorylation of Src family kinases (Lyn, Syk and Fyn) and phospholipase C-γ are followed by calcium mobilization and activation of protein kinase C, mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB (Galli et al., 2008). As a result, mast cells secret both preformed and newly synthesized mediators including histamine, eicosanoids, proteases, and several pro-inflammatory cytokines/chemokines. Using these products, mast cells contribute not only immediate-type hypersensitivity but also late reaction, like inflammatory responses.

Histamine released from activated mast cells is major mediator leading immediate-type hypersensitivity. Histamine causes vasodilation and increases permeability of vessels near the allergic site. By the release of chemotactic and pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-8, mast cells affect on the late phase responses of an allergic inflammation (Galli et al., 2008). These cytokines released from mast cells change terminal microenvironment and attract neutrophils and basophils. Therefore reduction of these pro-inflammatory cytokines is one of key indicators of relived allergic inflammatory symptoms.

MAPKs are mediators that respond to extracellular stimuli and regulate diverse cellular activities. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 are major subfamily of MAPKs. In mast cells, MAPKs play a crucial role in the regulation of pro-inflammatory cytokine (Scheller et al., 2011; Turner et al., 2012). NF-κB is a transcriptional factor which regulates expression of pro-inflammatory cytokines (Azzolina et al., 2003). For this reason, NF-κB is an obvious target of anti-inflammatory treatment (Kim et al., 2006). Caspase-1 is also involved in regulation of inflammatory cytokines. Caspase-1, a member of cysteine protease also known as IL-1β converting enzyme, is found predominantly in the cytoplasm of cells, where it proteolytically convert pro-IL-1β into mature form, that is involved in inflammation (Lamkanfi et al., 2011).

Abbreviations: HMC, human mast cell; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; PMA, phorbol 12-mystate 13-acetate; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase.

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Galangin (3,5,7-trihydroxyflavone) is a natural flavonol, which is constituent of propolis, a resinous mixture that made by honey bee from the juice of plants, vegetable and fruits (Schnitzler et al., 2010). Galangin has been demonstrated to possess anti-cancer effect by evaluating apoptotic activity or decreasing Bcl-2 and cyclin D3 (Bestwick and Milne, 2006; Murray et al., 2006; Tolomeo et al., 2008). Galangin has anti-oxidative activity by reducing NADH oxidation and as a scavenger of free radical (Russo et al., 2002). Some studies reported that galangin has anti-inflammatory effect caused by phospholipase A2 inhibition (Lattig et al., 2007) and decrease of adhesion molecule expression. Galangin also has anti-viral (Schnitzler et al.), anti-microbial (Cushnie et al., 2007), and vasorelexant activity (Morello et al., 2006). However, in spite of the beneficial biological effects, effect of galangin on the allergic inflammation was not elucidated. The aim of this study is to evaluate the effect of galangin on the mast cell-mediated allergic inflammation and determine the involving mechanism of these effects.

2. Materials and methods

2.1. Reagents and cell culture

Anti-DNP IgE, DNP-human serum albumin (HSA), phorbol 12-mystate 13-acetate (PMA), calcium ionophore A23187, *o*-phthaldialdehyde, galangin, and cromolyn were purchased from Sigma (St. Louis, MO, USA). Human mast cells (HMC)-1 were grown in IMDM (GIBCO, Grand Island, NY, USA) supplemented with 100 units/mL penicillin/streptomycin, and heat-inactivated 10% fetal bovine serum (GIBCO) in 5% CO_2 at 37 °C.

2.2. Animals

The male ICR mice (6 weeks) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The animals were housed in a laminar air flow room maintained under a temperature of 22 ± 2 °C and relative humidity of 55 ± 5% throughout the study. The care and treatment of the animal were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.3. Cell viability

Cell viability was assayed using XTT assay kit (WelGENE, Seoul, Korea). HMC-1 cells (2×10^4 cells/well in 96-well plates) were treated with galangin for 24 h followed by incubation with XTT plus phenazine methosulfate reagent for 4 h. Absorbance was read at 450 nm using an Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as relative absorbance compared with the control and expressed as percentage of the control.

2.4. Histamine contents

Histamine contents in cultured cell supernatants were measured by the *o*-phthaldialdehyde spectrofluorometric procedure as previously described (Singh et al., 2012). HMC-1 cells were preincubated with galangin at 37 °C for 30 min, and then stimulated with PMA plus A23187 for 8 h. The fluorescent intensity was measured at emission 438 nm and excitation 353 nm using spectrofluorometer.

2.5. Intracellular calcium

To determine intracellular calcium, Fluo-3/AM (Molecular Probes. Eugene, OR, USA) was used according to the manufacturer's protocol, a brief description of which follows. HMC-1 cells were preincubated with Fluo-3/AM for 30 min at 37 °C. After washing the dye from the cell surface, galangin (10 μg/mL) was pretreated 30 min prior to the PMA plus A23187 stimulation. The fluorescent intensity was measured using fluorometer (Molecular Devices, Sunnyvale, CA, USA) and visualized in a fluorescence microscope (Olympus BX51, Center Valley, PA, USA).

2.6. RNA extraction and PCR

The total cellular RNA was isolated from the cells (1×10^6/well in 24-well plate) after stimulation of PMA (40 nM) plus A23187 (1 μM) with or without galangin using a RNAiso Plus (Takarabio, Shiga, Japan) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using Maxmine RT Premix (iNtRON Biotech, Sungnam, Korea). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-α, IL-6, IL-1β, IL-8, and β-actin (internal control). H1 receptor mRNA level was determined by real-time PCR. The primer sets for cytokines and H1 receptor are summarized in Supplementary Table 1. Total RNA was isolated from PCA-reacted spots of skin in mice. PCA-reacted spots were detached and frozen in liquid nitrogen. Frozen skins smashed using a homogenizer with RNAiso Plus. Total RNA was extracted and dissolved in DEPC-water. The conditions for the reverse transcription and PCR steps were similar to those previously described (Kim et al., 2006). The primer sets were chosen by the Primer3 program (Whithead Institute, Cambridge, MA, USA). The cycle number was optimized in order to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a molecular imager gel doc XR system (Bio-Rad, Hercules, CA, USA).

After the PCA reaction, PCA-reacted ears were detached and total cellular RNA was isolated from the tissue. The expression level of H1R and β-actin were determined by quantitative real-time PCR. Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc.) according to the manufacturer's protocol. Briefly, 2 μL of cDNA (100 ng), 1 μL of sense and antisense primer solution (0.4 μM), 12.5 μL of SYBR Premix Ex Taq (Takarabio Inc.), and 9.5 μL of dH_2O were mixed together to obtain a final 25 μL reaction mixture in each reaction tube. The amplification conditions were 10 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, 15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C. Relative quantification of mRNA expression was performed using the TP850 software.

2.7. Nuclear protein extraction

After HMC-1 cell (3×10^6 in a 6-well plate) were activated for 2 h, cells were washed in 1 mL of ice-cold PBS, centrifuged at 1200*g* for 5 min, resuspended in 400 μL of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000*g* for 30 s. After several times of washing, pelleted nuclei were resuspended in 50 μL of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000*g* for 5 min at 4 °C, and supernatant gathered.

2.8. Western blotting

HMC-1 (3×10^6 in a 6-well plate) extracts were prepared by detergent lysis procedure (Lee et al., 2011). Samples of protein (50 μg) were electrophoresed using 8–12% SDS–PAGE, and then transferred to nitrocellulose membrane. The nucleus and cytosolic p65 NF-κB, IκBα, caspase-1, actin were assayed using anti-NF-κB (p65), anti-IκBα, anti-caspase-1 and actin antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA), respectively. The phosphorylation of JNK, p38 and ERK was determined using anti-phospho-JNK, anti-phospho-p38 and anti-phospho-ERK antibody (Cell Signaling, Beverly, MA, USA). Immunodetection was done using supersignal west pico chemiluminescent substrate (Thermo scientific, Waltham, MA, USA).

2.9. Caspase-1 activity assay

The enzymatic activity of caspase-1 was measured according to the manufacturer's specification using a caspase assay kit (R&D Systems Inc, Minneapolis, MN, USA). The cell lysate was centrifuged at 10,000*g* for 1 min. The protein supernatant was incubated with 50 μL reaction buffer and 5 μL substrates (WEHD-pNA) at 37 °C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Sigma).

2.10. Transient transfection and luciferase activity

HMC-1 cells were seeded at 1×10^6 cells in 12-well plate 4 h before the transient transfection. NF-κB luciferase reporter gene constructs (pNF-κB-LUC, plasmid containing NF-κB binding site; STANTAGEN, Grand Island, NY, USA) were transiently transfected into HMC-1 with FBS and antibiotics free IMDM containing 8 μL of lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 6 h of incubation, medium was replaced with IMDM containing 10% FBS and antibiotics. After transfected cells were incubated for 18 h, stimulated with PMA plus A23187 at 37 °C for 2 h. Galangin was pretreated to cells for 30 min prior to stimulation. Cells were harvested and washed in ice-cold PBS before lysis in 100 μL of cell lysis buffer (Luciferase assay kit; Promega), according to the manufacturer's protocol. Luciferase activity was determined using the luminometer.

2.11. Passive cutaneous anaphylaxis (PCA)

An IgE-dependent cutaneous reaction was carried out as described previously (Bae et al., 2011). The mice were injected intradermally into the ear with 0.5 μg of anti-DNP IgE. After 48 h, each mice were received an injection of 1 μg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Galangin and cromolyn were intraperitoneally administered at doses 1 and 10 mg/kg BW 1 h before the challenge. Thirty minutes after the challenge, the mice were killed and the ears were