Research Article

Study of the Molecular Mechanism of Anti-inflammatory Activity of Bee venom in Lipopolysaccharide Stimulated RAW 264.7 Macrophages

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Abstract

Purpose: Bee venom (BV) is traditionally used in many inflammatory chronic conditions but its mechanism of action at molecular level is not fully understood. This study was undertaken to elucidate the mechanism of action of bee venom at the molecular level.

Methods: We used lipopolysaccharide (LPS) stimulation in Raw 264.7 macrophage (RM) cells and studied the effect of BV on cell proliferation, inflammation related protein expression by western blotting and RNA expression by reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Bee venom was toxic to RM cells above 10 µg/ml but reduced the production of nitric oxide (NO) at 2–10 µg/ml in LPS stimulated RM cells by inhibiting the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 via nuclear factor (NF)-κB. However, bee venom also induced the pro-inflammatory cytokine, interleukin (IL)-1β via p38 mitogen activated protein kinase (MAPK) which is known to stimulate inflammatory activity.

Conclusion: It seems that NFκB and p38 MAPK signal pathways are involved in triggering the functional activation of LPS-stimulated macrophage. We suggest that some components of bee venom can cause inflammation by inducing IL-1β via p38 MAPK while others act as anti-inflammatory by suppressing iNOS and COX2 via NFκB.

Keywords: Bee venom, Cyclooxygenase-2, Interleukin 1beta, Inducible nitric oxide synthase, Lipopolysaccharide, Macrophage, Mitogen activated protein kinase, Nuclear factor kappa-B.

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INTRODUCTION

Bee venom (BV) from the sting of Honeybee (Apis mellifera) is traditionally used in China, Korea, Japan for arthritis, tendinitis, bursitis and other chronic conditions. It is reported to have proinflammatory [1] and anti-inflammatory effects [2-6]. Several studies have reported that following subcutaneous injection of a solution of BV into rat paw, as an experimentally-produced honeybee’s sting effect, the animal showed unique expressions of persistent nociception and inflammation [7-9]. Since the first report of anti-inflammatory effect of BV [10], many studies using diverse methodologies have supported this finding.

BV contains various peptides, including mellitin, apamin, adolapin and enzymes as well as non-peptide components such as histamine, lipid, carbohydrates [11-13]. Excessive production of nitric oxide (NO) and prostaglandin (PGE) play an important role in various inflammatory diseases [14]. In inflammatory reactions, NO is generated from arginine, mainly by inducible nitric oxide synthase (iNOS) while PGE is generated from arachidonic acid mostly by cyclooxygenase (COX-2). Recent studies have demonstrated that eukaryotic transcription factor nuclear factor-kappa B (NF-kB) is involved in the regulation of COX-2 and iNOS expression [15]. Accordingly, many substances developed to date to prevent inflammatory damage either by suppressing the activation of iNOS or COX-2 directly, or by inhibiting NFkB signalling, which regulates them in the transcriptional stage [16].

The biological functions of NFkB are involved in many pro-inflammatory cytokines such as TNF-α, TNF-β, IL-1β, IL-6 and IL-8 [17]. TNF-α plays a key role in the induction and maintenance of inflammation due to autoimmune reactions, by activating T cells and macrophages and by up-regulating other pro-inflammatory cytokines [18]. Likewise, IL-1β, one of the most important inflammatory cytokines secreted by macrophages, is induced by LPS. During inflammation, increased release of IL-1β leads to cell or tissue damage [19] and thus, reduction in IL-1β released from macrophages may retard inflammatory responses to LPS stimulation. Mitogen-activated protein kinase (MAPK) pathway, one of the most extensively studied intracellular signaling cascades, is also seems to have pro-inflammatory responses [20,21]

EXPERIMENTAL

Chemicals

Bee venom (99.5 % purity) was supplied by Chung-Jin Biotech Co. Ltd, Korea. Anti-iNOS, COX-2, IL-1β, NFkB, p38MAPK antibody and HRP were purchased from Santa Cruz Biotechnology Inc, Santa Crutz, CA, USA. The primers (see Table 1) were synthesised by Bioneer Corporation, Korea. Alamar blue was received from Invitrogen, USA while RAW 264.7 murine macophages were obtained from American Type Culture Collection, Rockville, MD, USA.

Experimental plan

We studied the effect of BV at different doses in the LPS stimulated RM cells by measuring NO production and the expression of different inflammatory enzymes and cytokines at protein level by Western blotting and at mRNA levels by RT-PCR in order to understand the mode of action of bee venom on inflammatory pathway.
Preparation of bee venom solution

Dried 0.01 g bee venom was diluted with 10 ml Dalbeco’s modified eagle’s medium (DMEM) medium (10 % foetal bovine serum (FBS), 1% penicillin) to make 1000 µg/ml, and then filtered. The solution was further diluted to 0. 5, 1, 2, 5, and 10 µg/ml before actual use.

Culture of Raw 264.7 cells and sample treatment

Mouse macrophage cell line Raw 264.7 cells were cultured in (DMEM) with 10 % faetal bovine serum and 1% penicillin in a humidified atmosphere of 5 % CO₂ at 37 °C. The cells were stimulated with LPS (1µg/well); 1 h later, they were treated with 0, 0.5, 1, 2, 5 and 10 µg/ml of BV and cultured for 24 h Various analyses were carried out on them as described in the following sections.

Cell proliferation assay

To determine the effect of BV on cell proliferation, the cells were incubated for 24h with BV at different concentrations (0, 0.5,1,2, 5, 10, 20 µg/ml). Cell proliferation was measured by alamar blue (AB) assay. A single plate was used for each assay and all measurements were conducted on a microplate reader (UVmax, Molecular Devices). In the assay, 10 µl AB reagent (Sorotec) was added to each well, and after 3 h of incubation at 37 °C under 5 % CO₂ in a humidified atmosphere, the absorbance difference was determined at 570nm using a spectrophotometer (Tecan Sunrise™ Absorbance Reader). The cell proliferation was calculated by comparing with control and expressed in percent.

Nitric oxide assay

To examine the effects of BV on NO production, we measured the levels of nitrite (an index of NO formation) produced by LPS. Thereafter, NO production assay was used to confirm the results. The RAW 264.7 cells were transferred to a 96-well plate (1000 cells/well) and incubated overnight. The cells were stimulated with LPS (1µg/well), and 1 h later, they were treated with 0, 0.5,1, 2, 5 and 10 µg/ml of BV and cultured for 24 h. One hundred microlitres of the culture supernatants were transferred into another 96-well plate and treated with 100 µl of Greiss reagent solution. After waiting for reaction to take place at room temperature, light absorption was measured at 570 nm with a spectrophotometer (Tecan Sunrise™ Absorbance Reader). NO was assessed with the aid of a standard curve prepared with standard sodium nitrite solutions (0, 3.75, 7.5, 15, 30, 60, 120 and 240 µM).

Protein assay

Cellular proteins were extracted from untreated and treated cells in cold lysis buffer (50 nM Hepes at pH 7.0, 250 nM NaCl and 5 nM EDTA), and the protein concentration of the samples were determined by Bio-protein assay in 96 wells. This solution was diluted with water to 1:4; proteins in the cell samples were also diluted with cold PBS to 1:4. After loading 10 µl of diluted protein samples into the wells and waiting for 10 min, the developed colour was measured in all 96 wells at 600 nm in a spectrophotometer (Tecan Sunrise™ Absorbance Reader). Protein content was computed with the aid of a standard curve prepared using bovine serum albumin (BSA) as a standard in a concentration range of 0.02 – 1.34 µg/ml.

Western blot analysis

After protein concentration was determined, 40 µg samples from treated and untreated cells extracts were separated on a 10 % sodium dodesyl sulfate (SDS)-polyacrylamide gel electrophoresis. The protein bands were transferred to a nitrocellulose membrane electrophoretically at 4 °C at 100 V for 1 h. The membrane was blocked with 5 % skimmed milk, washed 3 times with Tween 20-Tris-buffered saline (TBS-T), and transfer
of bands was confirmed by Ponceus solution at room temperature. The first antibody against iNOS, COX-2, NFkB, TNFα, IL1β, P38 MAPK and β-actin was added and incubated for 2 h. After washing with TBS-T 3 times (each time for 15 min), the membrane was incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membrane was again washed 3 times with TBS-T and then developed by enhanced chemi-luminescence (Amersham Life Science, Arlington Heights, IL, U.S.A) on an x-ray film.

Isolation of RNA from Raw 264.7 cells

The cells (3x10^6 cells/ml) were taken in culture disks and pretreated with bee venom at 0, 0.5, 1, 2, 5, 10 µg/ml and after one 1 h, the cells were treated with LPS (1 µg/ml). After 24 h, the cells were harvested into a 1.5 ml Eppendorf tube using 1 ml of Trizol reagent. The samples were homogenised and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added and the tubes were shaken vigorously (manually) for 15 sec and incubated for 2 to 3 min. The samples were centrifuged at 13000 rpm for 15 min at 4 °C, and separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase was transferred to a fresh tube and RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. Subsequently, 0.5 ml of isopropyl alcohol was added and the samples were incubated at room temperature for 10 min and centrifuged at 13000 rpm for 10 min at 4 °C. The supernatant was removed, the RNA pellet was washed once with 75 % ethanol (1 ml), and centrifuged at 7500 rpm for 5 min at 4 °C. At the end of the procedure, the RNA pellet was briefly dried at room temperature for 5 min, 20 ul of RNase-free water was added to it, and then incubated for 10 min at 56 °C. Finally, the concentration of the RNA was measured with a spectrophotometer while its quality was assessed by agarose gel electrophoresis.

Reverse transcriptase polymerase chain reaction (RT-PCR)

cDNA was synthesized from RNA isolated from cells using a SuperScript™ II kit (Invitrogen, USA). Cycle parameters were initial denaturation for 2 min at 95 °C, annealing for 1 min at 57, 60, 60, 55, 60 and 54 °C for NFkB, COX-2, iNOS, TNFα, IL-1β and GAPDH, respectively, elongation for 1 min at 72 °C and final extension for 10 min at 72 °C. Primer sequence (Table 1) of the NFkB, COX-2, iNOS, TNFα, IL-1β and GAPDH (for standardization) were amplified out of each cDNA batch with 35, 30, 30, 30, 30 and 30 amplification cycle, respectively. The resulting PCR products were separated in 2 % agarose gel and visualised by ethidium bromide staining.

Statistical analysis

The data are presented as mean ± SEM. All data were analysed by one-way ANOVA and differences between the means were assessed with Duncan’s multiple range tests (DMRT). Differences were considered significant at P < 0.05. All analyses were carried out using SPSS Software ver.11.5 (Chicago, Illinois).

RESULTS

Effect of bee venom on cell proliferation

The results indicate that BV-treated cells increased proliferation up to a dose of 0.5 – 2.0 µg/ml. However, BV did not show any toxic effect at 5 - 20 µg/ml and the number of cells decreased more than untreated control.

Effect of BV on NO production and iNOS expression

The results (see Fig 1) indicate that NO production reduced significantly (p < 0.05) in a dose-dependent manner with BV treatment.
Table 1: Primer sequences used for RT-PCR

| Gene symbol | Primer sequence 5′→3′ | Expected fragment size (bp) |
|-------------|------------------------|----------------------------|
| NFκB        | 5′-GGCCTGCAAGGGTTATCGTT-3′  
5′-TGTCCTGAGTGTGCCGGCTTT-3′ | 300 |
| iNOS        | 5′-AATGGCAACATCAGTGCGCCCATC-3′  
5′-GCTGTGTGTCACAGAAGTCTCGAAACCT-3′ | 807 |
| IL-1β       | 5′-ATCGAGAGTTCCCCAAGATAC-3′  
5′-GTGCTGCTTAATGTGCCCTTGAATC-3′ | 387 |
| TNFα        | 5′-ATGAGAAGGACTGACTGATC-3′  
5′-TACAGGTTGTCACTCGAAAT-3′ | 351 |
| COX2        | 5′-GGAGGACTATCAAGATAGT-3′  
5′-ATGGTCAGTAGACTTTTACA-3′ | 721 |
| GAPDH       | 5′-CCATCAACAGACCCCTTCC-3′  
5′-GTCCTCAGTGTAGGCAAGA-3′ | 462 |

Figure 1: The effects of bee venom on NO production and iNOS protein expression in LPS-stimulated Raw 264.7 cells. Note: Values represent means ± SEM of seven samples. Means with different superscripts (a, b, c) are significantly different (p < 0.05).

Also, bee venom consistently inhibited the expression of iNOS protein in RAW 264.7 cells in a concentration-dependent manner. These results demonstrated that bee venom produced a concentration-dependent inhibition of NO production and iNOS protein expression in response to LPS.

Effect of BV on COX-2 expression

The results, shown in Fig 2, indicate that bee venom at low concentrations (0.5 – 2.0 µg/ml) had no significant effect (p < 0.05) on COX-2 expression, but at higher concentrations (5 - 10 µg/ml), bee venom significantly reduced COX-2 expression in the LPS-stimulated RM cells. Thus, bee venom inhibited COX-2 activity and protein expression in RAW 264.7 cells stimulated with LPS.

Effect of BV on P38-MAPK and IL-1β expressions

The results in Fig 3 showed that bee venom exerted a dose-dependent increase in the expression of both p38 MAPK and IL-1β. The trend was similar and the rise in expressions were significant (p<0.05) at doses of 5 - 10 µg/ml bee venom. Thus, these data established a pro-inflammatory effect of BV via p38 MAPK pathway.
Effect of BV on NFκB activation and mRNA cytokine expression

The results in Fig 4 and Table 2 show significant decreases in the expressions of iNOS, COX-2 and NFκB at concentrations of 2 - 10 µg/ml of bee venom in LPS-stimulated RAW cells. BV treatment also induced increased expression of IL-1β at lower concentration but the expression was significantly greater (p < 0.05) at higher BV concentrations (5-10 µg/ml). However, the effect on the expression of TNFα was not significant although expression appeared to increase in a dose-dependent.

DISCUSSION

In this paper, we have presented data to show that bee venom controlled NO production in activated macrophages via the inhibition of iNOS expression, and also suppressed the expression of COX-2 acting at a transcriptional level. It is also evident from the results that bee venom inhibited LPS-induced expression of iNOS and COX2 genes through the blocking of NFκB activation at mRNA and protein expression levels. Recently, it was reported that bee venom exhibits anti-arthritic effect by reducing the expression of iNOS and COX-2 through suppression of NFκB [3,15].

In this study, we observed increased activity of p38 MAPK which enhanced IL-1β expression in RM cells. This suggests that p38 MAPK is a critical mediator for the release of pro-inflammatory cytokines such as IL-1β in RM cells due to treatment with bee venom (BV).

Although BV has anti-inflammatory properties, however, it can also induce inflammation since the p38MAPK activated by several different stimuli positively regulates a variety of genes involved in inflammation, such as...
Table 2: Effect of bee venom (BV) on relative mRNA expression (mean ± SD) of iNOS, IL-1β, COX-2, TNF-α and NFκB in LPS (1 µg/ml)-stimulated RAW 264.7 cells

| Concentration of BV (µg/ml) | iNOS (INT/mm²) | IL-1β (INT/mm²) | COX2 (INT/mm²) | TNFα (INT/mm²) | NFκB (INT/mm²) |
|-----------------------------|----------------|-----------------|----------------|----------------|----------------|
| 0                           | 7.37x10^3 ± 542.2a | 5.39x10^3 ± 403b  | 2.23x10^3 ± 256.8a | 3.99x10^3 ± 81.5a | 3.94x10^3 ± 216a |
| 0.5                         | 6.31x10^3 ± 528.9ab| 5.90x10^3 ± 446.3ab| 2.39x10^3 ± 223.8a| 4.08x10^3 ± 82.3a| 4.11x10^3 ± 259a |
| 1                           | 6.19x10^3 ± 324.1ab| 6.12x10^3 ± 360.4ab| 2.56x10^3 ± 165.6a| 4.08x10^3 ± 30.2a| 4.08x10^3 ± 237a |
| 2                           | 6.06x10^3 ± 366.5a | 6.45x10^3 ± 305ab | 2.53x10^3 ± 299.3a| 4.20x10^3 ± 30.2a| 3.73x10^3 ± 172ab|
| 5                           | 1.09x10^3 ± 77.7c | 6.81x10^3 ± 366.5a| 1.36x10^3 ± 291.6b| 4.24x10^3 ± 90.6b| 3.03x10^3 ± 197ab |
| 10                          | 0.83x10^3 ± 86c   | 6.92x10^3 ± 469.4a| 1.07x10^3 ± 181.2b| 4.48x10^3 ± 161.2a | 1.96x10^3 ± 290c |

a, b, c: means with different superscripts are significantly different (p<0.05).

TNF-α and IL-1β [20,22]. These cytokines appear to be interlinked in a cascade, being produced serially by cells during an inflammatory response. Cumulative evidence indicates that an abnormality in the production or functions of TNFα and IL-1β play a role in many inflammatory lesions [23]. Bee venom induced an increase in the activity of p38MAPK which activated IL-1β expression at protein and mRNA levels. iNOS, TNFα, COX2 and IL-1β. Therefore, inhibitors of NFκB activation are important in anti-inflammatory activity. Our data show that bee venom can directly inhibit NFκB activation and suppress the expression of iNOS and COX2. Although bee venom inhibited NFκB activation, it also increased the activation of TNFα and IL-1β mRNA expression. The naturally occurring peptides of whole bee venom have various pharmacological potencies to produce local inflammation, nociception and pain hypersensitivity in mammals. Over 50 % of whole bee venom plays a central role in the production of local inflammation [24]; however, these components exhibit anti-inflammatory activity in inflammatory cells.

CONCLUSION

NFκB and p38 MAPK signal pathways are involved in triggering the functional activation of LPS-stimulated macrophage and play an important role in the release of pro-inflammatory and anti-inflammatory cytokines. We suggest that some components of bee venom can induce inflammation by inducing IL-1β via p38 MAPK but some other components act as anti-inflammatory by suppressing iNOS and COX2 via NFκB when there is already inflammation. Further study is required to confirm the activity of individual components of bee venom at cellular and molecular level.

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