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Molecular mechanisms of anti-inflammatory effect of chrysophanol, an active component of AST2017-01 on atopic dermatitis in vitro models

Hyun-Ja Jeong[a], Hee-Yun Kim[b], Hyung-Min Kim[b,*]

[a] Department of Food Science & Technology, Hoseo University, 20, Hoseo-ro 79beon-gil, Baebang-eup, Asan, Chungcheongnam-do 31499, Republic of Korea
[b] Department of Pharmacology, College of Korean Medicine, Kyung Hee University, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea

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ABSTRACT

AST2017-01 mainly consists of *Rumex crispus* and -*Cordyceps militaris* and has been widely consumed as an herbal medicine or functional food in Korea. Here we investigated the influences of AST2017-01 and its active component, chrysophanol on human mast cell (HMC-1 cell) and human keratinocyte (HaCaT cell)-mediated inflammatory reactions. Pretreatment with AST2017-01 or chrysophanol suppressed intracellular calcium levels and histamine release in phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-treated HMC-1 cells. Levels of phosphorylated-mitogen-activated protein kinase increased by PMACI stimulation were reduced by AST2017-01 or chrysophanol pretreatment. Protein levels of IκB kinaseβ and receptor-interacting protein 2 in PMACI-treated HMC-1 cells were decreased by AST2017-01 or chrysophanol pretreatment. Pretreatment with AST2017-01 or chrysophanol significantly blocked PMACI-induced activation of caspase-1 and nuclear factor-κB. In addition, pretreatment with AST2017-01 or chrysophanol significantly decreased the PMACI-induced levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and thymic stromal lymphopoietin (TSLP) on HMC-1 cells. In activated HaCaT cells, pretreatment with AST2017-01 or chrysophanol significantly reduced production of TSLP and activation of caspase-1. In conclusion, these findings indicate that chrysophanol is an active component of AST2017-01 and AST2017-01 acts as a novel potent anti-inflammatory herbal medicine or functional food.

1. Introduction

Allergic inflammatory disorders, including atopic dermatitis (AD), allergic rhinitis, anaphylaxis, and allergic asthma are caused by type 2 immune responses and characterized by the presence of IgE and Th2 cells [1]. Th2 cell-derived interleukin (IL)-4 is thought to promote IgE production by B cells in response to a particular allergen, and the allergen-specific IgE antibodies bind to the high-affinity Fcε receptor 1 (FcεR1) on mast cells [2]. Mast cell activation by IgE interaction with FcεR1 triggers the release of inflammatory mediators with diverse biological effects to control allergic inflammation through degranulation and accelerates the development of AD [2–4]. In addition, keratinocytes play a key pathogenic role in development of AD via abnormal releasing cytokines and chemokines as well as thymic stromal lymphopoietin (TSLP) [5]. In our previous study, we have reported that TSLP is a proliferative factor for mast cell [3].

Mast cell stimulation by extracellular stress triggers the increase of intracellular calcium, which is proposed as a second messenger for mast cell activation [6]. Increased intracellular calcium is required for activation of IκB kinase (IKK), receptor-interacting protein 2 (RIP2), mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and caspase-1 [7]. MAPKs play a significant role in cytokine expression and are activated to control allergic inflammatory reactions [8]. In particular, NF-κB and caspase-1 are candidates for transcription and production of inflammatory cytokines including IL-1β, IL-6, tumor necrosis factor (TNF)-α, and TSLP [9,10]. Overexpression of these mediators in AD-skin lesion has been reported to correspond with disease severity [11]. For this reason, signaling pathway of inflammatory mediator production is an obvious target for emerging anti-allergic inflammatory therapies.

In Korean medicine, various processing methods (Beopje) have been applied to herbal medicines in order to improve their therapeutic effects and safety in clinical trials. AST2017-01 mainly consists of processed-*Rumex crispus* and processed-*Cordyceps militaris* and has been widely consumed as an herbal medicine or functional food in Korea. *Cordyceps militaris* shows anti-inflammatory and anti-cancer effects [12,13]. *Rumex crispus* is widely used to treat inflammation, edema, jaundice, stroke, disinfestation, and diarrhea [14–16]. However, anti-inflammatory properties of AST2017-01 and its specific chemical compound have not yet been demonstrated. In this study, we tested

* Corresponding author.
E-mail address: hmkim@khu.ac.kr (H.-M. Kim).

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anti-inflammatory effect of AST2017-01 and its major compound, chrysophanol on human mast cell and human keratinocytes.

2. Materials and methods

2.1. Reagents

Chrysophanol (purity: ≥ 98%), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fura-2/AM, poly(I:C), phorbol 12-myristate 13-acetate (PMA), A23187, and bicinchoninic acid were obtained from Sigma Chemical Co (St. Louis, MO, USA). TSLP, IL-1β, IL-6, and TNF-α antibodies were from R&D Systems, Inc. (Minneapolis, MN, USA). Phosphorylated (p)-c-Jun N-terminal kinases (JNK, SC-6254), JNK (SC-571), p-p38 (SC-7973), p38 (SC-7149), p-extracellular signal-regulated kinases (p-ERK, SC-7383), ERK (SC-94), p-IκBα (SC-8404), NF-κB (SC-8008), Poly-ADP-ribose polymerase (SC-8007), RIP2 (SC-22763), IKKβ (SC-7607), and GAPDH (SC-32233) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Fetal bovine serum (FBS), Dulbeco's Modified Eagle Medium (DMEM), and Isocove's modified Dulbecco's medium (IMDM) were purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Preparation of AST2017-01

AST2017-01 was provided by Gahwa Well Food Co. (Chungbuk, Republic of Korea). AST2017-01 was composed of processed-*Rumex crispus* and processed-*Cordyceps militaris* in a ratio of 6:4. Processed-*Rumex crispus* and processed-*Cordyceps militaris* were prepared in order of washing, steam, dehydration, parch, and then dehydration. AST2017-01 was boiled with distilled water at 80 °C for 3 h. The AST2017-01 extracts were filtered and concentrated in vacuo at 60°C. And then these were lyophilized. The extract yields of herbs were about 15–20% (w/w). The powders were dissolved in distilled water and filtered using a 0.22 μm syringe filter and kept at 4 °C. The concentration of chrysophanol in the AST2017-01 was approximately 0.13% [17]. Chrysophanol was dissolved in DMSO and diluted in distilled water (DMSO final concentration ≤ 0.1%). DMSO (0.1%) did not affect levels of inflammatory mediators as compared with PMA and A23187 (PMACI) stimulation [10].

2.3. Human mast cell line (HMC-1 cell) and human keratinocyte cell line (HaCaT cell) culture

HMC-1 cells were incubated in IMDM supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS at 37 °C in 5% CO_2 atmosphere at 95% relative humidity. HaCaT cells were cultured in DMEM supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS at 37 °C in 5% CO_2 with 95% humidity.

2.4. Fluorescent measurements of intracellular calcium levels

To evaluate the intracellular calcium level, HMC-1 cells suspensions were pretreated with Fura-2/AM for 30 min and then were harvested. After washing twice with medium containing extracellular calcium chelator EGTA (0.5 mM), the cells (1×10^5) were placed into a 96-well plate and pretreated with AST2017-01 or chrysophanol for 1 h. HMC-1 cells were stimulated with PMACI for 100 s. Phosphate-buffered saline (PBS) was treated as a negative control. The intracellular calcium levels were determined at 440 nm (excitation 360 nm) in a spectrofluorometer.

2.5. Histamine assay

Histamine levels were measured using a histamine assay kit (Oxford Biomedical Research, Oxford, MI, USA).

2.6. Western blot analysis

Stimulated cells were lysed and proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes, which were then blocked and incubated with primary (1:500 dilution) and secondary (1:3000 dilution) antibodies. Finally, protein bands were visualized using an enhanced chemiluminescence assay (Amersham Co. Newark, NJ, USA).

2.7. Caspase-1 assay

The enzymatic activities of caspase-1 were assayed using a colorimetric assay kit (R&D Systems).

2.8. MTT assay

Cell viability was measured using a MTT assay. Briefly, 500 μl of cell (3×10^5) were pretreated with AST2017-01 or chrysophanol for 1 h and then stimulated with PMACI for 8 h. MTT solution (5 mg/ml) then added and incubated at 37 °C for 4 h. After removing the supernatant by washing, the insoluble formazan product so obtained was dissolved in DMSO. Optical densities were measured using an ELISA reader at 590 nm.

2.9. Cytokines assay

Levels of TSLP, IL-1β, IL-6, and TNF-α were determined using a sandwich ELISA method according to the manufacturer's instructions (R&D Systems).

2.10. RNA isolation and quantitative real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from HMC-1 cells using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea). Concentrations of total RNA were determined by NanoDrop spectrophotometry (Thermo Scientific, Worcester, MA, USA). Total RNA (2.5 μg) was heated at 75 °C for 5 min and then chilled on ice. Samples were reverse-transcribed to cDNA for 60 min at 42 °C using a cDNA synthesis kit (iNtRON Biotech, Sungnam, Korea). Quantitative real-Time PCR was performed using a SYBR Green master mix with primers (Table 1) and mRNA was analyzed using an ABI StepOne real-time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels of cytokine were normalized versus GAPDH. All data were analyzed using the ΔΔCT method.

2.11. Statistics

All results are representative of three independent experiments conducted in duplicate and are expressed as the mean ± standard errors of means (SEMs). The analysis was performed using an

Table 1
The sequence of primers used for real-time PCR analysis.

Genes		Sequences
TSLP	Sense	TATGAGTGGGACCAAAAGTACCG
	Antisense	GGGATTGAAGGTTAGGCTCTGG
IL-1β	Sense	AAACAGATGAAGTGCTCCTT
	Antisense	TGGAGAACACCACTTGTTGC
IL-6	Sense	AAATTCGGTACATCCTCGACGGCA
	Antisense	AGTGCCTCTTTGCTGCTTTCACAC
TNF-α	Sense	AGGACGAACATCCAACCTTC
	Antisense	TTTGAGCCAGAAGAGGTTGA
GAPDH	Sense	TCGACAGTCAGCCGCATCTTCTTT
	Antisense	CCAAATCCGTTGACTCCGACCTT