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The Anti-Inflammatory Effects of Bee Venom in Monosodium Urate Crystal-Induced THP-1 Cells

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Background: Although bee venom (BV) has clinical benefits in osteoarthritis and rheumatoid arthritis, it has not been tested as treatment for gouty arthritis. Moreover, in vitro, BV has been proven to exhibit anti-inflammatory and positive effects on osteoarthritis, but only limited evidence can confirm its beneficial effects on gout. Thus, this study aims to assess the anti-inflammatory effects of BV on monosodium urate (MSU)-induced THP-1 monocytes.

Methods: THP-1 monocytes were differentiated into mature macrophages using phorbol 12-myristate 13-acetate (PMA) and pretreated for 6 hours with BV and a Caspase-1 inhibitor in a physiologically achievable range of concentrations (BV, 0.1–1 μg/mL; Caspase-1 inhibitor, 1–10 μM), followed by MSU crystal stimulation for 24 hours. The secretions of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, IL-8, cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and nitric oxide (NO) were increased in the MSU crystal-stimulated THP-1 cells.

Results: Caspase-1 inhibitors suppressed the production of all mediators in a dose-dependent manner. BV worked on equal terms with Caspase-1 inhibitors and showed more satisfactory effects on TNF-α, PGE2, COX-2, and inducible nitric oxide synthase (iNOS). Moreover, the western blot analysis revealed that BV regulated the transcriptional levels of these mediators via the suppression of extracellular signal-regulated kinase (ERK) pathway activation.

Conclusion: The results of the present study clearly suggest that BV inhibits MSU-induced inflammation in vitro, suggesting a possible role for BV in gout treatment.

Keywords: Bee venom; ERK pathway; Gout; Macrophage; Monosodium urate; THP-1 cell

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INTRODUCTION

Gout is a chronic urate metabolism disorder that is mostly caused by the under-excretion of uric acid. It is also the most common cause of arthritis in men with osteoarthritis. Its prevalence has been increasing worldwide and comprises approximately 3.9% of the total United States population [1]. Acute gout is characterized by a sudden onset of extremely painful self-limited inflammatory attacks of mono- or oligo-articular arthritis. In addition to this type of attack, some patients may develop chronic arthritis refractory to treatment, which is characterized by the formation of tophi. This type of arthritis often results in joint destruction, bone erosion, disfigurement, and disability [2]. Although oral colchicine, nonsteroidal anti-inflammatory drugs (NSAIDs), and daily corticosteroids have been used as management options for acute gout attacks [1], they are also associated with the risk of intolerances or adverse effects [3]. Therefore, the identification of new medications for the treatment or prevention of gout flares is important.

Gout inflammation is initiated when monosodium urate (MSU) crystals are phagocytosed by macrophages or other cells in the joints [4]. This results in the assembly of the nucleotide oligomerization domain-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasomes, a multimeric protein complex responsible for activating Caspase-1, which in turn cleaves the pro-interleukin-1β (IL-1β), resulting in the production and secretion of active IL-1β [5]. Other factors are also upregulated during gout inflammation, including IL-6, IL-8, and tumor necrosis factor-α (TNF-α) [4].

Bee venom (BV) contains a variety of peptides, including melittin (a major component of BV), phospholipase A2, apamin, adolapin, and mast cell degranulating peptides [6]. It is clinically beneficial as treatment for osteoarthritis [7] and rheumatoid arthritis [8]. Moreover, it has anti-inflammatory and positive effects on osteoarthritic chondrocytes, synoviocytes, and subchondral bone osteoblasts [9,10]. However, its treatment effects on gout need to be further investigated. The present study assessed the anti-inflammatory effects of BV on MSU-induced THP-1 monocytes.

MATERIALS AND METHODS

1. Chemicals and reagents

The MSU crystals were obtained from InvivoGen and dissolved in sterile phosphate-buffered saline (PBS) at 5 mg/mL. Phorbol 12-myristate 13-acetate (PMA) was purchased from Enzo Life Sciences and dissolved in dimethyl sulfoxide (DMSO). Caspase-1 inhibitor was purchased from Merck Millipore and used in DMSO. All the glassware used during the preparation of the chemicals or reagents were sterilized by autoclaving and dried at 180℃ for 2 hours.

2. Cell culture

The human THP-1 cell line was distributed from the Korean Cell Line Bank and grown in a Roswell Park Memorial Institute (RPMI) 1640 medium, with the addition of 10% fetal bovine serum (FBS), 2-mercaptoethanol 0.05 mM 4.5 g/L D-glucose, 2 mM L-glutamine, 10 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES), 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and antimicrobial agents (100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mg/mL gentamicin, sulfate, and 2.5 mg/mL amphotericin B) at 37℃ in a humidified atmosphere with 5% carbon dioxide (CO_2). These cells were induced to differentiate into mature macrophages using PMA at a concentration of 0.5 μM for 3 hours. After induction, the cells were washed with RPMI and incubated for 18–20 hours in normal media.

3. Cell viability assay

The cytotoxic effects of BV and Caspase-1 inhibitors were also assessed. Purified BV was purchased from Chungjin Biotech. PMA-disposed THP-1 cells (0.5 μM, 3.0 × 10^5 cells/well) were seeded and incubated overnight in 24-well microplates. Subsequently, they were exposed to varying concentrations of BV (0, 1, 10, 100, 1,000, 3,000, 5,000, and 10,000 ng/mL) and Caspase-1 inhibitors (0, 0.1, 1, 5, 10, and 20 μM) and incubated for 24 hours. Water-soluble tetrazolium-1 (WST-1) was then added, and the cells were continuously cultured for an additional 3 hours at 37℃. The absorbance was measured at 450 nm.

4. Macrophage activation studies

Different concentrations of MSU crystals in the physiological range (concentrations of serum uric acid that are possible in humans, i.e., up to 20 mg/dL) [11] were initially tested to establish the conditions for inducing proinflammatory cytokines from activated macrophages. MSU crystals (2.5, 5.0, 10.0, and 20 mg/dL) were added to differentiated cells and incubated for 24 hours. Culture supernatant was centrifuged (1,500 rpm, 5 minutes), and the upper layer was extracted and stored at temperatures of –80℃. In this study, the enzyme-linked im-

munosorbent assay (ELISA) was used in the measurement of the IL-1β /IL-1F2, IL-6, IL-8/CXCL8, and TNF-α levels. Differentiated cells also underwent the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for IL-1β, IL-6, IL-8, TNF-α, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS).

5. RNA extraction and RT-PCR analysis

0.5 μM PMA-disposed THP-1 cells were seeded and incubated overnight in 6-well plates (2.0×10^6 cells/well). They were then treated with varying concentrations of BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM) and incubated for 6 hours. Subsequently, MSU (20 mg/mL) was added and the cells were continuously cultured for 24 hours. The cells were homogenized using 1 mL of TRIzol reagent (Invitrogen) and centrifuged. The supernatant was then extracted, mixed with chloroform, and centrifuged. The upper layer of the recentrifuged solution was sampled, added with isopropanol, centrifuged, and washed with 70% ethanol. The pellet was dissolved in diethyl pyrocarbonate (DEPC). Absorbance was measured at 260 nm to determine the RNA quantity. Subsequently, reverse transcription was performed using a PrimeScript 1st strand cDNA synthesis kit (TAKARA) in accordance with the manufacturer's instructions. A PCR analysis was conducted using synthesized cDNA (1 μL), Power SYBR Green PCR master mix (2 × 10 μL, Applied Biosystems), DEPC-treated distilled water, and the following primers: iNOS, sense primer 5′-TGG ATG CAA CCC CAT TGT C-3′ and antisense primer 5′-CCC GCT GCC CCA GTT T-3′; COX-2, sense primer 5′-CAA ATC CTT GCT GTT CCC ACC CAT-3′ and antisense primer 5′-GTG CAC TGT GTT TGG AGT GGG TTT-3′; IL-1β, sense primer 5′-TGA TGG CTT ATT ACA GTG GCA ATG-3′ and antisense primer 5′-GTA GTG GTG GTC GGA GAT TCG-3′; IL-6, sense primer 5′-TTT CTG ACC AGA AGA AGG AA-3′ and antisense primer 5′-CTG GTC TTT TGG AGT TTG AG-3′; IL-8, sense primer 5′-ACT TTC AGA GAC AGC AGA GC-3′ and antisense primer 5′-GTG GTC CAC TCT CAA TCA CT-3′; TNF-α, sense primer 5′-CTA CAG CTT TGA TCC CTG AC-3′ and antisense primer 5′-CGG TCT CCC AAA TAA ATA CA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense primer 5′-ACC ACA GTC CAT GCC ATC AC-3′ and antisense primer 5′-TCC ACC ACC CTG TTG CTG TA-3′. The thermocycling programs consisted of 40 cycles at 95℃ for 15 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds (for all instances).

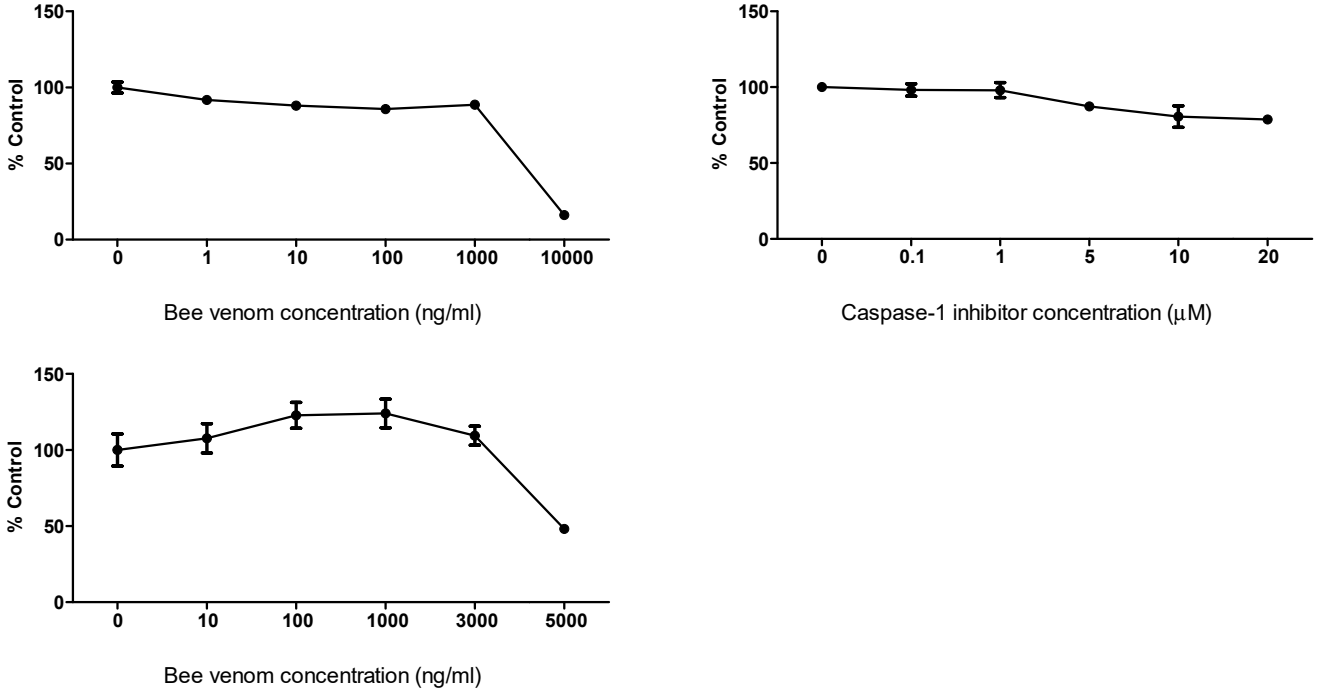

Fig. 1. Macrophages were exposed to varying concentrations of bee venom (BV) and Caspase-1 inhibitors to evaluate the cytotoxic effect. 0.5 μM phorbol 12-myristate 13-acetate-disposed THP-1 cells (3.0×10^5 cells/well) were exposed to varying concentrations of BV (0, 1, 10, 100, 1,000, 3,000, 5,000, and 10,000 ng/mL) and Caspase-1 inhibitors (0, 0.1, 1, 5, 10, and 20 μM) and incubated for 24 hours. Subsequently, the absorbance at 450 nm was measured. The concentrations used in the subsequent experiments were determined as BV (0.1 and 1 μg/mL) and Caspase-1 inhibitor (1 and 10 μM).

6. Western blot analysis

0.5 μM PMA-disposed THP-1 cells were seeded and incubated overnight in 6-well plates (2 × 10^6 cells/well). Subsequently, they were treated with varying concentrations of BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM) and incubated for 1 hour. Moreover, MSU (20 mg/mL) was added and the cells were continuously cultured for 24 hours. The cells were washed twice with ice-cold PBS and a lysis buffer (Pro-prep extraction solution) was added. The lysate was separated using 4–12% Bis-Tris Mini gels and transferred to polyvinylidene difluoride (PVDF) membranes at 30 V for 2 hours. Following the blockage of non-specific binding sites with 5% skim milk for 16–20 hours, the membranes were incubated overnight at 4℃ with appropriate dilutions of primary antibodies. They were then incubated with secondary antibodies (horseradish peroxidase [HRP]-conjugated anti-rabbit immunoglobulin G [IgG]) for 1 hour. Finally, the bands were visualized using enhanced chemiluminescence (ECL) reagents. The relative intensity of each protein was quantified and compared with the β-actin signals from the same sample. The primary antibodies used in this study were #9102 (p44/42 mitogen-activated protein kinase [MAPK] antibody) and #9101 (phospho-p44/42 MAPK antibody) that were obtained from Cell Signaling Technology. The secondary antibody used in this study was 65-6120 (goat anti-rabbit antibody), which was obtained from Invitrogen.

7. Enzyme-linked immunosorbent assay

PMA-disposed THP-1 cells (0.5 μM) were seeded and incubated overnight in 6-well plates (2.0 × 10^6 cells/well). Subsequently, they were treated with varying concentrations of BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM) and incubated for 6 hours. Moreover, MSU (20 mg/mL) was added and the cells were continuously cultured for 24 hours. Culture supernatant was centrifuged (1,500 rpm for 5 minutes) and the upper layer was extracted and stored at temperatures of −80℃. In this study, the ELISA was used in the measurement of the IL-1 β/IL-1F2, IL-6, IL-8/CXCL8, TNF-α, and prostaglandin E2 (PGE2) levels (R&D systems).

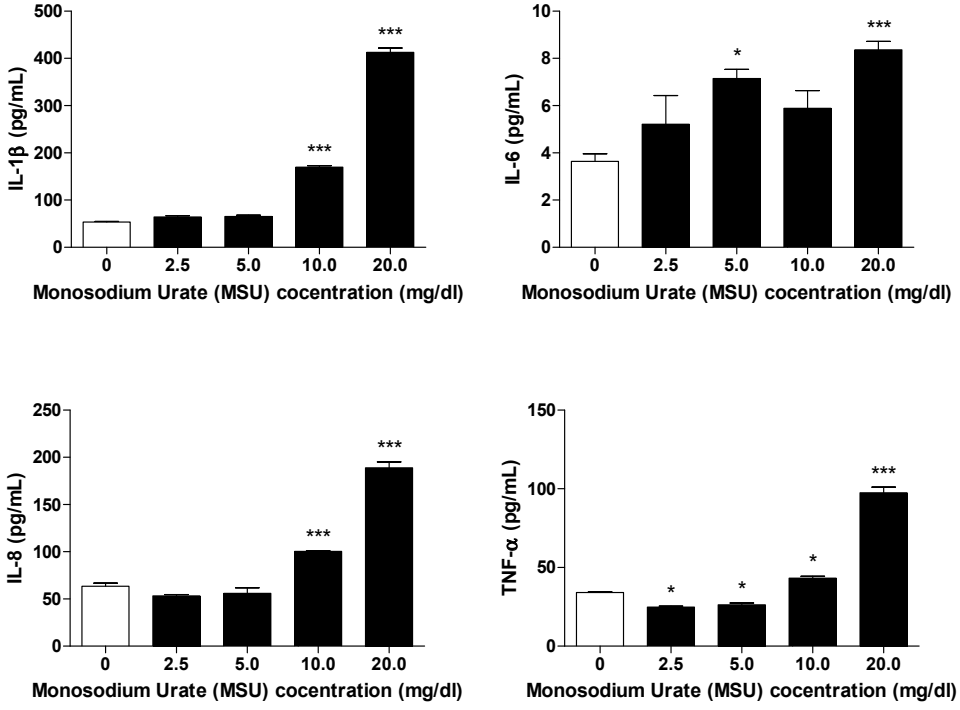

Fig. 2. The ELISA results revealed that the exposure of macrophages to high physiological concentrations of monosodium urate (MSU) crystals induced the production of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). THP-1 macrophages were exposed to MSU crystals of varying concentrations (0, 2.5, 5.0, 10.0, and 20.0 mg/dL) for 24 hours. MSU concentrations of ≥ 10 mg/dL consistently induced the production of IL-1β, IL-6, IL-8, and TNF-α, while 2.5 mg/dL of MSU-induced IL-6 production. Thus, MSU concentrations of 20 mg/dL were used for the subsequent experiments. The leftmost bar is the negative control (with no MSU). ***$p < 0.001$ vs. control group; *$p < 0.05$ vs. control group.

RESULTS

The cytotoxic effects of BV and Caspase-1 inhibitors were evaluated using a conventional WST assay (Fig. 1). Consequentially, this prompted us to choose 0.1 and 1 μg/mL for BV and 1 and 10 μM for the Caspase-1 inhibitors for all subsequent experiments.

Both the ELISA and RT-PCR results revealed that increasing concentrations of MSU crystals led to the increase in the production of IL-1β, IL-6, IL-8, TNF-α, iNOS, and COX-2 (Figs. 2, 3). Specifically, MSU concentrations of 20 mg/dL mostly increased the production of IL-1β, IL-6, IL-8, TNF-α, iNOS, and COX-2 by the macrophages. Thus, a concentration of 20 mg/dL was used in the subsequent experiments.The cells were stimulated with a high concentration of MSU (20 mg/dL) with preincubation according to varying concentrations of the Caspase-1 inhibitors (1 and 10 μM) and BV (0.1 and 1 μg/mL).

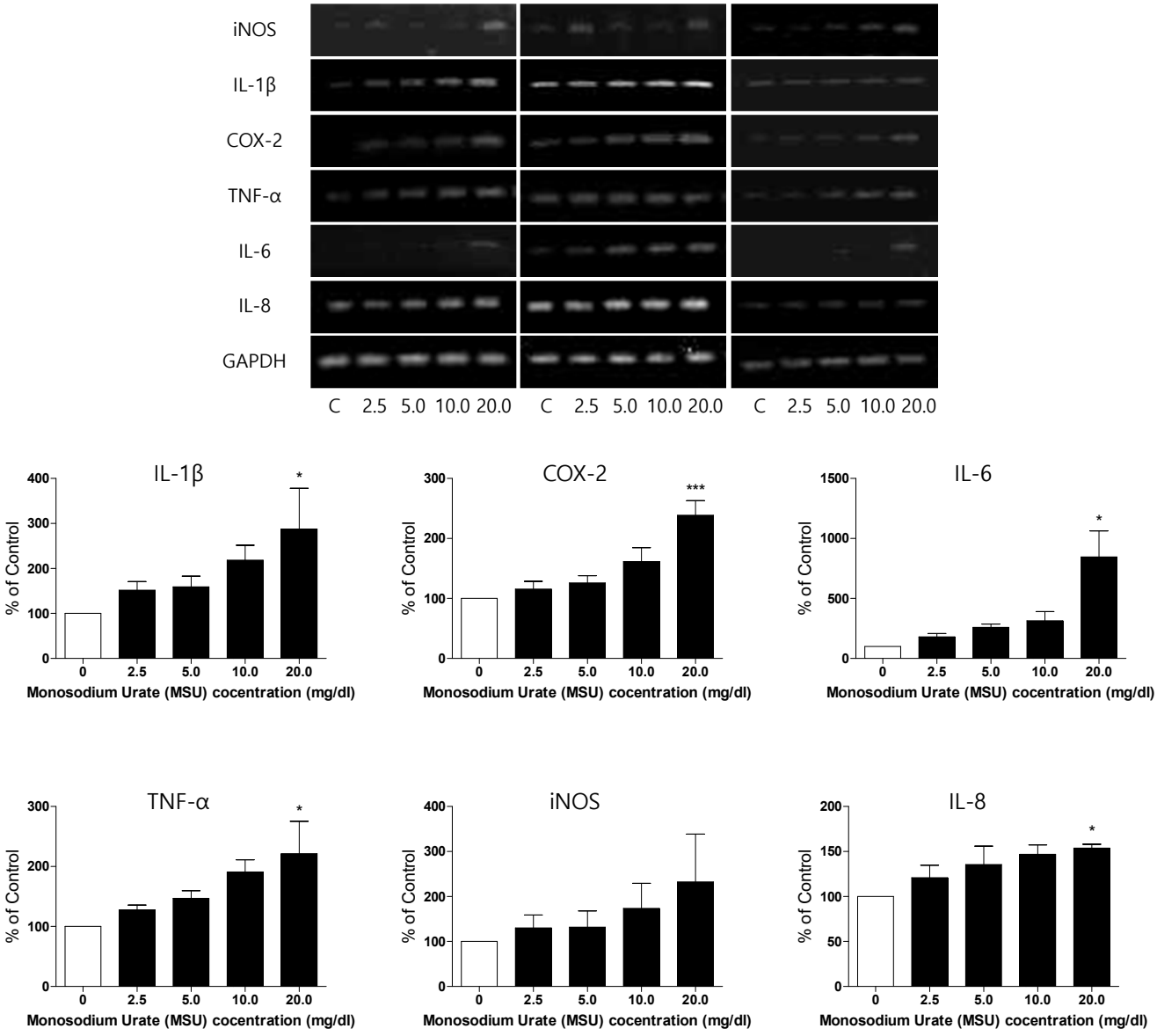

Fig. 3. The RT-PCR results revealed that the exposure of macrophages to high physiological concentrations of monosodium urate (MSU) crystals induced the production of IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). THP-1 macrophages were exposed to MSU crystals of varying concentrations (0, 2.5, 5.0, 10.0, and 20.0 mg/dL) for 24 hours. MSU induced the production of IL-1β, IL-6, IL-8, TNF-α, and iNOS in a dose-dependent manner, while MSU concentrations of ≥ 20 mg/dL induced IL-6 production. Thus, MSU concentrations of 20 mg/dL were used for the subsequent experiments. The leftmost bar is the negative control (with no MSU). ***$p < 0.001$ vs. control group; *$p < 0.05$ vs. control group.

RT-PCR analysis was performed for the measurement of the IL-1β, IL-6, IL-8, TNF-α, COX-2, and iNOS levels (Fig. 4). Phospho-ERK (P-ERK) was measured via western blot analysis (Fig. 5), while the PGE2 levels were measured using ELISA (Fig. 6). Six cytokines associated with gout inflammation, i.e., IL-1β, IL-6, IL-8, COX-2, iNOS, and PGE2, were fully inhibited by the Caspase-1 inhibitors in a dose-dependent manner, confirming inflammasome activation as the source of this cytokine. However, TNF-α was not inhibited by the Caspase-1 inhibitors (Figs. 4, 5). Macrophage exposure to BV for 6 hours prior to MSU led to a significant dose-dependent decrease in the production of IL-1β, IL-6, IL-8, COX-2, iNOS, TNF-α, and PGE2. BV worked on equal terms with Caspase-1 inhibitors and showed more satisfactory effects on TNF-α, PGE2, COX-2, and iNOS (Figs. 4, 5). Phospo-ERK produc-

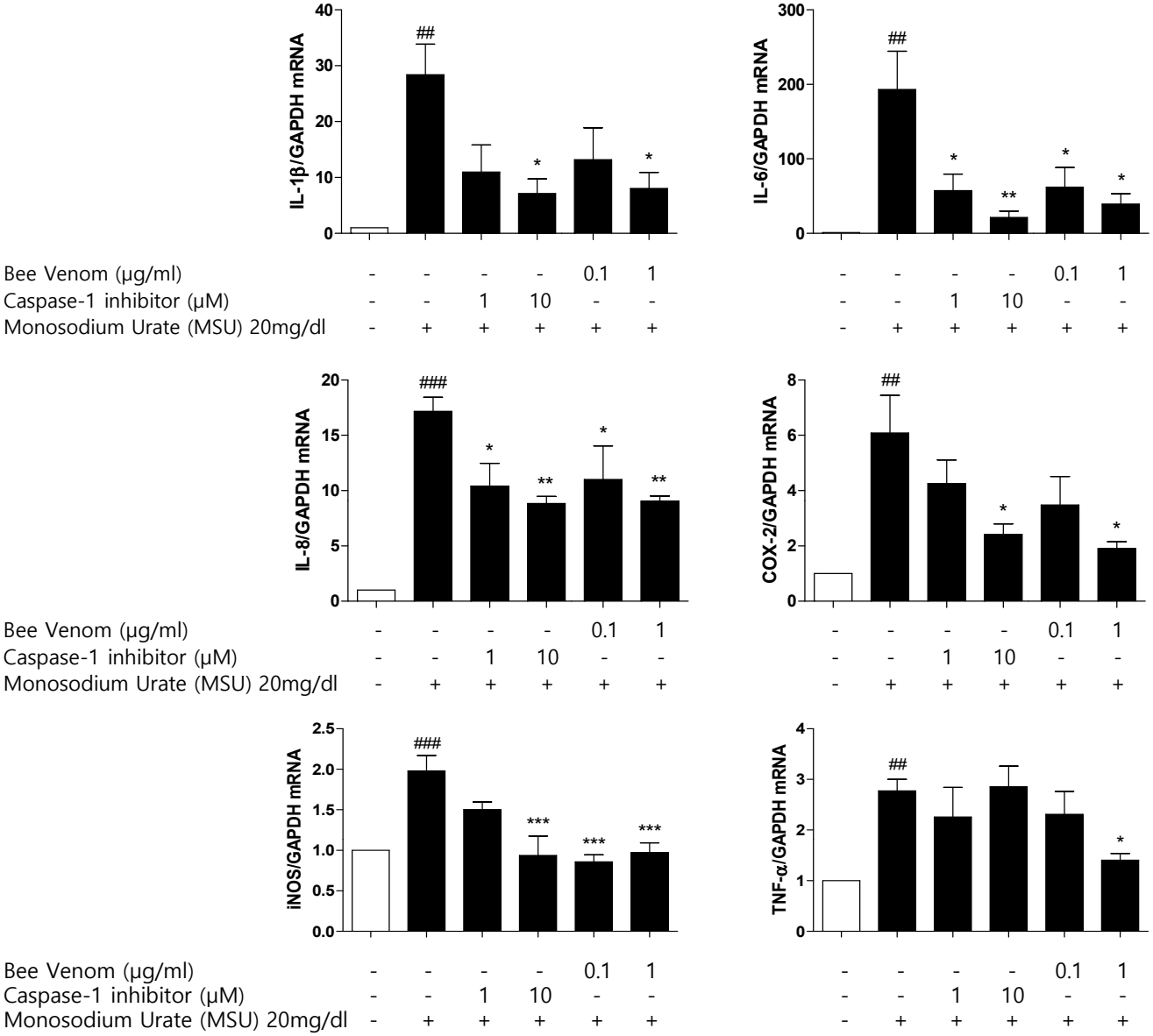

Fig. 4. Bee venom (BV) inhibited the monosodium urate (MSU)-induced cytokine production. THP-1 macrophages were pretreated for 6 hours with BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM), followed by the stimulation with MSU (20 mg/mL) for 24 hours. The cells were analyzed via reverse transcriptase-polymerase chain reaction. BV led to a significant dose-dependent decrease in the production of interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-α (TNF-α). BV worked on equal terms with Caspase-1 inhibitors and showed more satisfactory effects on TNF-α, COX-2, and iNOS. The leftmost bar is the negative control (with no MSU), whereas the second bar is the positive control (only MSU). $^{###}p < 0.001$ vs. control group; $^{##}p < 0.01$ vs. control group; $^{***}p < 0.001$ vs. MSU group; $^{**}p < 0.01$ vs. MSU group; $^{*}p < 0.05$ vs. MSU group.

tion was inhibited by both the Caspase-1 inhibitors and BV.

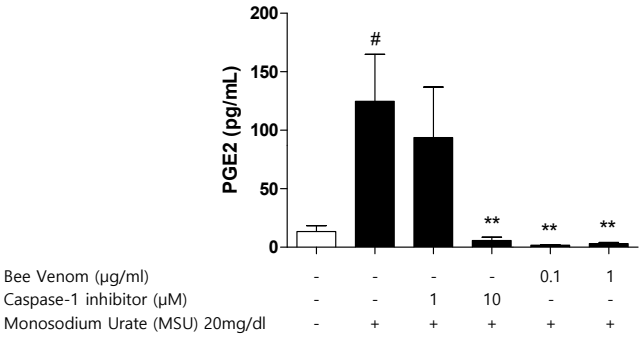

Fig. 5. Bee venom (BV) inhibited the monosodium urate (MSU)-induced prostaglandin E2 (PGE2) production. THP-1 macrophages were pretreated for 6 hours with BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM), followed by the stimulation with MSU (20 mg/mL) for 24 hours. The cells were analyzed via ELISA. BV led to a significant decrease in the production of PGE2 and showed more satisfactory effects compared with the Caspase-1 inhibitors. The leftmost bar is the negative control (with no MSU), whereas the second bar is the positive control (only MSU). $^{\#}p < 0.05$ vs. control group; $^{**}p < 0.01$ vs. MSU group.

DISCUSSION

Our results revealed that the anti-inflammatory effects of BV affect the transcription of various cytokines.

To date, IL-1β signaling is considered to be the initiatory event that induces gout inflammation and promotes the recruitment of numerous neutrophils at the site of inflammation [12]. The main cytokines with secondary involvement in this process are IL-8, IL-6, and TNF-α. IL-8 plays an important role in neutrophil recruitment and gout activation [4], whereas IL-6 is a proinflammatory cytokine whose production may be triggered by the exposure of synoviocytes and monocytes to MSU particles. Furthermore, it is involved in innate immunity and associated with the T helper 2 (Th2) pathway in the adaptive immune system. It is also involved in synovial activation and the regulation of osteoclastogenesis [13], and its association with bone damage in other inflammatory diseases is well-documented. This is presumed to be related to chronic gout arthritis [14]. TNF-α is involved with proinflammatory activation, maturation, and an increased monocyte to macrophage transformation [4]. Prostaglandins (PGs) are a product of the arachidonic acid metabolism of the COX pathway. PGE2 likely contributes to inflammation by increasing local blood flow

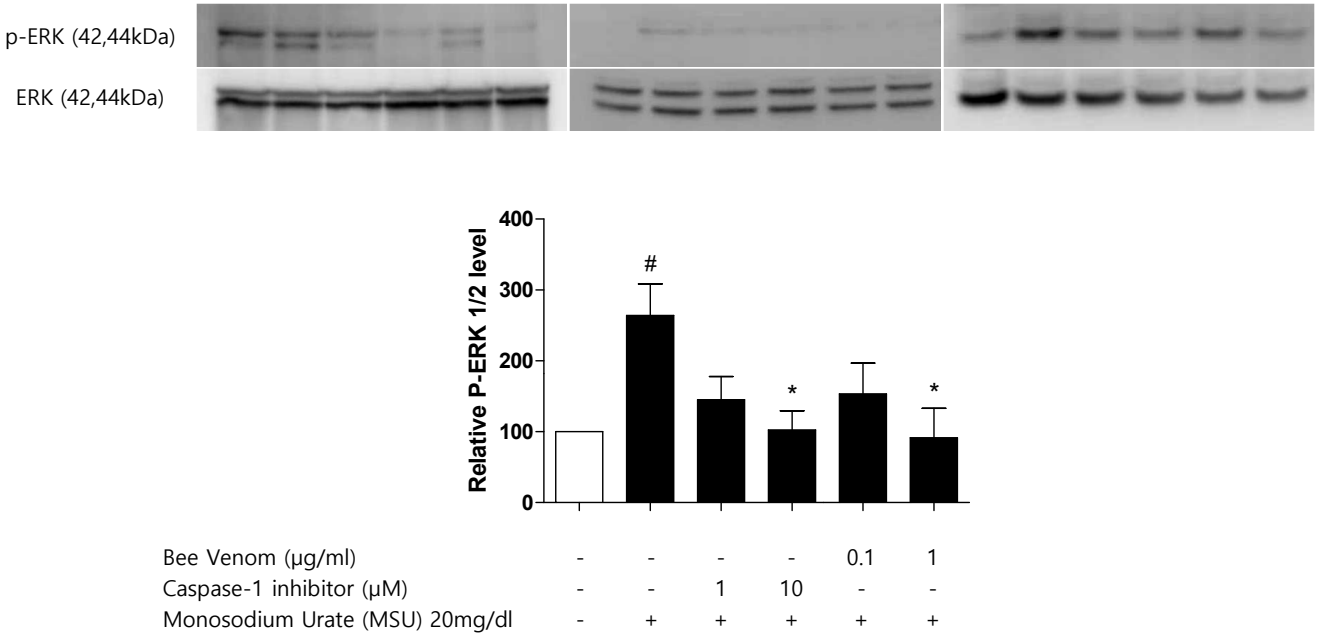

Fig. 6. The effect of bee venom (BV) on extracellular signal-regulated kinase 1/2 (ERK1/2) in monosodium urate (MSU) crystal-activated THP-1 cells. THP-1 macrophages were pretreated for 1 hour with BV (0.1 and 1 μg/mL) and Caspase-1 inhibitors (1 and 10 μM), followed by the stimulation with MSU (20 mg/mL) for 24 hours. Cellular proteins were prepared and analyzed for ERK1/2 and phospho-ERK 1/2 (P-ERK1/2), respectively. BV successfully inhibited the relative P-ERK1/2 level and Caspase-1 inhibitors. The leftmost bar is the negative control (with no MSU), whereas the second bar is the positive control (only MSU). $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ vs. MSU group.

and potentiating the effects of mediators, such as bradykinin and IL-1, which induce vasopermeability [15]. PGE2 has also been proven to trigger osteoclastic bone resorption, suggesting its influence on the pathophysiology of joint erosion in chronic arthritic diseases [16]. As mentioned previously, the COX pathway produces PGs. Two isoforms of the COX enzyme have recently been identified: COX-1 and COX-2. Moreover, COX-2 is the product of an immediate-early response gene in inflammatory cells. MSU activates iNOS and generates high concentrations of NO through the activation of inducible nuclear factors [17]. NO induces vasodilatation in the cardiovascular system and is involved in immune responses via cytokine-activated macrophages, which release NO at high concentrations [18].

Our data revealed that BV reduced the secretion of IL-1β, IL-6, IL-8, TNF-α, iNOS, and COX-2 as effectively as the Caspase-1 inhibitors, while BV also had more satisfactory effects on TNF-α, PGE2, COX-2 and iNOS. This suggests that BV attenuates MSU crystal-induced acute inflammation in THP-1 cells.

In gout inflammation, cytokines and chemokines lead to the activation of various MAPK signaling networks, thereby contributing to the induction of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and transcription of proinflammatory factors [19]. Notably, the activation of ERK has been shown to be an essential temporal regulator of NF-κB activity and the NF-κB-induced gene expression in response to IL-1β [20]. The removal of ERK1/2 by specific inhibitors reduced the activation of NF-κB and suppressed the transcription of NF-κB-dependent genes [21].

In the present study, BV suppressed the P-ERK production. The anti-inflammatory effects of BV may be mediated through the ERK signaling pathway. The findings of the present study imply that MSU crystals can induce macrophages to secrete inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, iNOS, and COX-2) and that BV will likely be more effective than Caspase-1 inhibitors in the treatment of gouty arthritis.

One of the limitations of our study is that the cells were pretreated with both BV and Caspase-1 inhibitors. Further co-treatment or post-treatment studies should be conducted to determine the additional treatment effects of BV on gout. Another limitation is the use of THP-1 cells, which were derived from the peripheral blood of a one-year-old male patient with acute monocytic leukemia. They are often used because of their ease of use. Moreover, it would be useful to repeat these experiments using primary peripheral monocytes or primary synovial macrophages. Furthermore, observing the effect of BV in an in vivo gout model would be challenging in revealing more about the effect of BV on joint destruction, bone erosion, disfiguration, and gout disability.

CONCLUSION

Both the ELISA and RT-PCR results revealed that increasing concentrations of MSU crystals led to the increase in the production of IL-1β, IL-6, IL-8, TNF-α, iNOS, and COX-2.

Six cytokines associated with gout inflammation, i.e., IL-1β, IL-6, IL-8, COX-2, iNOS, and PGE2, were fully inhibited by Caspase-1 inhibitors in a dose-dependent manner. However, TNF-α was not inhibited by the Caspase-1 inhibitor.

BV worked on equal terms with Caspase-1 inhibitors and showed more satisfactory effects on TNF-α, PGE2, COX-2, and iNOS.

Phospo-ERK production was inhibited by both Caspase-1 inhibitors and BV, indicating that the anti-inflammatory effect of BV may be induced through the ERK signaling pathway.

These results clearly suggest that BV inhibits MSU-induced inflammation in vitro, suggesting the potential of BV as an effective treatment for gout.

AUTHOR CONTRIBUTIONS

Conceptualization: YCP, FYC. Funding acquisition: YCP. Methodology: YCP, FYC. Investigation: SYC, DML. Data analysis: SYC, DML. Writing – original draft: SYC, DML. Writing – review & editing: All authors.

CONFLICTS OF INTEREST

There are no conflicts of interest regarding the publication of this manuscript.

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ETHICAL STATEMENT

This research did not involve any human or animal experiment.

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