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Original Article

Compound K Inhibits Interleukin-1β-induced Expression of Inflammatory Mediators and Matrix Metalloproteinases by Inhibiting Mitogen-activated Protein Kinase Activation in Chondrocytes

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Objective. This study examined the anti-inflammatory and chondroprotective effects of compound K (CK), a ginsenoside metabolite, on chondrocytes from osteoarthritis (OA) patients following stimulation with interleukin (IL)-1 β. **Methods.** Articular cartilage samples were obtained from six OA patients undergoing total knee replacement surgery. Nitric oxide (NO) production was measured by the Griess reaction. Subsequently, the mRNA and protein levels of matrix metalloproteinases (MMPs), inducible NO synthase (iNOS), and mitogen-activated protein kinases (MAPKs) were examined by a reverse transcription-polymerase chain reaction and western blot analysis. Cartilage degradation was assessed using a glycosaminoglycan (GAG) assay. **Results.** CK inhibited IL-1 β-induced NO production and iNOS expression in a dose-dependent manner. In addition, it inhibited the IL-1 β-stimulated release of MMP-1, -3, and -13 and tissue inhibitor of matrix metalloproteinase-1 from OA patient chondrocytes. In addition, CK effectively suppressed the IL-1 β-induced phosphorylation of p38, extracellular signal-regulated kinase-1/2, and c-Jun N-terminal kinase MAPKs. Moreover, the IL-1 β-mediated release of GAG was inhibited by CK in a dose-dependent manner. **Conclusion.** CK inhibited the IL-1 β-induced expression of inflammatory mediators and MMPs by, at least in part, inhibiting MAPK activation, and has potential as a therapeutic agent for the treatment of OA. **(J Rheum Dis 2018;25:188-196)**

Key Words. Panax, Ginsenosides, Nitric oxide, Matrix metalloproteinases, Osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of pain and disability in the growing elderly population [1]. It is characterized by an active and complex process involving inflammatory, mechanical, and metabolic factors, which ultimately disrupt the structure of the synovial joint, leading to its failure [2]. The pathologic changes seen in OA joints include degradation of the articular cartilage, thickening of the subchondral bone, formation of osteophytes, varying degrees of synovial inflammation, and degeneration of ligaments [3].

Of the cytokines involved in the pathogenesis of OA, the inflammatory cytokine interleukin (IL)-1 β is considered key. Patients with OA have an elevated level of IL-1 β in the synovial fluid, synovial membrane, cartilage, and subchondral bone [4,5]. Furthermore, expression of IL-1 receptor type 1 has been reported to be increased on the surface of chondrocytes from OA patients compared to those from healthy individuals [6]. IL-1 β plays a crucial role in the destruction of the cartilage matrix by upregulating the production of proteases, such as matrix metalloproteinases (MMPs) and zinc-dependent endopeptidases, which are specifically controlled by tissue inhibitors of metalloproteinases (TIMPs) [7,8]. MMPs are synthesized and secreted by resident chondrocytes, particularly under

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arthritic conditions. Among the various MMPs, collagenases such as MMP-1, -8, and -13 are particularly important, and are known to be upregulated during joint inflammation and degeneration [8,9].

Collagenase expression in chondrocytes is regulated by the activation of mitogen-activated protein kinase (MAPK) family members, including c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK), and several transcription factors, including nuclear factor-κB (NF-κB) [9-11]. IL-1β induces heightened inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in chondrocytes, leading to elevated production of NO and prostaglandin E_2 (PGE_2), which accelerate cartilage degradation by inhibiting proteoglycan biosynthesis [6,12].

Treatments for OA aim to relieve pain, decrease joint stiffness and swelling, prevent cartilage loss, and maintain the patient's quality of life [13]. Since targeting NO and PGE_2 is beneficial in OA therapy, non-steroidal anti-inflammatory drugs (NSAIDs) have been used as treatments for this disease for several years. However, long-term use of NSAIDs is known to induce gastrointestinal, renal, and cardiovascular toxicity [14]. Therefore, there remains a need for a safer, yet effective, treatment option for OA.

Ginseng, especially *Panax ginseng* Meyer, is one of the most widely used herbal medications globally, and is employed in the treatment of various diseases [15]. It is taken as an oral supplement, and its biological activities derive from ginsenosides, its major pharmacologically active components [16]. Compound K (CK) (Supplementary Figure 1) is a bacterial metabolite of the ginsenoside Rb1. The uptake and rate of absorption of CK in the human gastrointestinal tract, and ultimately its passage into systemic circulation, are rapid. It has been suggested that CK suppresses receptor activator of NF-κB ligand-induced osteoclastogenesis and tumor necrosis factor-α (TNF-α)-induced production of MMPs by fibroblast-like synoviocytes in rheumatoid arthritis [17]. CK also attenuates collagen-induced arthritis via suppression of the humoral immune response and modulation of mediators of joint destruction [18].

However, very few studies have reported the effects of CK in OA. In this study, we evaluated the anti-inflammatory and chondroprotective effects of CK on chondrocytes from OA patients following IL-1β stimulation and examined the underlying mechanisms.

MATERIALS AND METHODS

Preparation of CK

CK was obtained from the Korea Ginseng Corporation (Daejeon, Korea). Its purity was higher than 97%, based on high-performance liquid chromatography analysis.

Chondrocyte culture

Articular cartilage samples were obtained from six OA patients who fulfilled the American College of Rheumatology criteria for OA [19] and were undergoing total knee replacement surgery. Primary chondrocytes were isolated from articular cartilage as described previously [20]. The cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C in the presence of 5% CO_2. Cells from passages 1 to 3 were used in this study. The study was approved by Institutional Review Boards and ethics committees of Seoul National University Hospital (no. 1603-146-751), and was conducted in accordance with good clinical practice guidelines and the Declaration of Helsinki.

Enzyme-linked immunosorbent assay (ELISA)

Chondrocytes were pretreated with CK or ginsenoside Rb1 1 hour prior to IL-1β (2 ng/mL) stimulation for 24 hours. Culture supernatants were collected and stored at −20°C until analysis. The concentrations of MMP-1, -3, and -13 and TIMP-1 in cell culture supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measurement of NO

Chondrocytes were pretreated with CK or Rb1 1 hour prior to IL-1β (2 ng/mL) stimulation for 24 hours. The NO concentration in the culture supernatant was measured using Griess reagent according to the manufacturer's instructions. Briefly, cell culture supernatant was collected and mixed with an equal volume of Griess reagent, and optical density at 540 nm was measured 30 minutes later.

Western blot analysis

Chondrocytes were pretreated with the indicated concentrations of CK 1 hour prior to IL-1β (2 ng/mL) stimulation for 30 minutes. Nuclear and cytoplasmic proteins were extracted from the cells using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific, Waltham, MA, USA). Protein concentration was de-

termined with a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12% gel and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% non-fat milk at room temperature for 1 hour and probed with primary antibodies (all diluted 1:1,000) against the following proteins: iNOS, ERK1/2, phosphorylated (p-)ERK1/2, JNK, p-JNK, p38, and p-p38. After being washed three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Antibody binding was visualized using a chemiluminescent substrate (SuperSignal West Femto; Thermo Scientific).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA was synthesized by reverse transcription using 1 mg of DNase-treated total RNA and an iScript Reverse Transcriptase Kit (Bio-Rad Laboratories). RT-PCR was performed using primers targeting sequences encoding iNOS, MMP-1, -3, and -13, and TIMP-1. Actin was used as the internal control.

Zymography

Serum-starved cells were cultured in the presence of CK for 48 hours. Cell culture supernatant (10 μL) was mixed with 10 μL of sample buffer (0.5 M Tris-HCl, 20% glycerol, 10% SDS, and 0.1% bromophenol blue) and incubated for 10 minutes at room temperature. The samples were loaded on gelatin (1 mg/mL)-containing SDS-polyacrylamide gels and electrophoresed under a constant voltage (125 V). Subsequently, the gels were incubated in renaturation buffer (2.5% Triton X-100) with gentle agitation for 30 minutes at room temperature. The gels were then equilibrated for 30 minutes in developing buffer (50 mM Tris base, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35), and incubated in fresh developing buffer overnight at 37°C. Zymographic activity was detected by staining the gels with 0.2% Coomassie blue, before destaining them with a destaining solution (7.5% acetic acid in 20% ethanol). Areas affected by protease activity appeared as clear bands against a dark blue background, representing substrate digestion. Molecular weights were estimated by comparison with prestained SDS-PAGE markers and a human pro-MMP-2 standard (AnaSpec, Fremont, CA, USA).

Glycosaminoglycan (GAG) assay

Cartilage degradation was assessed by measuring the amount of proteoglycan released into the culture medium, as described previously [21]. Briefly, culture medium was added to a solution of 1,9-dimethylmethylene blue (DMB) (Sigma, St. Louis, MO, USA), a metachromatic dye that binds sulfated GAG, a major component of proteoglycans. The quantity of GAG-DMB complex formed was measured in a 96-well plate using a plate reader (Infinite M200; TECAN, Männedorf, Switzerland) at a wavelength of 595 nm. Loss of GAG was calculated and expressed as the total GAG (μg) released per mg (wet weight) of cartilage.

Thiazolyl blue tetrazolium bromide (MTT) assay

An MTT assay was used to determine the effects of CK and Rb1 on chondrocyte viability. Briefly, chondrocytes were seeded in a 96-well plate at a density of 6×10^3 per well. The cells were treated with 0 to 10 μM CK or 0 to 200 μM Rb1 for 1 hour, and stimulated with IL-1β (2 ng/mL) for 24 hours. Subsequently, 20 μL MTT (5 mg/mL) was added to each well, and the cells were incubated for an additional 4 hours. The supernatant was then removed, and the formazan crystals formed were dissolved using 150 μL of dimethyl sulfoxide. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad Laboratories).

Statistical analyses

Data are presented as the mean±standard error of the mean of three independent experiments. Statistical analysis was carried out using the Kruskal–Wallis test or Wilcoxon signed-rank test, as appropriate, and p-values of 0.05 or less were considered statistically significant.

RESULTS

CK inhibited IL-1β-induced NO production

To investigate the anti-inflammatory effect of CK, its influence on IL-1β-induced NO production was evaluated. Levels of NO in the supernatant increased after IL-1β treatment. However, CK inhibited IL-1β-induced NO production in a dose-dependent manner from a concentration of 1 μM. NO concentration was decreased by 95.8% when 5 μM CK was administered (Figure 1A). Rb1 at and above a concentration of 5 μM also sup-

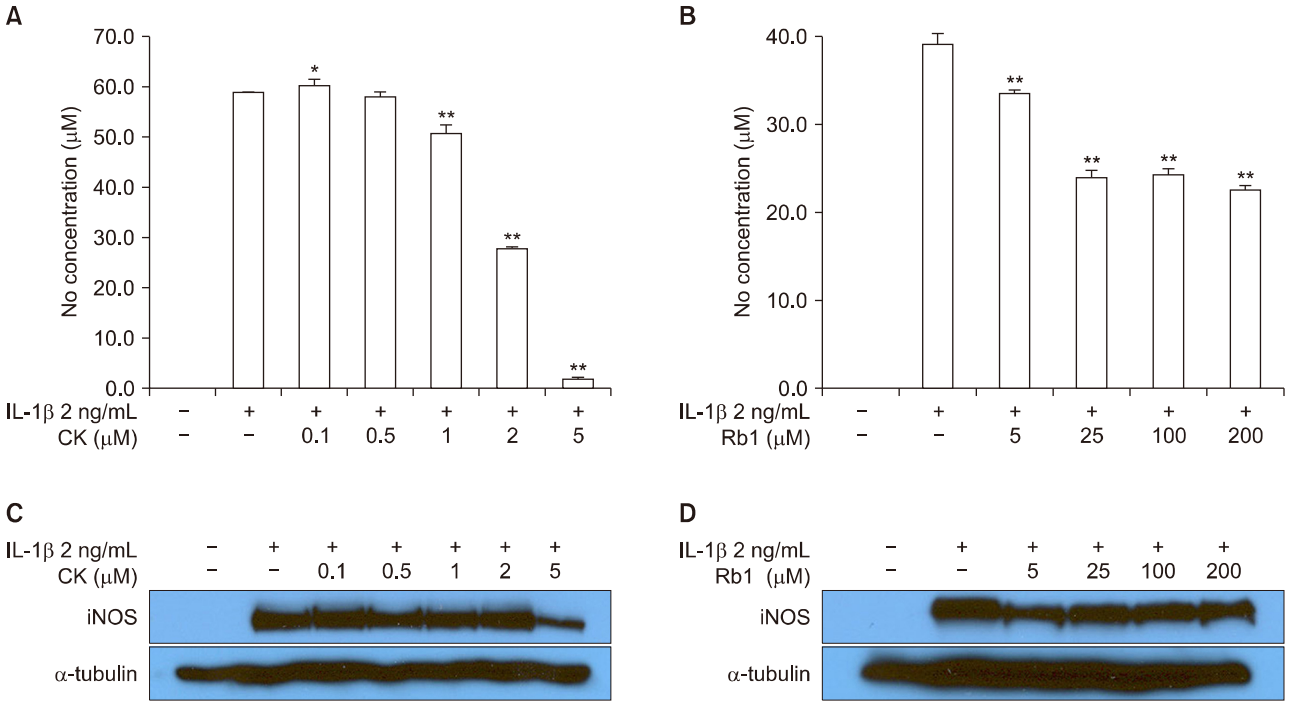

Figure 1. Interleukin (IL)-1 β-induced nitric oxide (NO) production inhibited by (A) compound K (CK) and (B) Rb1. (C) Strong inhibitory effect of CK on NO production is related to decreased expression of induced NO synthase (iNOS), (D) but the expression of iNOS was not inhibited by Rb1. The values presented are the means ± standard error of mean. of three independent experiments. *$p < 0.05$, **$p < 0.005$.

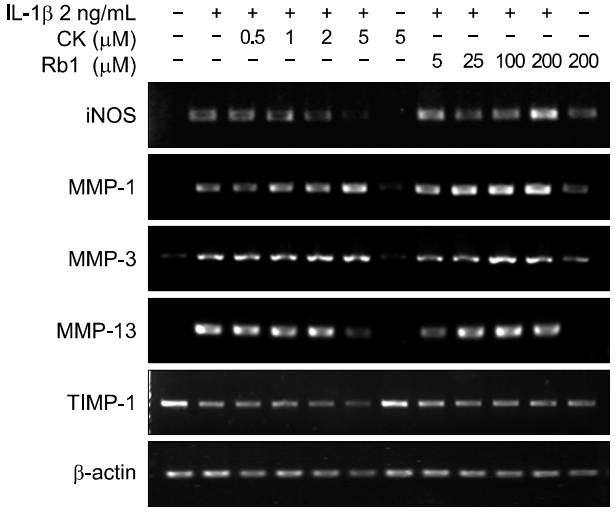

Figure 2. Effects of compound K (CK) and Rb1 on interleukin (IL)-1 β-induced mRNA expression of induced nitric oxide synthase (iNOS), matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases-1 (TIMP-1). The cells were pretreated with different concentrations of CK or Rb1 for 1 hour before incubation with IL-1 β (2 ng/mL). Following 24 hours of treatment, total RNA was isolated, and reverse transcription-polymerase chain reaction was performed using iNOS, MMP-1, MMP-3, MMP-13 and TIMP-1 primers.

pressed IL-1 β-induced NO production (Figure 1B). However, its inhibitory effect in this respect was far weaker than that of CK. Rb1 resulted in a 41.0% decrease in NO release when used at a concentration of more than 20 times the highest dose of CK.

CK inhibited IL-1 β-induced iNOS expression

The effect of CK on IL-1 β-induced iNOS expression was tested by western blotting. CK (5 μM) significantly inhibited iNOS expression triggered by IL-1 β (Figure 1C). However, Rb1 did not reduce iNOS levels, even when used at 200 μM (Figure 1D). Next, RT-PCR was performed to ascertain whether expression of iNOS mRNA was consistent with that of the corresponding protein. CK also suppressed IL-1 β-induced iNOS mRNA expression in a dose-dependent manner (Figure 2).

CK inhibited IL-1 β-induced MAPK activation in chondrocytes

The effect of CK on MAPK activation resulting from IL-1 β stimulation was assessed by western blot analysis. CK suppressed IL-1 β-induced activation of p38, ERK1/2, and JNK MAPKs in a dose-dependent fashion, and this

was particularly evident in the case of ERK1/2 (Figure 3). Interestingly, the attenuation of IL-1β-induced phosphorylation of MAPK signaling proteins by CK was evident from a concentration of 2 μM, comparable to that at which NO production began to be inhibited.

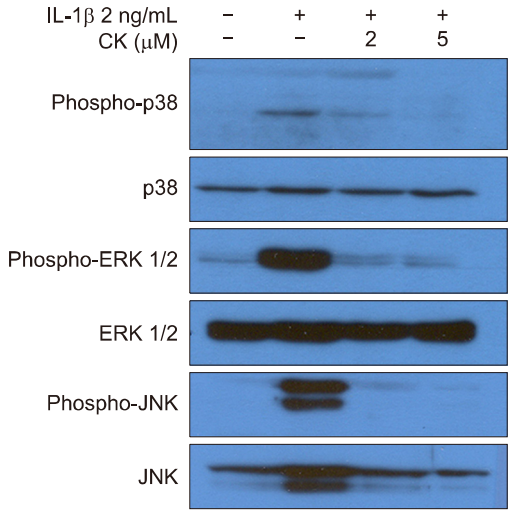

Figure 3. Compound K (CK) inhibits interleukin (IL)-1β-induced mitogen-activated protein kinase activation. ERK: extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase.

CK suppressed IL-1β-induced MMP expression and activity

Since MMPs are responsible for damaging the cartilage matrix, we examined the effects of CK on IL-1β-induced MMP expression and activity. CK suppressed IL-1β-induced MMP-13 mRNA expression (Figure 2). However, both CK and Rb1 had little effect on mRNA levels of MMP-1 and MMP-3, and no effect on those of TIMP-1. IL-1β-induced MMP activity (Figure 4A) was also inhibited by CK, especially at a concentration of 5 μM.

CK inhibited IL-1β-induced MMP-1, -3, and -13 and TIMP-1 production

The effects of CK on IL-1β-induced production of MMP-1, -3, and -13 and TIMP-1 were evaluated by ELISA. Treatment with CK reduced the elevated levels of MMP-13, -1, and -3 caused by IL-1β stimulation, especially at a concentration of 5 μM (Figure 4B). Furthermore, CK inhibited TIMP-1 production in a dose-dependent manner. In contrast, release of MMPs increased

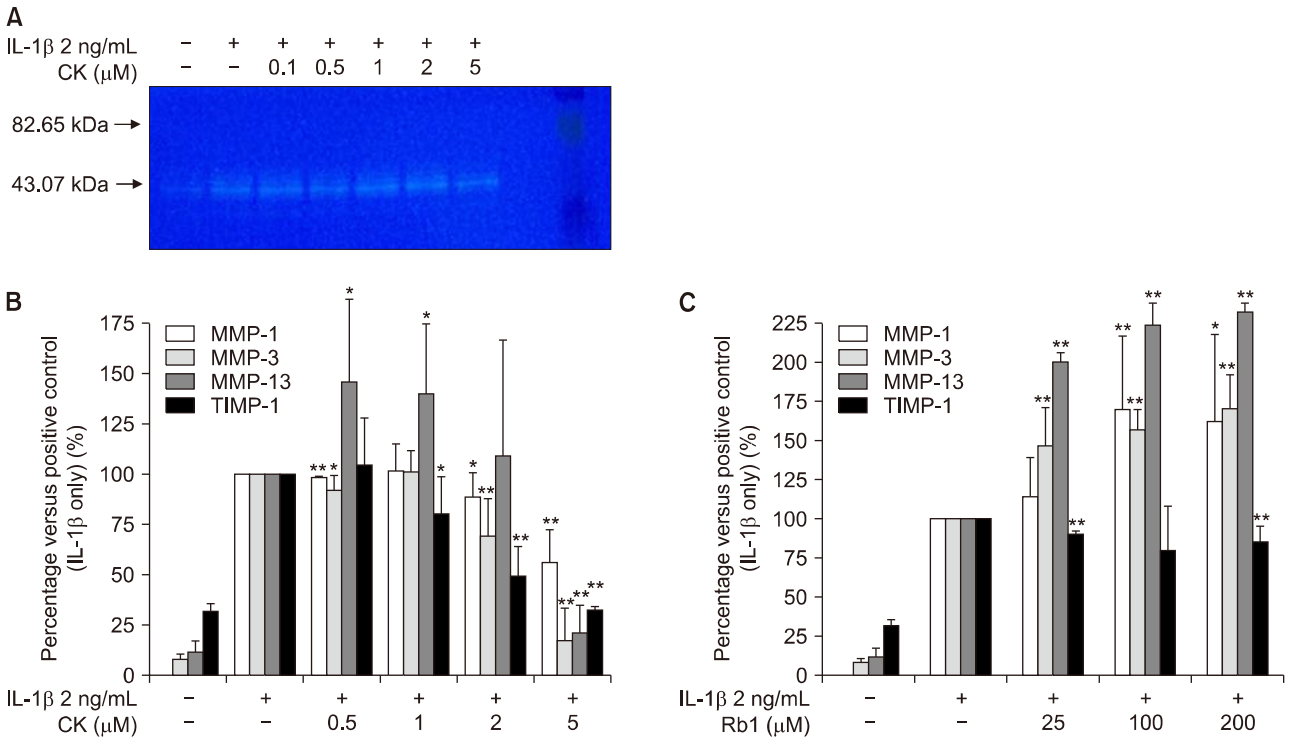

Figure 4. Activity of matrix metalloproteinases (MMPs) was inhibited by (A) compound K (CK) on the zymographic study. Production of MMP-1, MMP-3, MMP-13, and tissue inhibitor of metalloproteinases-1 (TIMP-1) measured by enzyme-linked immunosorbent assay was inhibited by (B) CK in a dose-dependent manner but not by (C) Rb1. IL: interleukin. The values presented are the means±standard error of mean of three independent experiments. *$p<0.05$, **$p<0.005$.

as the dose of Rb1 was raised (Figure 4C).

CK inhibited chondrocyte degeneration

The net effect of CK on chondrocyte degeneration was analyzed using a GAG assay. CK dose-dependent suppression of IL-1 β-mediated GAG release was observed (Figure 5A), whereas Rb1 failed to prevent GAG loss, even at higher doses (Figure 5B).

Effects of CK on cell viability

The cytotoxicity of CK to chondrocytes was evaluated with an MTT assay. Cell viability was not affected by CK at concentrations up to 10 μM (Figure 6). Therefore, the effects of CK on chondrocytes were not attributable to cytotoxicity.

DISCUSSION

In this study, CK effectively suppressed the IL-1 β-mediated release of the inflammatory mediator NO and MMPs including MMP-1, -3, and -13, at least in part by blocking MAPK activation. CK decreased IL-1 β-induced NO production by attenuating the expression of its upstream molecule, iNOS, at both the mRNA and protein level. In addition, CK was capable of inhibiting MMP production and activity triggered by IL-1 β.

It is widely accepted that excess production of inflammatory cytokines plays an important role in the pathogenesis of OA. Of such cytokines, IL-1 β has been shown to have a pivotal function in this disease due to its

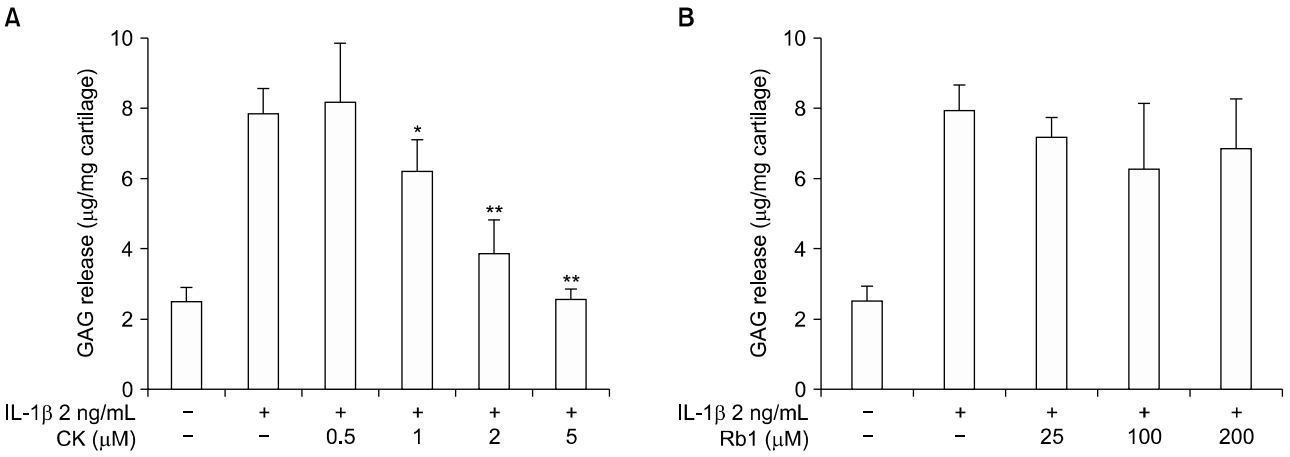

Figure 5. Effect of (A) compound K (CK) and (B) Rb1 on chondrocyte degeneration analyzed through the glycosaminoglycan (GAG) assay. IL: interleukin. The values presented are the means ± standard error of mean of three independent experiments. *$p < 0.05$, **$p < 0.005$.

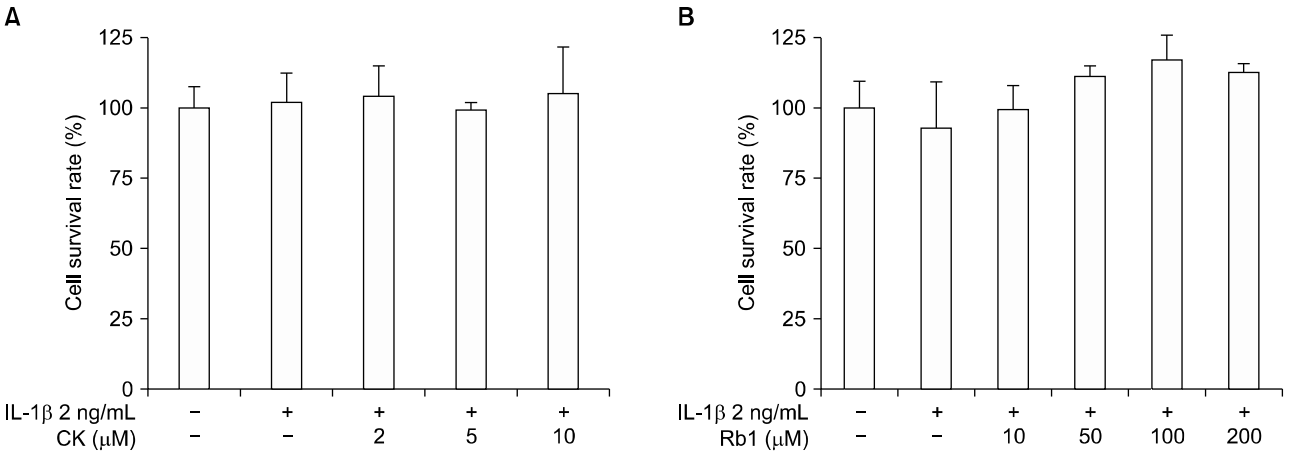

Figure 6. Effects of (A) compound K (CK) and (B) Rb1 on the cell viability of chondrocytes. The cells were cultured with different concentrations of CK (0 ~ 10 μM) or Rb1 (0 ~ 200 μM) for 24 hours. The cell viability was determined by MTT assay. IL: interleukin. The values are means ± standard error of mean of three independent experiments.

upregulation of MMP production, which leads to decreased synthesis of proteoglycan and collagen [22]. IL-1 β also induces the expression of iNOS and COX-2, resulting in elevated production of NO and PGE_2, respectively [6]. IL-1 β-mediated overproduction of NO has been reported to be an important proinflammatory factor in the development of OA due to its association with chondrocyte and synoviocyte death [23]. NO also upregulates the production of MMPs and other inflammatory cytokines [24]. In the present study, CK effectively inhibited chondrocyte NO production and iNOS expression induced by IL-1 β, suggesting that it has an anti-inflammatory effect on IL-1 β-stimulated chondrocytes.

MMPs are capable of degrading all of the components of the extracellular matrix (ECM) [25]. In particular, collagenases, including MMP-1, -8, and -13, specifically degrade type II collagen and proteoglycans through other MMPs in the ECM of cartilage, generating a microenvironment favoring the development of OA [8]. Whereas MMP-1 is expressed ubiquitously, MMP-13 is preferentially expressed by articular chondrocytes and is more closely linked to the degradation of type II collagen. The latter has thereby long been regarded as a key mediator of cartilage degradation in joint disorders [9]. MMP-3, responsible for the degradation of non-collagenous matrix components in OA, has been specifically linked to proteoglycan loss [26]. Our study demonstrated that CK effectively suppressed IL-1 β-stimulated MMP-1, -3, and -13 release by chondrocytes, which was associated with the inhibition of MMP mRNA expression, MMP-13 in particular. This indicates that CK treatment has a significant impact on cartilage homeostasis. CK administration had a greater effect on the release of MMP-13 than that of MMP-1, and also inhibited TIMP-1 production. Notably, the balance between MMPs and TIMPs has been reported to be disturbed in OA [8,27].

A growing body of evidence indicates that MAPK signaling pathways are critical in the regulation of inflammatory mediators [11,28]. Furthermore, they have been found to play a distinct role in cartilage matrix homeostasis, and alterations to such signaling, especially the p38 and ERK1/2 pathways, are associated with chondrocyte dysfunction as part of the pathogenesis and progression of OA [29]. Stimulating chondrocytes with IL-1 β results in phosphorylation and activation of ERK1/2, JNK, and p38 MAPKs [30]. In our study, CK inhibited the IL-1 β-induced activation of these proteins, implying that attenuation of MAPK activity partly accounts for the inhibitory effects of CK on the expression of genes encoding MMPs and iNOS, although other signaling pathways also exert an important influence on OA-related gene expression.

Ginsenoside Rb1 has been reported to inhibit TNF-α production in lipopolysaccharide-stimulated RAW264.7 macrophages [31], suppress activation of NF-κB [32], and reduce IL-1 β-induced NO production [33]. However, the results of our study revealed the anti-inflammatory effect of Rb1 to be minimal, even at much higher concentrations than those at which CK was used. Moreover, the dose of Rb1 required to achieve an anti-inflammatory effect may not be feasible in real-world clinical settings. Rb1 and CK are structurally similar but differ in the number of sugar moieties, and their effects on chondrocytes also vary.

CK is thought to have numerous biological and pharmacological properties, including anti-cancer, anti-diabetic, anti-tumor, anti-inflammatory, and anti-oxidant activity [34]. Previous studies have reported that the anti-inflammatory effects of CK are mainly brought about by reductions in iNOS, COX-2, and proinflammatory cytokine levels [32,35]. CK hinders inflammatory responses by controlling both ROS generation and MAPK, NF-κB, and activator protein-1 activity [35]. The anti-inflammatory activities of CK have also been explored in a variety of animal models of inflammation-associated disease [32,36]. In the present study, compared to Rb1, CK exerted a greater anti-inflammatory effect on IL-1 β-stimulated chondrocytes at much lower concentrations, suggesting that it may constitute a therapeutic option for OA patients.

Certain aspects of the subject under investigation merit further study. First, the mechanism connecting NO inhibition to the maintenance of cartilage homeostasis needs to be clarified, since Bezerra et al. [37] have reported that NOS reduces inflammation while paradoxically enhancing cartilage damage. Furthermore, given that previous attempts targeting inflammation, such as anti-TNF treatment, were not successful in OA treatment [38], whether the anti-inflammatory activity of CK on IL-1 β-stimulated osteoarthritic chondrocytes leads to a successful OA treatment also needs to be elucidated. However, CK possesses not only anti-inflammatory effects but also chondroprotective effects, which were proven by its ability to suppress the IL-1 β-mediated release of MMP-1, -3, and -13, and inhibit chondrocyte degeneration as analyzed by GAG assay. Hence, CK may serve as

a potential therapeutic agent in the treatment of OA; further in vivo studies are warranted. We demonstrated here that CK suppressed IL-1β-induced MAPK activation; however, the key MAPK signaling pathway factors involved remain to be elucidated. hypoxia-inducible factor (HIF)-2α is a transcription factor with catabolic effects in the osteoarthritic process, and acts as a central regulator of endochondral bone formation through direct regulation of collagen X, MMP-13, and vascular endothelial growth factor expression [39]. Overexpression of HIF-2α is known to directly cause progressive cartilage damage by upregulating the expression of various degradative enzymes, including MMP-1, -3, and -13 and ADAM metallopeptidase with thrombospondin type 1 motif 4 [40]. Further investigation to establish whether CK inhibits HIF-2α expression would therefore also be beneficial and interesting. Finally, additional studies are warranted to test the effect of CK during the various stages of OA.

CONCLUSION

The results of this study demonstrate that CK suppresses IL-1β-induced expression of NO, iNOS, and MMP-1, -3, and -13 in chondrocytes from OA patients. Moreover, this effect is mediated, at least in part, by the regulation of MAPK signaling pathways. Considering its anti-inflammatory and chondroprotective effects on IL-1β-stimulated osteoarthritic chondrocytes, CK may serve as a therapeutic agent in the treatment of this disease.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

SUPPLEMENTARY DATA

Supplementary data can be found with this article online at https://doi.org/10.4078/jrd.2018.25.3.188.

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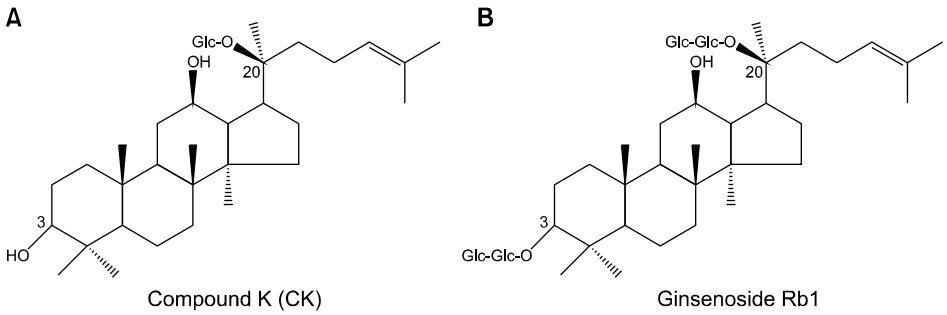

Supplementary Figure 1. Chemical structure of (A) compound K (CK) and (B) ginsenoside Rb1.