Cordycepin prevented IL- β -induced expression of inflammatory mediators in human osteoarthritis chondrocytes

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Abstract

Purpose Cordycepin, a nucleoside derivative isolated from Cordyceps, has been reported to exert anti-inflammatory, antitumor, antidiabetic and renoprotective effects. Osteoarthritis (OA) is a degenerative joint disease with an inflammatory component that drives the degradation of cartilage extracellular matrix. This study aimed to assess the effects of cordycepin on human OA chondrocytes.

Methods In this study, human OA chondrocytes were pretreated with cordycepin at 10, 50 or 100 μ M and subsequently stimulated with interleukin-1 β (IL-1 β) (5 ng/ml) for 24 h. Production of prostaglandin E₂ (PGE₂) and nitric oxide (NO) were evaluated by the Griess reaction and an enzyme-linked immunosorbent assay (ELISA). Gene expression of matrix metalloproteinase (MMP)-13, IL-6, inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) was measured by real-time polymerase chain reaction (PCR). MMP-13 and IL-6 proteins in culture medium were determined using cytokine-specific ELISA. Western immunoblotting was used to analyse the iNOS and COX-2 protein production in culture medium. Nuclear factor kappa-B (NF- κ B) activity regulation was explored using Western immunoblotting. **Results** Pretreatment with cordycepin significantly inhibited the production of PGE₂ and NO induced by IL-1 β . Cordycepin also significantly decreased the IL-1 β -stimulated gene expression and production of MMP-13, IL-6, iNOS and COX-2 in OA chondrocytes. Pretreatment with cordycepin

attenuated IL-1 β -induced activation of NF- κ B by suppressing degradation of its inhibitory protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B- α) in the cytoplasm.

Conclusions We show for the first time the anti-inflammatory activity of cordycepin in human OA chondrocytes. Thus, with this unique profile of actions, cordycepin may prove to be a potentially attractive and new therapeutic/preventive agent for OA.

Keywords Cordycepin · Interleukin-1 β (IL-1 β) · Nitric oxide · Osteoarthritis

Introduction

Osteoarthritis (OA) is a degenerative disease of the joints leading to progressive cartilage damage, osteophyte formation and subchondral bone sclerosis, producing pain and loss of function. Although its aetiology is unknown, hereditary, developmental and abnormal metabolic and mechanical processes are all considered to contribute to joint degeneration. In OA, cell matrix signaling is disrupted by depletion of collagen and proteoglycan due to the production of matrix metalloproteinases (MMPs), aggrecanases (ADAMTSs) and other proteases, resulting in matrix degradation and mechanical loss of tissue resilience [1]. Production of these substances is induced by inflammatory cytokines and mediators, such as interleukin-1 β (IL-1 β), tumor necrosis factor alpha (TNF- α), IL-6, nitric oxide (NO), and prostaglandins, which are found at elevated levels in OA joint tissues [2–4].

IL-1 β appears to play a central role in OA pathogenesis. It is implicated in the degeneration of articular cartilage due to its induction of proteoglycan loss and matrix degradation [5]. Elevated levels of IL-1 occur in the synovial fluid and cartilage tissue of patients with OA [6]. IL-1 receptor antagonist, a

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natural competitor of IL-1, suppresses cartilage degradation, further supporting the role of IL-1 in cartilage breakdown [7]. Therefore, there is an interest in developing disease-modifying OA drugs (DMOADs) for treating OA, which may inhibit both IL-1 β -mediated inflammation and extracellular matrix (ECM) degradation.

Cordyceps militaris is the rare Chinese caterpillar fungus [8] and has benefits to the human body, including the circulatory, immune, respiratory and glandular systems. Extract of *Cordyceps* exhibits antitumour effects on cancers and inhibitory effects on the production of inflammatory mediators [9–11]. Cordycepin, a nucleoside derivative isolated from *Cordyceps*, is reported to exert inhibitory effects on macrophages based on its anti-inflammatory property [12]. Cordycepin has also been studied for antitumor [13], antidiabetic [14], and renoprotective effects [15]. Noh et al [16] demonstrated that cordycepin has anti-rheumatic and anti-inflammatory effects on IL1 β -stimulated rheumatoid arthritis synovial fibroblasts (RASFs). Shin et al [17] discovered that cordycepin could suppress expression of diabetes-regulating genes by inhibiting lipopolysaccharide-induced inflammation in macrophages.

The excellent therapeutic properties of cordycepin have been demonstrated in various cell types [15, 18–21]. However, the role of cordycepin in inflammatory responses in articular chondrocytes is still unclear. In this study, we investigated whether cordycepin exerted anti-inflammatory activities in human OA chondrocytes.

Materials and methods

Chondrocyte isolation and cultures

Tissue collection was in accordance with the terms of the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical College, and followed the guidelines of the Declaration of Helsinki and Tokyo. OA cartilage tissues were obtained from three OA patients (aged 53–68 years, two men and one woman) who underwent total knee replacement at the Second Affiliated Hospital of Wenzhou Medical College. The patients met the American College of Rheumatology (ACR) classification criteria for OA diagnosis [22]. Full ethical consent was obtained from all patients. Cartilage was separated from underlying bone and connective tissues, and joint cartilage slices were isolated with hyaluronidase (Sigma) for five minutes, similar to previously published methods [23, 24]. Chondrocytes were plated at 1×10^5 cells/well in six well plate, serum starved overnight, pretreated with various concentrations of cordycepin (10, 50 or 100 μ M) for two hours prior to IL-1 β (5 ng/ml) for 24 hours. Conditioned media was collected for NO, prostaglandin E₂ (PGE₂), MMP-13, IL-6, cyclo-oxygenase (COX-2) and inducible nitric oxide

synthase (iNOS) measurement. In another study, cells were pretreated with cordycepin (10, 50 or 100 μ M) for two hours and then stimulated with IL-1 β for one hour. Then, cells were harvested for analysis of nuclear factor kappa B (NF- κ B) activation.

Cell viability assay

Effects of cordycepin on cell viability in OA chondrocytes was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT). After incubation with various concentrations of cordycepin in a 96-well plate at a density of 6×10^3 /well for 24 hours, cells were incubated with 20 μ l of 0.5 % MTT for four hours. The supernatant was removed and 150 μ l of dimethylsulfoxide (DMSO) was added. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). The culture medium was used as a blank.

NO and PGE2 measurement

Nitrite levels in culture medium were assessed by Griess reaction, as previously described [25]. PGE₂ levels were investigated using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). All assays were performed in duplicate.

RNA isolation and real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was used to detect the expression of MMP-13, IL-6, COX-2 and iNOS. Total RNA (tRNA) was extracted using TriZol (Invitrogen), according to the manufacturer's instructions, and quantified. Its concentration was determined spectrophotometrically at 260 nm (HP 8452A Diode Array Spectrophotometer). First-strand complementary DNAs (cDNAs) were synthesized from 0.3 mg of the isolated RNA by oligo(deoxythymidine) [oligo(dT)] using DyNamoTM cDNA Synthesis Kit (Fermentas) and used as templates for real-time PCR. The expression of messenger RNAs (mRNAs) was determined quantitatively using DyNamo SYBR1 Green qPCR kit (Takara, Japan). The PCR was performed on a final volume of 25 μ l containing 2 μ l cDNA, 7.5 pmol of each primer, 1 μ l 6-carboxyl-x-rhodamine (ROX) reference dye and 12.5 μ l of SYBR Green Master Mix (Tiangen), with ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). The samples underwent 40 cycles consisting of the following steps: initial denaturation at 95 °C for five minutes, followed by a set cycle of denaturation at 94 °C for ten seconds different annealing temperatures for each pair of primers (ranging between 53 and 62 °C) for ten seconds, extension at 72 °C for 28 seconds and a final elongation at 72 °C for five minutes. Fold increment of

any assayed gene was calculated by normalizing its expression level to that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, which was used as an internal control. Each gene analysis was performed in triplicate. Primer sequences of the targeted genes are listed in Table 1.

Measuring MMP-13 and IL-6 by ELISA

Human OA chondrocytes were pretreated with cordycepin (10, 50 or 100 μ M) for two hours and then stimulated or not with IL-1 β (5 ng/ml) for 24 hours. The effect of IL-1 β and/or cordycepin on the level of MMP-13 and IL-6 secreted by chondrocytes in the culture medium was further confirmed by sandwich ELISA (R&D Systems), and all assays were performed according to the manufacturer's instructions. Results were rounded to one decimal place.

Western blot analysis

Stimulated cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by a scraper and cytoplasmic proteins were isolated using an extraction kit (Beyotime Institute of Biotechnology, Jiangsu, China). The protein was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with blocking buffer (5 % w/v skimmed milk powder in 1 $\times$ Tris-buffered saline (TBS) containing 0.1 % Tween-20 for one hour then incubated with rabbit polyclonal antibodies for iNOS, COX-2, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B- α), mouse monoclonal antibody (mAb) phospho-I κ B- α , mouse mAb phospho-p65, mouse mAb p65 and β -actin overnight at 4 °C (Santa Cruz Biotechnology, CA, USA). Membranes were washed and incubated for one hour at room temperature with horseradish-peroxidase (HRP)-linked secondary antibodies. After washing, membranes were detected by enhanced

chemiluminescence (ECL) and exposed to X-ray films (Kodak, China).

Statistical analysis

All experiments were performed independently at least three times, and data, expressed as mean $\pm$ standard deviation (SD), from a representative experiment are shown. Results are expressed as mean $\pm$ SD. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test; values of $P < 0.05$ were considered significant.

Results

Potential cytotoxic effects of cordycepin

Cytotoxicity of cordycepin on human OA chondrocytes has not yet been reported. Therefore, we used MTT to assess the effect of cordycepin on cellular toxicity of human OA chondrocyte. As shown in Fig. 1, cell viability of human OA chondrocytes was not significantly impaired under the concentration of cordycepin (10, 50 or 100 μ M) after cultured for 24 hours.

Effect of cordycepin on IL-1 β -induced NO and PGE2 production

The effect of cordycepin on IL-1 β -induced NO and PGE2 production is shown in Fig. 2a, b. Human OA chondrocytes were pretreated with cordycepin (0, 10, 50 or 100 μ M) for two

Table 1 Primer sequences used in real-time polymerase chain reaction (PCR)

Gene		Primer sequences (5'-3')
<i>MMP-13</i>	Forward	CGC CAG AAG AAT CTG TCT TTA AA
	Reverse	CCA AAT TAT GGA GGA GAT GC
<i>IL-6</i>	Forward	GAC AGC CAC TCA CC TCTT CA
	Reverse	TTC ACC AGG CAA GTC TCC TC
<i>iNOS</i>	Forward	CCT TAC GAG GCG AAG AAG GAC AG
	Reverse	CAG TTT GAG AGA GGA GGC TCC G
<i>COX-2</i>	Forward	GAG AGA TGTATC CTC CCA CAG TCA
	Reverse	GAC CAG GCA CCA GAC CAA AG
<i>GAPDH</i>	Forward	TCT CCTCTG ACT TCA ACA GCG AC
	Reverse	CCC TGT TGC TGT AGC CAA ATT C

Fig. 1 Effects of cordycepin on cell viability in osteoarthritis (OA) chondrocytes was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT). Chondrocyte viability was not significantly impaired under 10-, 50- or 100- μ M concentrations of cordycepin cultured for 24 h. Values are mean $\pm$ standard deviation (SD)

Fig. 2 Effects of cordycepin on the production of nitric oxide (NO) and prostaglandin E_2 (PGE $_2$). Human osteoarthritis (OA) chondrocytes were pretreated with cordycepin at 10, 50 or 100 μ M for 2 h, then stimulated or not with interleukin-1 β (IL-1 β) (5 ng/ml) for 24 h. **a** Nitrite levels in culture medium were assessed by Griess reaction. **b** PGE $_2$ levels were assessed using the enzyme-linked immunosorbent assay (ELISA) kit. Values are mean $\pm$ standard deviation. * P <0.05, ** P <0.01 compared with IL-1 β group

hours and stimulated or not with IL-1 β (5 ng/ml) for 24 hours. Stimulation with IL-1 β significantly increased the production of NO (p <0.05) and PGE $_2$ (p <0.05) compared to unstimulated control. Pretreatment with cordycepin 50 or 100 μ M partly blocked upregulation of NO and PGE $_2$ induced by IL-1 β (P <0.05).

Effect of cordycepin on IL-1 β -induced iNOS and COX-2 mRNA and protein expression in OA chondrocytes

The mRNA expression of iNOS (Fig. 3a) and COX-2 (Fig. 3b) were quantitatively determined by real-time PCR. We found cordycepin exhibited a broad spectrum of inhibitory effects on the expression of proinflammatory mediators.

Fig. 3 Effect of cordycepin on interleukin-1 β (IL-1 β)-induced expression of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) in osteoarthritis (OA) chondrocytes pretreated with cordycepin at 10, 50 or 100 μ M for 2 h and stimulated or not with IL-1 β (5 ng/ml, 24 h) for **a** iNOS and **b** COX-2 mRNA. The expression of mRNA was analysed with real-time polymerase chain reaction (PCR). **c** Protein levels of iNOS and COX-2 were assayed using Western blot analysis. Values are mean $\pm$ standard deviation. * P <0.05 compared with IL-1 β group

Cordycepin (50 or 100 μ M) significantly reduced gene expression of iNOS and COX-2 induced by IL-1 β (P <0.05). Data concerning iNOS and COX-2 expression were

confirmed at the protein level. As shown in Fig. 3c, cordycepin inhibited upregulation of iNOS and COX-2 protein induced by IL-1 β in OA chondrocytes by Western blot analysis.

Effect of cordycepin on IL-1 β induced MMP-13 and IL-6 mRNA and protein expression in OA chondrocytes

OA chondrocytes, stimulated with IL-1 β , showed a significant expression of MMP-13 and IL-6 mRNA in comparison with unstimulated cells. Pretreatment with cordycepin (10, 50 or 100 μ M) downregulated MMP-13 (Fig. 4a) and IL-6 (Fig. 4b) mRNA expression in IL-1 β -stimulated OA chondrocytes during 24 h ($P < 0.05$). We then used ELISA to assay the effect of cordycepin on IL-1 β -induced protein production of MMP-13 (Fig. 4c) and IL-6 (Fig. 4d). Protein expression of MMP-13 and IL-6 was up-regulated

significantly in IL-1 β -stimulated OA chondrocytes ($P < 0.05$). However, MMP-13 and IL-6 levels were significantly reduced after pretreatment with cordycepin.

Effect of cordycepin on NF- κ B activation

Activation of NF- κ B was investigated by Western blot analysis due to its importance in the upregulation of COX-2 and iNOS expression in the pathogenesis of inflammation. IL-1 β treatment activated the NF- κ B signaling pathway and increased I κ B- α phosphorylation. Also, we found the levels of the p65 subunit of NF- κ B were increased in the nucleus and decreased in the cytoplasm. In contrast, pretreatment with cordycepin (10, 50 or 100 μ M) inhibited I κ B- α phosphorylation and translocation of the p65 subunit (Fig. 5).

Fig. 4 Effect of cordycepin on interleukin-1 β (IL-1 β)-induced expression of matrix metalloproteinase-13 (MMP-13) and IL-6 in human osteoarthritis (OA) chondrocytes pretreated with cordycepin at 10, 50 or 100 μ M for 2 h and then stimulated or not with IL-1 β (5 ng/ml, 24 h) for **a** MMP-13 messenger RNA (mRNA) and **b** IL-6 mRNA. The mRNA

expression was analyzed by real-time polymerase chain reaction (PCR). Levels of **c** MMP-13 and **d** IL-6 in culture supernatants were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) for at 24 h. Values are mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$ compared with the IL-1 β group

Fig. 5 Effect of cordycepin on interleukin-1 β (IL-1 β)-induced activation of nuclear factor kappa B (NF- κ B) in IL-1 β -stimulated human osteoarthritis (OA) chondrocytes pretreated with cordycepin at 10, 50 or 100 μ M for 2 h and then stimulated or not with IL-1 β (5 ng/ml) for 1 h. Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B- α) degradation and phosphorylation and nuclear factor kappa B (NF- κ B) p65 activation were analysed by Western blotting

Discussion

Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is recommended for treating some chronic inflammatory diseases, such as OA. As individual response to drugs can vary, and as potential side effects remain one of the problems for long-term use of NSAIDs, phytochemicals are potential candidates as a novel agent to prevent inflammation for the treatment of many diseases, including OA [26]. Cordycepin, a nucleoside derivative isolated from Cordyceps, is reported to exert inhibitory effects on macrophages and RA synovial fibroblasts due to its anti-inflammatory effect [12, 16]. In this study, we evaluated the role of cordycepin in inflammatory responses in human articular chondrocytes. The most important finding of this study was that cordycepin could prevent the expression of key molecules, such as PGE2, NO, MMP-13, IL-6 and COX-2 in IL-1 β -stimulated human OA chondrocytes. We found the anti-inflammatory effects of cordycepin are at least in part due to its inhibitory effect on the NF- κ B pathway.

We found that cordycepin had no significant effect on chondrocyte viability at the concentrations used in 24 hour cultures. Our results were consistent with the research from Noh et al. [16], who found no significant change RA synovial fibroblast cell viability following treatment with cordycepin (50 μ M or 100 μ M). Also, Seulmee et al. [17] showed that the exposure of mouse leukaemic monocyte macrophage cell line (RAW 264.7) to cordycepin at 5–40 μ g/ml for 24 hours showed no significant adverse effect on cell viability versus the untreated control.

Overproductions of NO and PGE2 are correlated with the pathophysiology of OA. NO is an inflammatory mediator produced by the NOS family of enzymes [27]. There are three types of NOS: iNOS, neuronal NOS (nNOS) and endothelial

NOS (eNOS). The latter two are constitutive NOS, whereas iNOS is induced by cytokines to produce NO. In this study, we demonstrated that cordycepin prevented IL-1 β -induced NO production as well as iNOS expression at the mRNA and protein levels in human OA chondrocytes. Results were consistent with Seulmee et al.'s results [17]. They found that cordycepin could inhibit NO production as well as iNOS expression on lipopolysaccharide-induced inflammation in RAW 264.7 cells. PGE2 is also an inflammatory factor elevated by COX-2. There are two isoforms of COX enzymes: COX-1 and COX-2. COX-1 contributes to gastric defense and COX-2 is the target in OA treatment. In this study, we also found that the induction of PGE2 production and COX-2 expression by IL-1 β were blocked by cordycepin. Our results are consistent with previous study showing that cordycepin prevents PGE2 production and COX-2 expression on lipopolysaccharide-induced inflammation in RAW 264.7 cells [17].

MMPs are a family of enzymes normally required for degradation of ECM components, including type II collagen, during cartilage remodeling [28]. Recent studies demonstrate elevated MMP-13 in arthritic joints [29], and this protease is considered to be the major MMP responsible for cartilage breakdown [30]. Thus, blocking MMP-13 activity is a therapeutic approach to OA. IL-1 β is a proinflammatory cytokine that plays a synergistic effect with IL-1 β in OA [31]. The study reported here shows that addition of cordycepin effectively suppresses overexpression of MMP-13 and IL-6 in IL-1 β -stimulated OA chondrocytes, indicating that cordycepin may act as a potent anti-inflammatory agent in chondrocytes.

We also investigated molecular mechanisms by which cordycepin inhibited inflammatory mediators in response to IL-1 β in chondrocytes. We focused on NF- κ B due to its importance in inflammatory process [32]. NF- κ B is retained in the cytoplasm with I κ B- α inactivity. IL-1 β activates NF- κ B by triggering I κ B- α degradation. NF- κ B activation resulted in the up-regulation of a group of responsive genes that contributed to inflammation, including *iNOS* and *COX-2* [33, 34]. In our study, IL-1 β induced I κ B- α degradation in chondrocytes; this degradation was blocked by cordycepin. Our results are partly supported by Seulmee et al. [17], who found that cordycepin inhibits lipopolysaccharide-induced *COX-2* and *NO* gene expression through down-regulating NF- κ B in RAW 264.7 cells. However, Noh et al. [16] found that cordycepin inhibited MMP-1 and -3 production by suppressing the activator protein 1 (AP-1) but not NF- κ B in RASFs. We speculated that the differences maybe attribute to the different types of cells. In RASFs, AP-1 is an essential component for MMP-1 synthesis, and cooperation between mitogen-activated protein kinase (MAPK) and NF- κ B signaling pathways is necessary for IL-1 β -induced synthesis of MMP-1 [35]. Ren et al. [36] found that cordycepin inhibited NF- κ B signaling by suppressing NF- κ B and I κ B- α activity in

human embryonic kidney (HEK)-293 T cells. However, the exact mechanism implicated in the regulation of inflammation in chondrocytes by cordycepin is still unclear. Further studies are needed to elucidate the precise mechanism of cordycepin regulation on inflammatory processes in OA chondrocytes.

This study is limited in terms of its in vitro experimental design and that extrapolations to in vivo conditions should be carried out with caution. Future studies are necessary to define in vivo actions of cordycepin using animal models of OA and further delineate the molecular mechanisms evoked by cordycepin.

Conclusions

In conclusion, we demonstrated that cordycepin prevented PGE₂, NO, MMP-13, IL-6 and COX-2 production on IL-1 β -stimulated human OA chondrocytes. With this unique profile of actions, cordycepin may prove to be a potentially attractive and new therapeutic/preventive agent for OA.

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