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Inhibition of COX-2 Activity and Proinflammatory Cytokines (TNF-α and IL-1β) Production by Water-Soluble Sub-Fractionated Parts from Bee (*Apis mellifera*) Venom

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Bee venom is used as a traditional medicine for treatment of arthritis. The anti-inflammatory activity of the *n*-hexane, ethyl acetate, and aqueous partitions from bee venom (*Apis mellifera*) was studied using cyclooxygenase (COX) activity and pro-inflammatory cytokines (TNF-α and IL-1β) production, *in vitro*. COX-2 is involved in the production of prostaglandins that mediate pain and support the inflammatory process. The aqueous partition of bee venom showed strong dose-dependent inhibitory effects on COX-2 activity (IC_{50} = 13.1 μg/mL), but did not inhibit COX-1 activity. The aqueous partition was subfractionated into three parts by molecular weight differences, namely, B-F1 (above 20 KDa), B-F2 (between 10 KDa and 20 KDa) and B-F3 (below 10 KDa). B-F2 and B-F3 strongly inhibited COX-2 activity and COX-2 mRNA expression in a dose-dependent manner, without revealing cytotoxic effects. TNF-α and IL-1β are potent pro-inflammatory cytokines and are early indicators of the inflammatory process. We also investigated the effects of three subfractions on TNF-α and IL-1β production using ELISA method. All three subfractions, B-F1, B-F2 and B-F3, inhibited TNF-α and IL-1β production. These results suggest the pharmacological activities of bee venom on anti-inflammatory process include the inhibition of COX-2 expression and the blocking of pro-inflammatory cytokines (TNF-α, and IL-1β) production.

Key words: Bee venom, Cyclooxygenase-2, Anti-inflammatory activity, Tumor necrosis factor-α, Interleukin-1β

INTRODUCTION

Major efforts are being directed towards the identification of new classes of pharmacological agent with the capacity to inhibit the mediators of inflammation and tissue destruction as exemplified by the pro-inflammatory cytokines and prostanoids. Bee venom (BV) continues to be used as a folk medicine for the treatment of arthritis in Korea. The medicinal use of BV sourced substances has been practiced since ancient times. In the modern world, BV is widely used in the treatment of arthritis and other inflammatory and degenerative diseases. Bee sting or BV therapy has been reported to be effective in the treatment of rheumatic diseases (Zurier *et al.*, 1973). In animal models, adjuvant induced arthritis has been shown to be successfully suppressed by long-term BV treatment. BV or its substances have also been reported to be effective in the treatment of rheumatoid arthritis in humans. Recently, Kwon *et al.*, (2001) showed that BV treatment produced antinociceptive and anti-inflammatory effects in an adjuvant induced arthritis model.

BV contains at least 18 active substances (Habermann, 1972). Melittin, the largest component of BV, is a hydrophobic peptide containing 26 amino acid residues, and is a powerful direct hemolytic agent, which possess

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antibacterial and antifungal activity (Habermann, 1972). Apamin is a basic peptide containing 18 amino acids and is a powerful neurotoxin. Apamin blocks calcium-dependent potassium channels, thus enhancing nerve transmission (Gauldie *et al.*, 1976). Adolapin has an anti-inflammatory effect, and has been suggested to inhibit the prostaglandin synthase system (Shkenderov and Koburova, 1982). Adolapin also exhibited a potent analgesic effect. Mast cell degranulating peptide (MCD-peptide) is a basic peptide containing 22 amino acid residues. It was reported that in both the rat-paw edema test and in adjuvant arthritis in rats, this peptide has a powerful anti-inflammatory effect (Billingham *et al.*, 1973; Hanson *et al.*, 1972; Gauldie *et al.*, 1976). Other substances, such as Compound X, hyaluronidase, phospholipase A_2 and histamine are involved in the inflammatory effect of the venom, and are associated with the softening of tissue and the facilitation of the flow of the other BV components. Measurable amounts of the neurotransmitters dopamine and norepinephrine have also been detected in BV (Habermann, 1972).

Studies on BV have, therefore, demonstrated that it is a complex mixture of enzymes, peptides and active amines, and that it has a wide variety of biological and pharmacological activities. BV contains only small amounts of adolapin and MCD-peptide but much larger amounts of phospholipase A_2 and the peptides, melittin and apamin. Although it does not appear to be very suitable as a therapeutic agent, it is believed BV possesses anti-inflammatory properties the modes of action of which are unknown.

Prostaglandins (PGs) have long been known to contribute to pain and inflammation in rheumatic disease (Liang *et al.*, 1999). PGE_2 is the major cylooxygenase metabolite in macrophages and has complex pro-inflammatory and immunoregulatory properties (Lin *et al.*, 1999). COXs are the first rate-limiting enzyme in the synthesis of prostacyclins, prostaglandins and thromboxane from arachidonic acid (Creminon *et al.*, 1995). COX-1, the predominantly constituted form of the enzyme, is expressed throughout the body and provides certain homeostatic functions. In contrast, COX-2, the inducible form, is expressed in cells exposed to inflammatory and other physiologic stimuli and growth factors and is involved in the production of those prostaglandins that mediate pain and support the inflammatory process (Simon, 1999).

Cytokines are regulators of the host responses to infections, immune responses, inflammation and trauma. Some cytokines act to exacerbate disease (pro-inflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory). IL-1β and TNF-α are pro-inflammatory cytokines and the blocking of IL-1β or TNF-α has proved to be highly successful in patients with rheumatoid arthritis, and inflammatory bowel disease (Carteron, 2000; Dinarello, 1997).

In this study, we investigated the effect of BV partitions and subfractions from the aqueous partition of BV on the activities of COX-2 in LPS-activated murine macrophages, J774A.1 cell line and TNF-α and IL-1β production in a PMA-treated U937 cell line. This study may help to explain the molecular mechanisms of the action of BV in inflammatory conditions, such as rheumatoid arthritis and other inflammatory diseases.

MATERIALS AND METHODS

Cell culture

The mouse macrophage cell line (J774A.1 cells), human airway epithelial cell line (A549 cells) and the human myelomonocytic cell line (U937 cells) were purchased from the American Type Culture Collection. Cells were grown at 37°C in a humidified atmosphere (5% CO_2) in RPMI-1640 medium containing 5% fetal bovine serum.

Preparation of bee venom partitions

Lyophilized whole bee venom was obtained from Sigma (Catalog No. V-3375). Lyophilized whole bee venom was dissolved in distilled water, and partitioned three times with an equal volume of *n*-hexane at room temperature, and the resulting aqueous layer was then partitioned three times with an equal volume of ethylacetate. The *n*-hexane and ethylacetate partitions were concentrated to dryness by evaporation *in vacuo*, and aqueous layer was freeze-dried.

Preparation of sub-fractions from bee venom aqueous partition

A portion of the freeze-dried aqueous partition was dissolved in 2% acetic acid solution, and passed through using Minitan Filter plates (Millipore), with a molecular weight cut-off points of 10,000 (10 KDa). The resulting two fractions (below 10 KDa fraction and above 10 KDa fraction) are freeze-dried. The fraction (below 10 KDa) was referred to as B-F3. The other fraction (above 10 KDa) was freeze-dried and applied to the Sephadex G-100 column and washed with 2% acetic acid solution at a flow rate of 6 mL/h. Eluted fractions were collected and examined using 17% SDS-PAGE, and fractions containing molecular weights above 20 KDa were collected and freeze-dried. This fraction was named B-F1. The remaining portions (between 10 KDa and 20 KDa) were also freeze-dried, are referred to as B-F2.

Gel electrophoresis

SDS-PAGE was performed by the method of Shagger and von Jagow (1991) in a Mini-Protean II apparatus (Bio

Rad, USA). PLA_2 (Catalog No. P-9279) and melittin (Catalog No. M-4171) were purchased from Sigma and three subfractions (B-F1, B-F2, and B-F3) were prepared as described above. Each sample (10 μg) was separated by 17% (w/v) SDS-PAGE. Gels were stained with Coomassie blue 0.25% in methanol: water: acetic acid (50:40:10) for 4 h, followed by destaining in 30% methanol and 10% acetic acid.

COX-1 enzyme assay

A radiometric COX-1 assay was performed using a modification of the procedure described by Warner *et al.* (1999). Cultured A549 cells were plated at 2×10^5 cells per well in a 24-well plate and incubated for 24 h at 37°C in a humidified atmosphere (5% CO_2) with test samples or vehicle solvent (final 0.5% DMSO). The plate contents were centrifuged, the supernatant was removed and the cells were resuspended in 200 μL of PBS. The resuspended cells were incubated for 10 min at 37°C in the presence of 1 μM calcium ionophore (A23187) and [1-^{14}C]arachidonic acid solution (0.05 μCi) and then acidified with 10 μL of 1 M citric acid to pH 3.5. After shaking the 24-well plate, 200 μL of the reaction mixture was transferred to a microcentrifuge tube and extracted twice with 500 μL of ethyl acetate to isolate the metabolic products of cyclooxygenase. The ethyl acetate layers were dried *in vacuo*, resuspended in 10 μL of ethyl acetate and then applied to aluminum foil-backed silica gel TLC sheets. TLC was performed at room temperature using a solvent containing chloroform-methanol-acetic acid-water (v/v, 90:10:1:1). The [^{14}C]-labeled prostanoids, were detected by autoradiography, by placing the TLC plate in contact with a Fuji BAS-IIIs imaging plate for 24 h. The imaging plate was scanned using a BAS-1500 bio-imaging analyzer (Fuji Film) and the radioactivities of the prostanoid bands were quantified. The inhibitory effects of test samples on COX-1 activity were determined by measuring the amount of ^{14}C-labeled prostanoids produced versus the DMSO control, and are expressed as percentages of the control.

COX-2 enzyme assay

J774A.1 macrophages were cultured in a 150 cm^2 tissue culture flask until confluent. Cultured J774A.1 macrophages were plated at 2×10^5 cells/well in a 24-well plate and preincubated for 24 h at 37°C in a humidified atmosphere (5% CO_2). Cells were incubated for 24 h, added to an aspirin solution (final concentration, 0.4 μg/mL) so as to exclude the basal COX-1 effect and further incubated for 24 h. Teh cells pretreated with aspirin were then added to the test samples (final 0.5% DMSO) and LPS (2.5 μg/mL) for 18 h to induce COX-2 (Osullivan *et al.*, 1992). The inhibitory effects of the test samples upon COX-2 were determined using the COX-1 assay method as described above. The inhibitory activity was expressed as IC_{50} (50% inhibitory concentration) and each experiment was performed at least twice.

Determination of COX-1 and COX-2 mRNA expressions using RT-PCR

The expression of COX-1 and COX-2 mRNA was analyzed using a reverse transcription-polymerase chain reaction (RT-PCR) based method. COX-1, COX-2 and β-actin mRNA were amplified using the primer pairs: 5-CTCATAGGGGAGACCATCAAG-3 (sense primer) and 5-CCTTCTCTCCTACGAGCTCCTG-3 (antisense primer) for COX-1, 5'-CTCGAGTTGTCAAACTGCGAGCTAA-3' (sense primer) and 5'-GTGTTGAATTCAGAGGCAATGCG-3' (antisense primer) for COX-2 and 5'-GACCCAGATCATGTTTGAGA-3' (sense primer) and 5'-GCTTGCTGATCCACATCTGC-3' (antisense primer) for β-actin. Total RNA was extracted using Ultraspec™ solution (BIOTECX, USA) according to the manufactures instructions, and cDNA was synthesized from 4 μg of RNA using a Titan™ one tube RT-PCR Kit (Boehringer Mannheim, Germany) according to the manufactures instructions with some modification. Briefly, a mixture of 4 μg of RNA and 1 μL of antisense primer (20 pmol) was heated at 95°C for 5 minutes and cooled rapidly on ice. The mixture was adjusted to a volume of 50 μL with 1×RT-PCR buffer, 0.2 mM deoxynucleotide triphosphate (dNTP), 5 mM DTT, 5 units of RNase inhibitor, 20 pmol sense primer and 1 μL enzyme mix, and RT was conducted at 50°C for 30 min, followed by 35°C cycles of 94 for 1 min, 60°C for 2 min, and 68°C for 2 min. The PCR products were then electrophoresed in 1% agarose gel and stained with SYBR® Green I (FMC BioProducts, USA).

TNF-α and IL-1β ELISA

TNF-α and IL-1β assays were performed by a modification of the procedure described by Barrios-Rodiles *et al.* (1999). U937 cells at a density of 3.5×10^4/well were pretreated in 96-well microtiter plates for 6 h with PMA (final 200 nM). Test samples and LPS (final 1 μg/mL) were then added simultaneously and incubated for a further 18 h at 37°C in humidified atmosphere (5% CO_2). The supernatants were removed and TNF-α and IL-1β contents were measured by ELISA.

MTT assay

Cytotoxicity was measured by the mitochondrial-dependent reduction of MTT to formazan (Mosmann, 1983). Briefly, cells were seeded at a density 1×10^6 cells/ml in 96 well-plates. After incubating for 24 h, cells were treated with test samples. The cells were incubated for an additional 18 h, and the medium was replaced with fresh medium containing MTT (final concentration: 0.5 mg/mL),

and incubation continued for 1 h at 37°C. The medium was then removed by aspiration and MTT-formazan production was solubilized in 200 μL DMSO. The extent of MTT reduction to formazan within the cells was quantified by measuring absorbance at 570 nm using an ELISA reader.

RESULTS AND DISCUSSION

The bee venom is a very complicated mixture of enzymes, polypeptides, peptides and other various ingredients. From the ancient times in both Eastern and Western societies, bee venom had been used as a traditional remedy for arthritis and inflammation, and many investigations have been undertaken on its ingredients and their activities. In this study, we tried to determine the active anti-inflammatory components of bee venom and to identify mode of action. Initially, bee venom was separated using the three kinds of solvent, *n*-hexane, ethyl acetate, and water. The bee venom was first partitioned in a water/*n*-hexane system, and the resulting aqueous layer was then extracted with ethyl acetate. Of the 3 g of bee venom so obtained, 0.081 g was partitioned in *n*-hexane, 0.129 g in ethyl acetate and 2.79 g in the aqueous phase. COX-2 enzyme assay was performed on these fractions in order to test their mechanisms of action, and the aqueous partition was found to have a significant effect in a dose-dependent manner (IC_{50} = 13.1 μg/mL) (Table I). However these fractions had no effects upon COX-1 activity (Table I). In addition, no significant cytotoxic effects were observed at the maximum concentration used (20 μg/mL). Because aqueous extract is a major component of bee venom and the effective extract in terms of COX-2 activity, it was subfractionated into three parts by molecular weight difference, namely B-F1 (above 20 KDa), B-F2 (between 20 KDa and 10 KDa) and B-F3 (below 10 KDa) (Fig. 1). The effects of these subfractions were investigated on COX-2 enzyme activity and their mRNA expression levels to test the mechanism of action. B-F2 and B-F3 decreased COX-2 enzyme activities and their mRNA expression levels, in a dose-dependent manner, but B-F1 had no effect upon COX-2 enzyme activity and mRNA expression level (Table II, Fig. 2). The inhibitory effects of B-F2 and B-

Table I. Inhibitory effects of solvent partitions from whole bee venom on the activities of COX-1 in human airway epithelial cell line, A549 cells and COX-2 in LPS-activated murine macrophages, J774A.1 cells

	COX-1 activity IC_{50}[a] (μg/mL)	COX-2 activity IC_{50}[a] (μg/mL)
Hexane partition	> 20	> 20
Ethylacetate partition	> 20	18.1±1.0
Aqueous partition	> 20	13.1±1.1

[a]IC_{50} is defined as the concentration (μg/mL) giving 50% inhibition versus the control (final 0.5% DMSO). The table shows the means± S.E. of two independent experiments performed in duplicate.

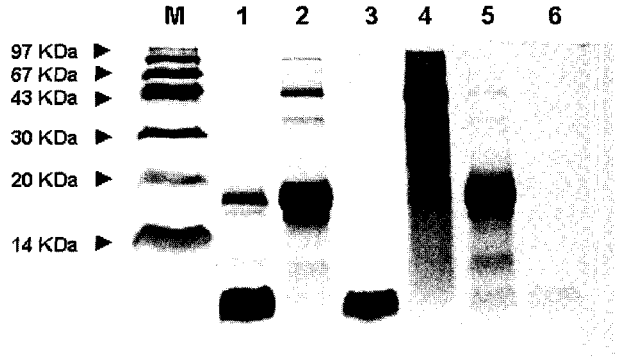

Fig. 1. SDS-PAGE gel electrophoresis of aqueous partition of bee venom and its subfractions. Each sample (10 μg) was electrophoresed through a 17% SDS-PAGE, stained with Coomassie blue. M: molecular marker (KDa); 1: bee venom aqueous partition; 2: pospholipase A_2; 3: melittin; 4: B-F1 (above 20 KDa); 5: B-F2 (between 20 KDa and 10 KDa); 6: B-F3 (below 10 KDa). Results are representative of three independent experiments.

Table II. Inhibitory effect of subfractions from aqueous partition of bee venom on the activities of COX-1 in human airway epithelial cell line, A549 cells and COX-2 in LPS-activated murine macrophages, J774A.1 cells

	COX-1 activity IC_{50}[a] (μg/mL)	COX-2 activity IC_{50}[a] (μg/mL)
B-F1	> 20	> 20
B-F2	> 20	0.42±0.4
B-F3	> 20	0.16±0.6

[a]IC_{50} was defined as the concentration (μg/mL) giving 50% inhibition versus the control (final 0.5% DMSO).
The aqueous partition of bee venom was subfractionated into three parts by molecular weight differences, namely, B-F1 (above 20 KDa), B-F2 (between 20 KDa and 10 KDa) and B-F3 (below 10 KDa). The table shows the means±S.E. of two independent experiments performed in duplicate.

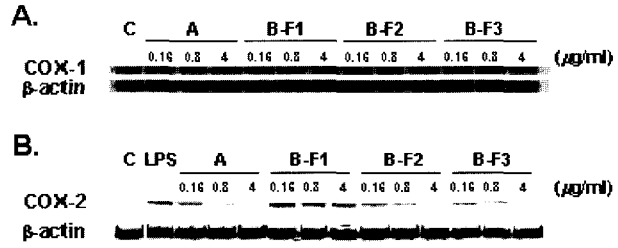

Fig. 2. The effects of the subfractions from aqueous partition of bee venom on COX mRNA expression. The production of mRNAs coding COX-1 and COX-2 were evaluated in A549 cells (for COX-1, A) and in LPS-induced J774A.1 cells (for COX-2, B) treated with subfractions from aqueous partition of bee venom. Control (C) cells were treated with 0.5% DMSO (final concentration). Results are representative of two independent experiments.

Table III. Inhibitory effects of subfractions from aqueous partition of bee venom on the production of LPS induced TNF-α and IL-1β in PMA-treated U937 cells

	TNF-α IC_{50}[a] (μg/mL)	IL-1β IC_{50}[a] (μg/mL)
B-F1	0.7±0.2	1.8±0.6
B-F2	0.7±0.2	2.0±1.0
B-F3	0.6±0.1	1.6±0.9

[a] IC_{50} was defined as the concentration (μg/mL) giving 50% inhibition versus the control (final 0.5% DMSO).

The aqueous partition of bee venom was subfractionated into three parts by molecular weight differences, namely, B-F1 (above 20 KDa), B-F2 (between 20 KDa and 10 KDa) and B-F3 (below 10 KDa). The table shows the means±S.E. of three independent experiments performed in duplicate.

F3 upon COX-2 enzyme activity correlated with COX-2 mRNA expression. B-F3 contained small amount of peptides with molecular weights below 10 KDa, which included, melittin (M.W., 2,840 daltons), apamin (M.W., 2,036 daltons), MCD-peptide (M.W., 2,588 daltons) and minimine (M.W., 6,000 daltons). Of these peptides, MCD-peptide has been reported to have anti-inflammatory properties (Billingham *et al.*, 1973; Hanson *et al.*, 1972; Gauldie *et al.*, 1976). B-F2 was less effective than B-F3 in terms of its effect upon COX-2 enzyme activity and mRNA expression. It contained phospholipase A_2 (M.W., 19,000 daltons) and adolapin (M.W., 11,500 daltons). Adolapin has been reported to have anti-inflammatory effects (Shkenderov and Koburova, 1982).

TNF-α and IL-1β are released primarily by monocytes/macrophages in response to stimulation by activating factors, which bind to receptors on the cell surface. This type of reaction occurs during the early phase of inflammatory and immunological reactions. Therefore, TNF-α and IL-1β can be viewed as early mediators of the inflammatory reactions and are early indicators of the inflammatory process. We investigated the effects of the aqueous partition and its subfractionated parts (B-F1, B-F2 and B-F3) on LPS-induced TNF-α and IL-1β production in PMA-treated U937 cells. The aqueous partition was found to strongly inhibit TNF-α and IL-1β production and B-F1, B-F2 and B-F3 also showed inhibitory effects (Table III). In contrast to the inhibitory action for the COX-2 mRNA expression and enzyme activity, B-F1 had a strong inhibitory activity against TNF-α and IL-1β production. It has been reported that both TNF-α and IL-1β stimulates COX-2 mRNA expression in mouse osteoblasts and bovine endothelial cells (Wadleigh and Herschman, 1999). Moreover, the transcriptional regulation of COX-2 by TNF-α and IL-1β in murine osteoblasts involves the activation of the transcription factor NF-κB. Recent studies have shown that NF-κB is also involved in the induction of COX-2 expression in response to LPS in human and mouse macrophages. However, Mitchell *et al.* (1997) reported that COX-2 activity was regulated independently by NF-κB activation. Subfraction B-F1 contained mainly high molecular weight enzymes, such as hyaluronidase and glucosidase. The mode of action of B-F1 may differ from that of B-F2 and B-F3, which contain small peptides. Our studies showed that the pharmacological effects of bee venom are related to the inhibition of COX-2 activity, and TNF-α and IL-1β production. Subfraction B-F2 and B-F3, which contained low molecular weight peptides, showed the strong inhibitory effects against COX-2 activity and its mRNA expression, and all three subfractions showed the inhibitory effects on TNF-α and IL-1β production. We believe that the identification of the active principles of bee venom warrants further investigations for the treatment of inflammatory diseases.

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