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Effects of bee venom on the pro-inflammatory responses in RAW264.7 macrophage cell line

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Abstract

The purpose of this study is to elucidate the molecular mechanism of anti-inflammatory effect of bee venom (BV), which has been used for the treatment of various inflammatory diseases in oriental medicine. With this aim, we examined the effects of BV on the nitric oxide (NO) production by lipopolysaccharide (LPS) or sodium nitroprusside in RAW264.7 macrophages. We further investigated the effects of BV on the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) with RT-PCR in LPS-stimulated RAW264.7 cells. BV suppressed the NO production and decreased the levels of iNOS, COX-2, NF-κB and MAPK mRNA in a dose-dependent manner. These results suggest that BV has an anti-inflammatory effect by inhibiting iNOS and COX-2 expression, possibly through suppression of NF-κB and MAPK expression.

Keywords: Bee venom; Nitric oxide synthase; Cyclooxygenase-2; Nuclear factor-κB; Mitogen-activated protein kinase

1. Introduction

Bee venom (BV) is the venom that bees store within their venom sacs for self-defence, and has traditionally been used in oriental medicine to relieve pain and treat inflammatory diseases (Kwon et al., 2001). Since Billingham et al. (1973) first discovered the anti-inflammatory effects of BV, many studies have supported it using diverse methodologies. BV contains various peptides including mellitin, apamin, adolapin and mast cell degranulation (MCD) peptide. It also contains enzymes (e.g. phospholipase A2) and non-peptide components (e.g. histamine, lipids, carbohydrates) (Habermann, 1971; Banks and Shipolini, 1986; Lee et al., 2001). Although adolapin and MCD peptide have anti-inflammatory activities, these substances are present in very small quantities in the whole BV (Martin and Hartter, 1980; Koburova et al., 1985; Kwon et al., 2001). It was reported that mellitin, a major component of BV, inhibits the enzymatic activity of phospholipase A2, an inflammatory trigger (Saini et al., 1997). On the other hand, Hartman et al. (1991) reported that mellitin injection induced paw edema in mice. In addition, whole BV into hind paw produces local inflammation (Lariviere and Melzack, 1996). Therefore, it is necessary to investigate further the molecular mechanism of the anti-inflammatory effects of whole BV.

It has been reported that the excessive production of nitric oxide (NO) and prostaglandin (PG) plays an important role in causing various inflammatory diseases (Higgs et al., 1984). NO in inflammatory reactions is generated from argi-

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nine mainly by inducible nitric oxide synthase (iNOS), while PGs are generated from arachidonic acid mostly by inducible cyclooxygenase (COX-2). Recent studies have demonstrated that eukaryotic transcription factor nuclear factor-kappa B (NF-κB) is involved in the regulation of COX-2 and iNOS expression (Surh et al., 2001). Accordingly, many candidate substances developed to date to prevent inflammatory damage either suppress the activation of iNOS or COX-2 directly, or inhibit NF-κB signalling, which regulates the transcriptional stage (Ogden and Moore, 1995; Suh et al., 1998). Mitogen-activated protein kinase (MAPK) pathway is also one of the most extensively studied intracellular signalling cascades deemed to be pro-inflammatory responses (Surh et al., 2001).

In the present study, we examined the effects of BV on the NO generation by lipopolysaccharide (LPS) or sodium nitroprusside (SNP) in RAW264.7 macrophages, a cell line well known for being involved in the inflammation. We further investigated the effects of BV on the expression of iNOS, COX-2, NF-κB and MAPK with RT-PCR in LPS-stimulated RAW264.7 cells.

2. Materials and methods

2.1. Cell culture

Mouse macrophage cell line RAW264.7 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM medium with 5% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Bee venom preparation

BV was obtained by provoking the bee sac of *Apis mellifera* using a microchip-installed electromagnetic extractor. Dried BV 0.1 g was diluted in 99.9 ml of distilled water to make BV solution of 1 mg/ml. After sterilizing the BV solution in an autoclave for 20 min at 121 °C, it was diluted to 1, 5, 10 and 20 μg/ml to be used in the experiments. Major components of BV in this preparation are maintained after sterilizing (Lee et al., 2000).

2.3. NO generation analysis

To examine the effects of BV on the NO formation, we measured the levels of nitrite, an index of NO formation, produced by LPS. Thereafter, a NO donor SNP was used to confirm the results. The RAW264.7 cells were divided into 10^3 cells/well on a 96-well plate and incubated overnight. Then they were stimulated with LPS 100 ng/well or SNP 500 M for 2 h, and treated with 0, 1, 5, 10, and 20 μg/ml concentration levels of BV for 5 h. One hundred microlitres of the culture supernatants were transferred into new 96-well plate, and treated with 50 μl of Greiss reagent solution. After having waited for its reaction at room temperature, the light absorption was observed in 570 nm and standard solutions of 0, 1, 10, 20, 50, 100, 150 μM sodium nitrite were applied to a chromophoric group and analyzed.

2.4. RT-PCR

The cells were placed on a 6-well plate, divided to 10^5 cells/well and cultivated overnight. They were treated with LPS 100 ng/ml and then with 0, 1, 5, and 10 μg/ml BV. After 5 h, the cells were gathered into a 1.5 ml eppendorf tube, and were centrifuged at 15,000 rpm for 5 min. RNA was isolated using RNAzol, chloroform, followed by ethanol precipitation. Total RNA (1 μg) was reverse transcribed into DNA in a total volume 20 μl using random primer and a first-strand cDNA synthesis kit (Roche, Gipt-Oberfrick, Switzerland). PCR amplification was conducted in a total volume of 50 μl of 1× PCR buffer containing 1.0 μl of the first-strand cDNA, 0.25 μM of each dNTP. Two micromolar of each primer and 2.5 μl of Taq DNA polymerase (Takara Shuzo). The following oligonucleotides were used: MAPK sense primer 5′-aaaatttcgaggttgagagt-3′; MAPK antisense primer 5′-tattaaaggctctatttc-3′; NF-κB sense primer 5′-ggcctgcaaaggttatcgtt-3′; NF-κB antisense primer 5′-tgtctgtgagttgccggtctt-3′; COX-2 sense primer 5′-gatacgtgttgacgtccaga-3′; COX-2 antisense primer 5′-gtctgtctagagtttcaccg-3′; iNOS sense primer 5′-gcctcatgccttgattcat-3′; iNOS antisense primer 5′-gagggtgaattccaga-3′; β-actin sense primer 5′-ccaaggccaaccgccgc-3′; β-actin antisense primer 5′-agggtacatggtgccgcc-3′. Cycle parameters were: annealing 45 s at 56, 57, 54, 55 and 54 °C for MAPK, NF-κB, COX-2, iNOS and β-actin, respectively, elongation 1 min at 72 °C, and denaturation 1 min at 94 °C. Resulting PCR products were separated in 1% agarose gel and visualized by ethidium bromide staining. Sequence of the MAPK, NF-κB, COX-2, iNOS and β-actin (for standardization) were amplified out of each cDNA batch with 30, 35, 35, 32, and 35 amplification cycle, respectively.

2.5. Statistical analysis

mRNA expression analysis using RT-PCR was conducted at least three times to ensure that the same result was derived, and this study used the representative diagram. Other experiments were also repeated at least three times and the results are indicated in mean ± S.D. Statistical analysis comparing each test group was done through Student's *t*-test verification. Significance in difference was recognized only when the *p*-value was below 0.05.

3. Results

3.1. NO generation analysis

In order to observe the effects of BV on the generation of NO, the RAW264.7 cells were stimulated with

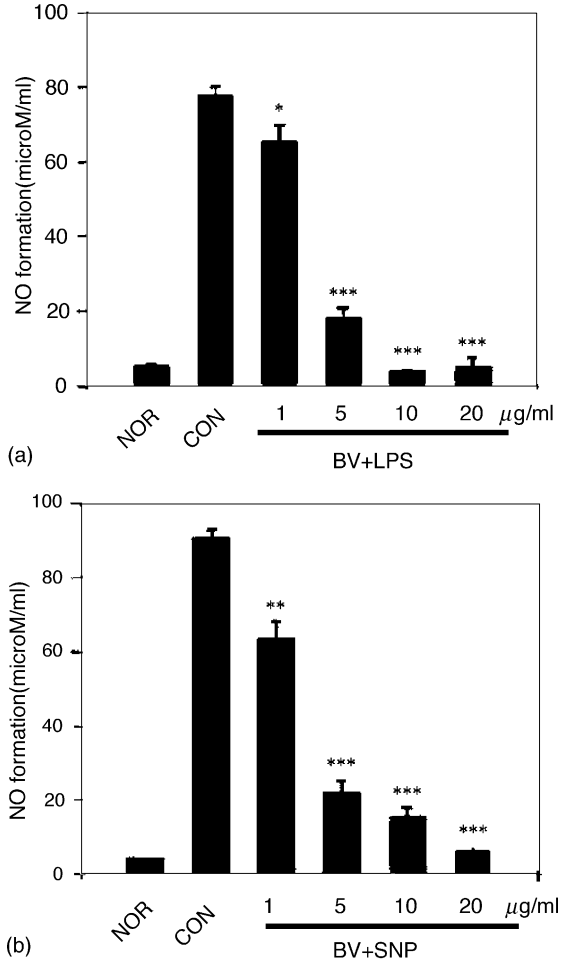

Fig. 1. The effects of BV on the generation of NO by LPS (a) or SNP (b) in RAW264.7 cells. Results are mean ± S.D. of triplicate determination in a typical experiment. Cells were sampled from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ by Student's *t*-test. NOR, normal, unstimulated RAW264.7 cells; CON: control, LPS only or SNP only.

LPS or SNP and then treated with BV. Thereafter, the levels of nitrite, an index of NO formation, were measured. The effects of BV on the NO generation by LPS are shown in Fig. 1a. BV dose-dependently reduced the concentration of nitrite ($p < 0.05$). In the normal group, which was not treated with LPS and BV, the nitrite level was 5.4 ± 0.5 μM, while that of the control group, which was treated with LPS only, was 78 ± 2.5 μM. The nitrite levels of the 1, 5, 10, and 20 μg/ml BV-treated groups, which were treated with LPS and BV, were 65.7 ± 4.0, 18.3 ± 2.5, 3.8 ± 0.4 and 5.0 ± 2.6 μM, respectively (Fig. 1a). The effects of BV on the NO generation by SNP are shown in Fig. 1b. BV dose-dependently reduced the concentration of nitrite ($p < 0.05$). The nitrite level of the normal group was about 4 μM, whereas that of the control group was 89 ± 4 μM. The nitrite levels of 1, 5, 10, 20 μg/ml BV-treated groups were 63 ± 4.5, 22 ± 3.5, 15 ± 2.5, 6 ± 1.5 μM, respectively (Fig. 1b). These data suggested that BV has an anti-inflammatory activity to inhibit the production of pathogen-induced NO.

3.2. RT-PCR analysis

RT-PCR analysis was performed in order to examine the effects of BV on the transcriptional expression of COX-2, iNOS, NF-κB and MAPK, which are index proteins in the inflammatory reactions of RAW264.7 cells. All doses of BV (1, 5, 10 μg/ml) reduced the expression of COX-2 mRNA. The expression of iNOS, NF-κB, and MAPK mRNA was not reduced by a 1 μg/ml dose of BV, but was reduced by 5 and 10 μg/ml. The β-actin employed in this experiment was an internal control (Fig. 2). This result indicated that BV possesses an inhibitory effect on the expression of iNOS, COX-2, NF-κB and MAPK in RAW264.7 macrophages.

4. Discussion and conclusions

Serious inflammation can cause septic shock, rheumatoid arthritis or other diseases in which the excessive production of NO and PG plays a pivotal role (Higgs et al., 1984). Therefore, it has been recognized that suppressing the excessive production of NO and PG is a significant goal in the treatment of inflammatory diseases. iNOS is induced rapidly and acutely by stimulus such as LPS or cytokines (Liang et al., 1999) and catalyzes the oxidative deamination of L-arginine to produce NO. COX-2 is barely detectable under normal physiological condition, but can be induced rapidly and transiently by pro-inflammatory mediators and mitogenic stimuli. COX-2 converts arachidonic acid via oxygenation to PG endoperoxide intermediates (Surh et al., 2001). It has been known that immune stimuli such as LPS activate cellular gene expressions through MAPK pathway and NF-κB, a series of activations. NF-κB is a key transcription factor to regulate the expression of COX-2 and iNOS. D'Acquisto et al. (1997) showed that NF-κB is involved in COX-2 protein expression in LPS-stimulated J774 macrophages, and suggested that the inhibitors of NF-κB activation may be a useful tool for the pharmacological control of inflammation. The activation of

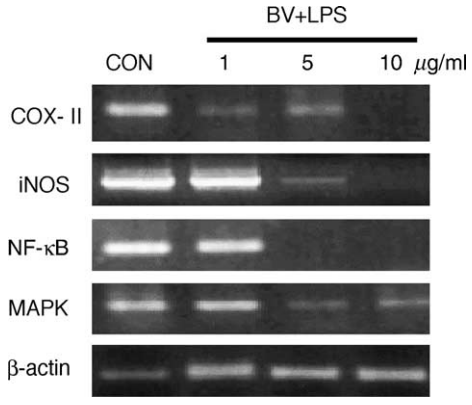

Fig. 2. RT-PCR analysis. PCR primers were located in exon 1 for COX-2, exon 2 for iNOS, exon 3 for NF-κB, exon 4 for MAPK and exon 5 for β-actin, resulting in the amplification of PCR products corresponding to 340, 289,300, 320, and 450 bp respectively. CON: control, LPS only.

NF-κB has been reported to be the most essential process to express iNOS by LPS. The MAPK pathway is known to be involved in COX-2 expression and regulate NF-κB activation (Surh et al., 2001).

The present study showed that the level of nitrite produced by LPS or SNP in RAW264.7 macrophages was reduced by BV treatment in a dose-dependent manner. It indicates that BV directly or indirectly suppresses the formation of NO. It was also demonstrated that BV decreased the levels of iNOS, COX-2, NF-κB and MAPK mRNA. These results suggest that the anti-inflammatory effects of BV are due to the inhibition of iNOS and COX-2 expression, possibly through the suppression of NF-κB and MAPK activation. Under normal conditions, NF-κB fuses with the inhibitors of NF-κB (i.e. IκB) and resides within the cytoplasm, but should there be a stimulus such as LPS, IκB degrades due to the activation of various kinases and the NF-κB released thence moves into the nucleus to induce various enzymes (Baeuerle, 1998; Ghosh et al., 1998). Therefore, it is possible that anti-inflammatory effects of BV observed in the present study may be associated with suppression of the dissociation of IκB from NF-κB.

BV is composed of various peptides, enzymes and non-peptide components. Peptides are mainly composed of apamin, melittin, mast cell degranulation peptide (MCD peptide) and adolapin. Enzymes consist of phospholipase A2, hyaluronidase, acid phosphomonoesterase, α-D-glucosidase and lypophospholipase. Non-peptide components consist of histamine, dopamine and noradrenaline (Habermann, 1971; Banks and Shipolini, 1986; Lee et al., 2001). Each BV substance has been the subject of many studies employing diverse methodologies in an effort to prove their anti-inflammatory effect (Martin and Hartter, 1980; Koburova et al., 1985; Somerfield et al., 1986; Saini et al., 1997). However, the major anti-inflammatory molecule has not yet been elucidated. In addition, our results could not rule out a potential scavenger activity of some of the BV compounds, although it has been reported that whole BV and mellitin by inhibiting superoxide production have anti-inflammatory effects that are not due to toxic or scavenging effects (Somerfield et al., 1984, 1986). Therefore, further studies are needed to identify the major components of BV that are responsible for the anti-inflammatory effects.

In conclusion, our study demonstrated that BV has an anti-inflammatory effect by inhibiting iNOS and COX-2 expression possibly through the suppression of NF-κB and MAPK activities. It thus seems that BV treatment may serve an effective anti-inflammatory therapy.

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