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Melittin has a chondroprotective effect by inhibiting MMP-1 and MMP-8 expressions via blocking NF-κB and AP-1 signaling pathway in chondrocytes

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ABSTRACT

Bee venom is a natural ingredient produced by the honey bee (*Apis mellifera*), and has been widely used in China, Korea and Japan as a traditional medicine for various diseases such as arthritis, rheumatism, and skin diseases However, the regulation of the underlying molecular mechanisms of the anti-arthritis by bee venom and its major peptides is largely unknown. In this study, we investigated the potential molecular mechanisms underlying the anti-arthritis effect of bee venom and its major peptides, melittin and apamin, in tumor necrosis factor-α (TNF-α) responsive C57BL/6 mice chondrocyte cells. The bee venom and melittin significantly and selectively suppressed the TNF-α-mediated decrease of type II collagen expression, whereas the apamin had no effects on the type II collagen expression. We, furthermore, found that the bee venom and melittin inhibited the protein expression of matrix metalloproteinase (MMP)-1 and MMP-8, which suggests that the chondroprotective effect of bee venom may be caused by melittin. The inhibitory effects of melittin on the TNF-α-induced MMP-1 and MMP-8 protein expression were regulated by the inhibition of NF-kB and AP-1. In addition, melittin suppressed the TNF-α-induced phosphorylation of Akt, JNK and ERK1/2, but did not affect the phosphorylation of p38 kinase. These results suggest that melittin suppresses TNF-α-stimulated decrease of type II collagen expression by the inhibiting MMP-1 and MMP-8 through regulation of the NF-kB and AP-1 pathway and provision of a novel role for melittin in anti-arthritis action.

1. Introduction

Arthritis is caused by the breakdown of articular cartilage. It has two classic varieties: rheumatoid arthritis (RA) and osteoarthritis (OA). RA is a systemic-autoimmune disease, caused by inflammation of multiple joints or synovial hyperplasia [1]. OA is called degenerative arthritis and is initiated by joint injury and infection, obesity, gene mutation, and aging [2]. It has a metabolically active and dynamic process that involves joint tissues such as cartilage, synovia, subchondral bones, and periarticular soft tissues. At the molecular level, arthritis is characterized by the degradation of cartilage, specifically extra-cellular matrix (ECM) components, which are maintained by the equilibrium between the anabolic and catabolic activities of the chondrocytes [3].

Chondrocytes are the only cell type present in the cartilage. They play an important role in articular cartilage remodeling and maintenance. They are affected by various growth factors and the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which inhibit the anabolic activities of ECM synthesis and trigger the catabolic pathways [1,3,4]. Particularly, TNF-α is a major pro-inflammatory cytokine the level of which increases in the synovial fluid of arthritis

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patients. It has been suggested as being produced by chondrocytes or infiltrating cells in OA [5]. Its release is associated with RA via the neutrophils and monocytes/macrophages that infiltrate the inflammation sites [6,7]. In addition, chondrocytes or chondrocyte-like cell lines have demonstrated the existence of functional TNF-α receptors [8]. TNF-α cleaves to different components of the cartilage ECM by inducing MMP expression in chondrocytes [9].

The cartilage matrix is degraded primarily by MMPs, which include collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), and stromelysins (MMP-3 and MMP-10). Among the MMPs, the levels of collagenases are elevated in the joint tissues of RA and OA patients [10,11]. Particularly, MMP-1 is known to degrade of Type II collagen, which is a major component of chondrocytes [12]. Also, there is a significant homology between MMP-8 and MMP-1, with a 57% likeness to, and a 72% chemical similarity with the deduced protein sequence [13]. Thus, it is very important to explain the regulation of MMP-1 and MMP-8 and the mechanisms involved in chondrocytes to develop new ways to protect cartilage in RA and OA.

Bee venom is a natural ingredient produced by the honey bee (*Apis mellifera*), and has been widely used in China, Korea and Japan as a traditional medicine for various diseases such as arthritis, rheumatism, and skin diseases [14,15]. It has many biologically active enzymes, peptides and biogenic amines, such as melittin, apamin, adolapin, and mast cell-degranulating peptide [16]. Additionally, bee venom has been reported as having biological and pharmacological activities, including anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer effects [17–19]. However, the regulation of the underlying molecular mechanisms of the anti-arthritis by bee venom and its major peptides is largely unknown.

To gain better insight on the anti-arthritis effects of bee venom, this study investigated the anti-arthritis mechanisms of bee venom and its peptides in mouse rib chondrocytes. The investigation showed that melittin suppressed TNF-α-mediated decrease of type II collagen expression by inhibiting MMP-1 and, MMP-8 expression, but it did not affect the type I collagen. Moreover, the regulatory effect of melittin on the collagen expression appeared to have blocked NF-κB by suppressing the Akt, JNK and ERK pathway in the chondrocytes.

2. Materials and methods

2.1. Cells and materials

Primary chondrocytes were isolated from the rib cages of C57BL/6 mice at ages of 3–6 days. Ribs were cleared of the surrounding tissues via treatment of 0.1% collagenase and 0.02% trypsin. The rib cartilage was cut off at a distance from the sternum and costochondral junction. After washing with PBS, the rib cartilage was treated with 0.1% collagenase in PBS, at 37 °C for 1 h to remove remnant of the perichondrium. Isolated chondrocyte cells (1×10^6 cells/dish) were incubated in DMEM containing 10% FBS and 1% antibiotic mixture in a 5% CO_2 at 37 °C. All assays were performed on chondrocyte cells at passages 1–3. All surgical and experimental procedures used in this study were approved by the Institutional Review Board Committee at Daegu Catholic University Medical Center which conforms to the US National Institutes of Health guidelines for care and use of laboratory animals. Bee venom was obtained from the National Institute of Agricultural Science and Technology (NIAST), Suwon, Korea. Chemicals were obtained from Sigma (St. Louis, MO), unless otherwise indicated.

2.2. Cell viability assays

Cells were plated in 96-well culture plates at 1×10^4 cells/well in DMEM culture medium and allowed to attach for 24 h. Media were then discarded and replaced with 100 μl of new medium containing various concentrations of ascofuranone and cultured for 24 h. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT) (Roche Molecular Biochemicals, Indianapolis, IN) was added to each well. The amount of formazan deposits was quantified according to the supplier's protocol after 4 h of incubation with MTT test solution in a 37 °C and 5% CO_2 incubator.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. For RT-PCR, a cDNA was synthesized from 1 μg of total RNA using AMV RNA PCR Kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: type I collagen (347 bp), 5′-GCTGGC TCCAAAGGAGAATC-3′ (sense) and 5′-GCCTTCTTTAC CAGATGGGC-3′ (antisense); type II collagen (288 bp), 5′-CAGATGGGAGTC ATGCAA GG-3′ (sense) and 5′-CTCTGTGACCCTTGACACCG-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

2.4. Western blot analysis

Cell lysates, SDS-PAGE, transfer to an Immonobilon-p-membrane (Millipore, USA), and immunoblotting were performed as described previously [20]. To determine the translocations of NF-κB (p65) and AP-1 (c-jun and c-fos), nuclear extracts of cells were subjected to describing as follows. Cells were suspended in tubes with 0.4 ml of lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. Cells were then allowed to swell on ice for 15 min, and 25 μl of 10% Nonidet P-40 was added. Homogenates were centrifuged at 4 °C for 2 min at 13,000 rpm. The nuclear pellets were resuspended in 50 μl of ice-cold nuclear extraction buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin, and incubated on ice for 15 min with intermittent mixing. Nuclear extracts were then centrifuged at 4 °C for 5 min at 13,000 rpm and supernatants were either used immediately. Specific antibodies for MAPKs and phospho-MAPKs were purchased from Santa Cruz (Santa Cruz, CA).

2.5. Statistical analysis

All in vitro-results presented here derived from at least three independent experiments performed in triplicate. The significances of differences between experimental and control values were calculated using analysis of variance with the Newman–Keuls multi-comparison test. P values of <0.05 were deemed to be significantly different.

3. Results

3.1. Effects of bee venom and its peptides on TNF-α-mediated decrease of type II collagen expression in chondrocytes

Before the pharmacological potential of bee venom and its major peptides in arthritis was investigated, the cytotoxicity of bee venom, melittin, and apamin on chondrocytes was analyzed through an MTT assay. Apamin showed more than 90% cell viability on the chondrocytes under the tested concentrations (0.5, 1, 2, and 4 μg/ml) after 24 h of treatment (Fig. 1A). However, the cell viability of bee venom and melittin decreased at 2 μg/ml concentration. Based on these results, non-cytotoxic concentrations of all the drugs were used for the further examination.

Next, to investigate the inhibitory effects of bee venom, melittin and apamin on arthritis, drugs with different concentrations (0.5 and 1 μg/ml) were tested on the TNF-α-stimulated chondrocyte. As shown in Fig. 1B, TNF-α significantly decreased the mRNA levels of types I and II