Cordycepin Suppresses Thymic Stromal Lymphopoietin Expression via Blocking Caspase-1 and Receptor-Interacting Protein 2 Signaling Pathways in Mast Cells

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Cordycepin (3′-deoxyadenosine) is one of the active components isolated from Cordyceps militaris, and has been shown to have anti-inflammatory, anti-oxidant, anti-aging, and anti-cancer effects. Mast cell-derived thymic stromal lymphopoietin (TSLP) plays an important role in the pathogenesis of allergic inflammatory reactions. Here, we investigated the regulatory effect and mechanisms of cordycepin on the expression of TSLP in the human mast cell line, HMC-1 cells, and in the human keratinocyte cell line, HaCaT cells. Cordycepin significantly decreased the production and mRNA expression of TSLP through the inhibition of caspase-1 and nuclear factor-κB activation. Cordycepin also significantly reduced the phosphorylation of receptor-interacting protein 2 and inhibitory kappa B (IκB) kinase β. Cordycepin significantly decreased the production and mRNA expression of interleukin (IL)-8, IL-1β, IL-6, and tumor necrosis factor-α in activated HMC-1 cells. Moreover, cordycepin significantly decreased the levels of TSLP in activated HaCaT cells. Our studies suggest that cordycepin can be applied to the treatment of allergic inflammatory diseases exacerbated by TSLP.

Key words cordycepin; thymic stromal lymphopoietin (TSLP); mast cell; caspase-1; receptor-interacting protein 2

Mast cell is a tissue-resident cell of hematopoietic origin, classically known as effector cell in immediate allergic and inflammatory responses. They are able to produce potential inflammatory mediators, such as cytokines, histamine, chemotactic factors, proteases, and arachidonic acid metabolites that act on the inflammatory, smooth muscle, mucous glands, connective tissue, and vasculature cells.1 Thymic stromal lymphopoietin (TSLP) is a cytokine that is involved in various biological functions, including regulating innate and acquired immune responses. TSLP is expressed and released by caspase-1 in activated mast cells.2 Caspase-1 plays a prominent role in several inflammatory disorders as the protease activates the pro-inflammatory cytokines.3

Receptor-interacting protein 2 (RIP2) is a functional factor for nuclear factor-kappa B (NF-κB) signaling pathway.4 RIP2, also known as caspase recruitment domain (CARD) 3, CARDICK, or RICK2, consists of an N-terminal serine/threonine kinase domain and a CARD domain for protein–protein interaction.5 RIP2 and caspase-1 lead to activation of the transcription factor, NF-κB.6 NF-κB was also activated by phosphorylation of inhibitory kappa B alpha (IκBα) via activation of IκB kinase β (IKKβ).7

Cordycepin (3′-deoxyadenosine) is isolated from the parasitic fungus Cordyceps militaris and has been proposed as an active compound of Cordyceps militaris that is reputed to alleviate various diseases.9 Cordycepin has the various biological activities, such as the induction of apoptosis, inhibition of cell proliferation, inhibition of platelet aggregation, inhibition of inflammation, and inhibition of cell invasiveness and migration.9 However, the detailed mechanism of cordycepin for allergic inflammatory reactions in activated mast cells has not yet been studied. Therefore, we investigated its effect and regulatory mechanism of cordycepin on phorbol myristate acetate (PMA) plus calcium ionophore A23187 (PMACI)-stimulated human mast cell line, HMC-1 cells and polyriboinosinic polyribocytidylic acid (poly(1:C))-stimulated human keratinocyte cell line, HaCaT cells.

MATERIALS AND METHODS

Reagents We purchased Isocove’s modified Dulbecco’s medium (IMDM) and Dulbecco’s modified Eagle’s medium (DMEM) from Gibco BRL (Grand Island, NY, U.S.A.); PMA, A23187 (calcimycin; C29H37N3O6), pyrrolidine dithiocarbamate (PDTC), poly(I:C), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co (St. Louis, MO, U.S.A.); TSLP, interleukin (IL)-8, IL-1β, IL-6, and tumor necrosis factor (TNF)-α antibodies from R&D Systems, Inc (Minneapolis, MN, U.S.A.); RIP2, caspase-1, NF-κB, poly-ADP-ribose polymerase, phosphorylated (p)IκBα, tubulin, IKKβ, pIKKβ, pRIP2, and actin antibodies from Santa Cruz Biotechnology (Dallas, TX, U.S.A.).
Extraction and Isolation of Cordycepin  The fruiting bodies of cultured *Cordyceps militaris* were purchased from Hwasan land Agricultural Co., Ltd. (Seoul, Korea) and identified by Roh, of College of Korean Medicine, Daejeon University. The voucher specimens (voucher No. 201400098) were deposited in the herbarium in the College of Korean Medicine, Daejeon University. The dried *Cordyceps militaris* (100 g) were chopped and extracted with ethanol for 2 d at room temperature to give the crude extract 8.4 g. The crude extract was suspended in distilled water and then chromatographed on a Diaion HP-20 gel column using 20, 40, 60, 80, 100% ethanol as eluent. Fraction 2 (650 mg) was subjected to column chromatography on silica gel, eluted with CH$_2$Cl$_2$–MeOH–H$_2$O (7 : 2 : 0.5) to yield subfractions 1–5. Subfraction 3 (140 mg) was chromatographed over Sephadex LH-20 eluted with methanol to give compound 1 (112 mg). Compound 1 was identified as cordycepin by direct comparison with an authentic sample. Cordycepin was dissolved in dimethyl sulfoxide (DMSO) and diluted with IMDM.

Cells Culture  HMC-1 cells were incubated in IMDM supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO$_2$ with 95% humidity. HaCaT cells were kindly provided by Prof. Sang-Hyun Kim (Kyungpook National University) and 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.

Cytokines Assay  The levels of TSLP, IL-8, IL-1β, IL-6, and TNF-α were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer’s instructions (R&D Systems).

MTT Assay  Cell viability was determined by MTT assay. Briefly, 500 µL of HMC-1 cells suspension (3×10^5 cells) was cultured in 24-well plates for 8 h after pretreatment by cordycepin. HaCaT cells were (2×10^5) cultured in 24-well plates for 24 h after pretreatment by cordycepin. Fifty microliters of MTT solution (5 mg/mL) was added and the cells were incubated at 37°C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using an ELISA reader at 540 nm.

Preparation of Nuclear and Cytosolic Extracts  Briefly, after activating the cells for the times indicated, we washed 5×10^6 cells in ice-cold phosphate-buffered saline (PBS) and centrifuged them at 15000×g for 1 min. We then resuspended them in 40 µL of a cold hypotonic buffer (10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (Hepes)/KOH, 2 mM MgCl$_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9). Next, we allowed the cells to swell on ice for 15 min; we lysed them gently with 2.5 µL of 10% Nonide P (NP)-40; and we centrifuged them at 15000×g for 3 min at 4°C. The supernatant was aliquots (cytosolic protein) and the pellets were gently resuspended in 40 µL of cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9) and then left on ice for 20 min. After conducting the centrifugation (15000×g for 15 min at 4°C), we froze the aliquots of supernatant containing the nuclear proteins in liquid nitrogen and stored them at −70°C until ready for analysis. Finally, we used the bicinchoninic acid protein assay (Sigma, St. Louis, MO, U.S.A.) to measure protein concentrations.

Western Blot Analysis  The cell extracts were separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the protein was transferred to nitrocellulose membranes and then the membranes were blocked and incubated with primary (1:500 dilution) and secondary (1:3000 dilution) antibodies. Finally, the protein bands were visualized by an enhanced chemilumi-
nesence assay (Amersham Co., Newark, NJ, U.S.A.) according to manufacturer’s instructions.

**Caspase-1 Assay** The enzymatic activity of caspase-1 was assayed using a colorimetric assay kit (R&D System Inc.) according to the manufacturer’s protocol.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)** Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea), we isolated the total RNA from HMC-1 cells in accordance with the manufacturer’s specifications. Total RNA (2.5 µg) was heated at 75°C for 5 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 60 min at 42°C using a cDNA synthesis kit (Bioneer, Daejeon, Korea). Quantitative real-time PCR was performed using a SYBR Green master mix and the detection of mRNA was analyzed using an ABI StepOne real-time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). We performed real-time with the following primers:

- **glyceraldehyde-3-phosphate dehydrogenase (GAPDH)** (5'-TCG ACA GTC AGC ATC TTC TTT-3'; 5'-ACC AAA TCC GTT GACTC CGAC CT-3');
- **TSLP** (5'-TAT GAG TGG GAC CGA GTT GCT CG-3'; 5'-ACC AAG TCC GTT GCA CAA CAA CTT-3');
- **IL-8** (5'-ATT AGC CAC CAT CTG CTA CAG T-3'; 5'-GTG CTT CCA CAT GTG CTC ACA-3');
- **IL-1β** (5'-AAA CAG ATG AAG TGC TCC TT-3'; 5'-AGG ACG AAC ATC CCA CTT TCA CAC-3');
- **IL-6** (5'-ATT GCT CTT TGG GGT CAC TAC-3'; 5'-AGG AAC AGA GCC CAA CTT GGT-3');
- **TNF-α** (5'-AGG ACG AAC ATC CCA CTT TCA CAC-3'; 5'-TTT GAG CCA GAA GAG GTT GA-3').

The level of the target mRNA was normalized to the level of the GAPDH and compared with the control. All data were analyzed using the ΔΔCT method.

**Statistics** All results are representative of three independent experiments with duplicate and expressed as the mean ± standard error of the mean (S.E.M.). The statistical evaluation of the results was performed by an independent t-test and an ANOVA with a Tukey post hoc test using SPSS statistical software (IBM Corporation, Armonk, NY, U.S.A.). Results with a p-value of <0.05 were considered significant.

**RESULTS**

**Cordycepin Decreased the TSLP Production and mRNA Expression on the Activated HMC-1 Cells** First, we estimated the effect of cordycepin on production of TSLP in the activated HMC-1 cells. Cordycepin (0.1, 1, 10 µM) significantly decreased the production of TSLP (Fig. 1A, p<0.05). The real-time PCR also demonstrated that the TSLP mRNA levels were significantly reduced by cordycepin in the activated HMC-1 cells (Fig. 1B, p<0.05). NF-κB inhibitor, PDTC inhibited the TSLP production and mRNA expression in the activated HMC-1 cells (Figs. 1A, B). However, cytotoxicity did not appear at doses of 0.1, 1, and 10 µM of cordycepin (Fig. 1C).

**Cordycepin Decreased the Levels of Caspase-1 in the Activated HMC-1 Cells** We next sought to determine how TSLP is regulated by cordycepin in activated HMC-1 cells. We performedWestern blotting for caspase-1 because TSLP was produced by caspase-1. The levels of caspase-1 were sig-

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Fig. 2. Cordycepin Decreased the Levels of Caspase-1 in Activated HMC-1 Cells

(A) HMC-1 cells (3×10^6) were pretreated with cordycepin for 1 h and then stimulated with PMACI for 2 h. The levels of caspase-1 were analyzed with Western blotting. (B) The caspase-1 activity was measured with caspase-1 assay kit. # p<0.05; significantly different from the unstimulated cells, * p<0.05, significantly different from the PMACI-stimulated cells.
significantly decreased by cordycepin in activated HMC-1 cells (Fig. 2A, $p<0.05$). In caspase-1 assay, cordycepin also significantly inhibited the caspase-1 activity in a concentration-dependent manner (Fig. 2B, $p<0.05$).

**Cordycepin Decreased the Levels of NF-κB, RIP2, and IKKβ in the Activated HMC-1 Cells**

We also examined the regulatory mechanisms of cordycepin on TSLP mRNA expression. As can be seen in Figs. 3A and B, cordycepin inhibited the NF-κB translocation to the nuclei and the IκBα phosphorylation in cytosol ($p<0.05$). Cordycepin inhibited the expressions and phosphorylation of RIP2 in the activated HMC-1 cells (Figs. 3C, D, $p<0.05$). In addition, cordycepin inhibited the expressions and phosphorylation of IKKβ in the activated HMC-1 cells (Figs. 3E, F, $p<0.05$). However, cordycepin alone had no effect on the RIP2 and IKKβ expression (data not shown).

**Cordycepin Decreased the Levels of Inflammatory Cytokines on the Activated HMC-1 Cells**

Caspase-1 and NF-κB regulate the production and mRNA expression of proinflammatory cytokine and cordycepin inhibited the caspase-1 and NF-κB activation. So, we estimated the regulatory effects of cordycepin on production of IL-8, IL-1β, IL-6, and TNF-α in the activated HMC-1 cells. PMACI significantly increased the production of IL-8, IL-1β, IL-6, and TNF-α in the acti-
Cordycepin showed many pharmacological activities, such as immune enhancing, anticancer, antiaging, and antioxidant effects. In this study, we first reported that cordycepin inhibited the level of TSLP by down-regulating caspase-1, translocation of NF-κB, and the expression of TSLP in the activated HMC-1 cells. Caspase-1 knockout macrophages have also decreased NF-κB activity. NF-κB is a transcription factor that has prominent roles in cell proliferation, immunity, inflammation, and apoptosis. NF-κB activation is closely modulated by IKKβ-mediated phosphorylation of IκBα. RIP2 is a serine/threonine kinase and a signal transducer for both the innate and adaptive immune responses. In this study, the stimulation with PMACI increased the level of RIP2 and IKKβ. Cordycepin decreased the activation of caspase-1, translocation of NF-κB and phosphorylation of IκBα. The expression and phosphorylation of RIP2 and IKKβ were also significantly suppressed by the treatment with cordycepin in the activated HMC-1 cells. In our previous study, TSLP production was inhibited by both caspase-1 inhibitor and NF-κB inhibitor and the expression of TSLP mRNA was inhibited by NF-κB inhibitor in the PMACI-stimulated HMC-1 cells. In this study, PDTC (NF-κB inhibitor) also decreased the production and expression of TSLP mRNA. Thus, this study can assume that cordycepin inhibits the production of TSLP through the blockade of the caspase-1 and NF-κB pathways in the activated mast cells.

Caspase-1 activation is mediated by cytosolic protein complexes called inflammasomes, a complex comprising several adaptors, such as RIP2. An upstream adaptor of caspase-1, RIP2, binds and oligomerizes caspase-1 zymogen, promoting its autoactivation. In previous study, PMACI increased the
ELISA. (B) HaCaT cells (1 × 10^5) were pretreated with cordycepin for 1 h and stimulated with poly(I:C) 10 μg/mL for 24 h. The production of TSLP was analyzed with the ELISA. (B) HaCaT cells (1×10^6) were pretreated with cordycepin for 1 h and stimulated with poly(I:C) for 2 h. The mRNA expression of TSLP was analyzed with the real-time PCR analysis. (C) Cell viability was analyzed with the MTT assay. *p<0.05; significantly different from the unstimulated cells, #p<0.05; significantly different from the poly(I:C)-stimulated cells.

Fig. 5. Cordycepin Decreased the TSLP Production and mRNA Expression on the Activated HaCaT Cells

(A) HaCaT cells (2×10^5) were pretreated with cordycepin for 1 h and stimulated with poly(I:C) 10 μg/mL for 24 h. The production of TSLP was analyzed with the ELISA. (B) HaCaT cells (1×10^6) were pretreated with cordycepin for 1 h and stimulated with poly(I:C) for 2 h. The mRNA expression of TSLP was analyzed with the real-time PCR analysis. (C) Cell viability was analyzed with the MTT assay. *p<0.05; significantly different from the unstimulated cells, #p<0.05; significantly different from the poly(I:C)-stimulated cells.

protein level of RIP2 in HMC-1 cells.15) That is, we showed PMACI activates caspase-1 depending on the activation of inflammasome. In this study, cordycepin decreased the caspase-1 activation and RIP2 expression. Therefore, we can assume that cordycepin decreased the caspase-1 activation through the inhibition of RIP2 expression and phosphorylation. However, the precise mechanism of cordycepin on RIP2 expression is not known at this time. Therefore, further investigations are required in order to elucidate the specifically mechanism of cordycepin on upstream mechanisms of RIP2.

PMACI-mediated activation of mast cells produces numerous chemotactic and proinflammatory cytokines such as IL-8, IL-1β, IL-6, and TNF-α.26,27) Blocking of these proinflammatory cytokines might provide new therapeutic approaches for the control of allergic inflammation.28) Cordycepin significantly inhibited the lipopolysaccharide (LPS)-induced release of TNF-α and IL-1β via the suppression of mitogen-activated protein kinases and NF-κB signaling pathways in RAW264.7 cells.13) Likewise, our results showed that cordycepin decreased the levels of inflammatory cytokines on the activated HMC-1 cells. Therefore, we can assume that the anti-inflammatory effects of cordycepin might be originated from inhibitory action for the PMACI-induced inflammatory cytokines.

Increased expression of TSLP has been revealed in skin keratinocytes from atopic dermatitis patients.29) Leyva-Castillo et al.30) suggested that blocking TSLP production in skin could be therapeutically useful in preventing or limiting allergen sensitization that is commonly developed in atopic dermatitis patients, and halting the progress of the “atopic march.” In this study, cordycepin inhibited the production and mRNA expression of TSLP in the activated human keratinocytes. Therefore, we suggest that the cordycepin has an anti-inflammatory effect through the down-regulation of TSLP in the activated keratinocytes.

In conclusion, the present study is first report that cordycepin inhibited the TSLP expression through blockage of RIP2, IKKβ, caspase-1, and NF-κB signal cascade in the activated mast cell. Cordycepin also suppressed the levels of inflammatory cytokines in the activated mast cells. In addition, cordycepin inhibited the levels of TSLP in the activated keratinocytes. Therefore, our studies provide that cordycepin can be applied to the treatment of allergic inflammatory diseases exacerbated by TSLP.

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Conflict of Interest The authors declare no conflict of interest.

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