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Bee Venom Suppresses Lipopolysaccharide-stimulated Expression of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Mouse BV2 Microglial Cells

Mi-Hyeon Jang[1] · Myoung-Hwa Lee[1] · Chang-Ju Kim[1] · Hye-Sook Shin[2] · Se-Keun Park[3] · Jeong-Seon Kim[3] · Ee-Hwa Kim[4,*]

[1]Dept. of Physiology, College of Medicine, Kyung-Hee University;
[2]College of Nursing Science, Kyung-Hee University;
[3]Dept. of Oriental Food & Nutrition,
[4]Dept. of Acupoint & Meridian, College of Oriental Medicine, Semyung University

봉독약침액이 BV2 세포에서 LPS로 유발된 염증반응에 미치는 영향

장미현[1], 이명화[1], 김창주[1], 신혜숙[2], 박세근[3], 김정선[3], 김이화[4,*]

경희대학교 의과대학 생리학교실[1] · 경희대학교 간호학과[2] · 세명대학교 한방식품영양학과[3] · 세명대학교 한의과대학 경혈학교실[4,]*

Abstract

목적 : 본 연구는 봉독 약침액이 BV2 microglial cell에서 LPS로 유발된 염증반응에 대한 억제효과를 관찰하고자 하였다.

방법 : 봉독 약침액의 항염증작용을 관찰하기 위하여 BV2 microglial cell에 봉독약침액을 1시간전에 농도별(0.1, 1, 100 ㎍/㎖)로 전처치한 후 LPS (5 ㎍/㎖)로 24시간동안 처리하여 RT-PCR, western blot, PGE_2 assay, NO synthesis assay등의 방법으로 관찰하였다.

결과 : LPS 염증유발에 의해서 BV2 microglial cell에서 COX-2 및 NOS 발현이 증가하였고, 이러한 증가는 prostaglandin E2 및 NO 합성을 증가시켰다. 이에 반하여 봉독약침액으로 전처치한 군에서는 COX-2 및 NOS 발현을 억제시켜 결과적으로 prostanglandin 합성 및 NO 합성을 억제시킴을 확인할 수 있었다. 또한 LPS 염증유발에 의해서 활성화된 NF-κB의 발현을 억제시켰다.

결론 : 봉독약침액은 LPS 염증유발에 의해서 증가된 prostaglandin E2 및 NO 합성을 억제시킴으로써 여러 가지 염증질환의 치료에 유효한 효과가 있을 것으로 사려된다.

Key words : Bee venom, Lipopolysaccharide, Prostaglandin E_2, Nitric oxide

I. Introduction

Bee venom has traditionally been used for the treatment of inflammatory diseases such as rheumatoid arthritis, and for the relief of pain. Bee venom is known to possess anti-inflammatory effects similar to non-steroidal anti-inflammatory drugs (NSAIDs). Recently, it was reported that the administration of bee venom produced both antinociceptive and anti-inflammatory effects in Freund's adjuvant-

· 교신저자 : 김이화, 충북 제천시 신월동 산21 세명대학교 한의과대학 경혈학교실, Tel. 043-649-1348, Fax. 043-649-1349, E-mail : kimeh@semyung.ac.kr
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induced rheumatoid arthritis model[1]. The antinociceptive effect of bee venom is known to be mediated by the central activation of α_2-adrenoceptors[2].

Brain inflammation has been implicated in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and ischemic brain injury. Anti-inflammatory drugs reduce the risk and progression of Alzheimer's disease, and the neuronal damage in animal models of Parkinson's disease. Microglia are the major inflammatory cells in the brain that are activated by brain injury[3].

Lipopolysaccharide (LPS) initiates a number of major cellular responses that play vital roles in the pathogenesis of inflammatory responses including activation of inflammatory cells and the production of cytokines and other mediators. Prostaglandin E_2 (PGE_2) is a key inflammatory mediator that is converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and the bacterial endotoxin, LPS. COX-2 produces a large amount of PGE_2 and this induces inflammation[4,5].

Nitric oxide (NO), endogenously generated from L-arginine by NO synthase (NOS), plays an important role in the regulation of many physiological processes. Several isoforms of NOS exist and these isoforms fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Of these, iNOS is an important enzyme involved in regulation of inflammation. It was reported that LPS up-regulates iNOS expression in macrophages[6] and microglial cells[7].

In addition, it has been documented that the process of inflammation is regulated by several transcription factors. Many recent studies have provided evidence that nuclear factor-kappa B (NF-κ B) regulates an important signaling in brain inflammation[8]. NF-κ B, a member of the Rel transcription factor family, is normally held in the cytoplasm as an inactive form that is bound to an inhibitory protein, Iκ B. It was reported that LPS leads to the activation of NF-κ B through phosphorylation of Iκ B protein[9].

In the present study, the effect of bee venom on the LPS-stimulated PGE_2 synthesis and NO production in mouse BV2 microglial cells was investigated.

Ⅱ. Materials & Methods

1. Cell culture

Bee venom was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The mouse BV2 microglial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2 and 95% O_2

in a humidified cell incubator.

2. RNA isolation and reverse transcription-polymerase chain reaction

To identify expressions of COX-2 and iNOS mRNA, reverse transcription-polymerase chain reaction (RT-PCR) was performed, and the exact primer sequences used in this study were designed according to Jang et al.[10]. For mouse *COX-2*, the primer sequences were 5′-TGCATGTGGCTGTGGATGTCATC AA-3′ (a 25-mer sense oligonucleotide) and 5′-CACTAAGACAGACCCGTCATCTCCA-3′ (a 25-mer anti-sense oligonucleotide). For mouse *iNOS*, the primer sequences were 5′-GTGTTCCACCAGGAGATGTTG-3′ (a 21-mer sense oligonucleotide) and 5′-CTCCTGC CCACTGAGTTCGTC-3′ (a 21-mer anti- sense oligonucleotide). For *cyclophilin*, the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTTCTTC GAC-3′ (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGCC ATGGACAAGATG-3′ (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp for *COX-2* and 500 bp for *iNOS*, and 299 bp for *cyclophilin*.

For *COX-2* and *iNOS*, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, with an additional extension step at the end of the procedure at 72°C for 5 min. For *cyclophilin*, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically by using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

3. Western blot analysis

To confirm the expression of COX-2 and iNOS protein, the cells were lysed in a lysis buffer, and the protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). Protein from 50 g was separated on SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Rabbit iNOS antibody and goat COX-2 antibody were used as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit antibody for iNOS and anti-goat antibody for COX-2 were used as the secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA).

4. Measurement of PGE_2 synthesis

Assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc.). Cells were

lysed and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plate was incubated at room temperature and shook for 1 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 nm.

5. Measurement of NO synthesis

In order to determine the effect of bee venom on NO synthesis, the amount of nitrite in cell-free culture supernatant was measured using a commercially available NO detection kit (Intron Biotech., Seoul, Korea). After collection of 100 ㎕ of supernatant, 50 ㎕ of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

6. Statistical analyses

The results are expressed as the mean ± standard error mean (SEM). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS(Ver 10.0). Difference was considered statistically significant at $P < 0.05$.

Ⅲ. Results

1. Effect of bee venom on the COX-2 and iNOS mRNA expression

RT-PCR analysis of the mRNA expressions of COX-2 and iNOS was performed to provide an estimate of the relative levels of expression of these genes. In the present study, the mRNA levels of COX-2 and iNOS in the control cells were used as the control value of 1.00.

The expression of COX-2 mRNA following a treatment with 5 ㎍/㎖ LPS for 24 h was significantly increased to 11.96 ± 0.96, but then decreased to 4.59 ± 0.56, 3.45 ± 0.68, and 3.12 ± 0.41 in cells treated with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ acetylsalicylic acid (ASA), respectively (Fig. 1, left).

The expression of iNOS mRNA was markedly increased to 9.47 ± 0.58 following treatment with 5 ㎍/㎖ LPS for 24 h, while it was decreased to 5.01 ± 0.67, 4.21 ± 0.28, and 3.39 ± 0.57 in cells treated with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ ASA, respectively (Fig. 1, right).

2. Effect of bee venom on the COX-2, iNOS, NF-κB, and IκBα protein expression

From the Western blot analysis of COX-2 and iNOS and protein, the increased

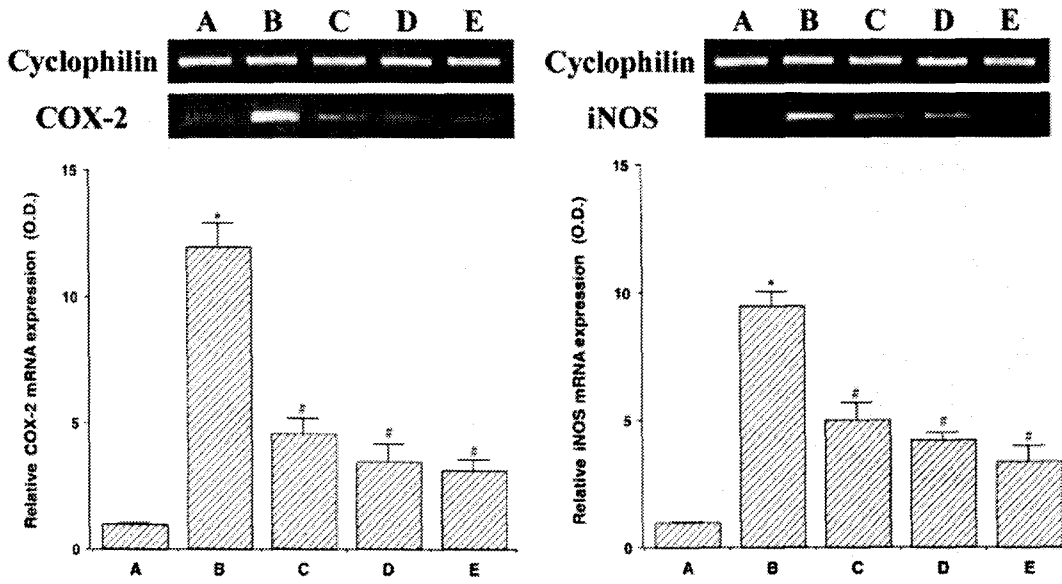

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Cells were pre-treated with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ acetylsalicylic acid (ASA) for 1 h followed by 5 ㎍/㎖ lipopolysaccharide (LPS) treatment for 24 h. Cyclophilin was used as the internal control. * represents $P < 0.05$ compared to the control. # represents $P < 0.05$ compared to the LPS-treated group. (M) Marker, (A) control, (B) LPS-treated group, (C) LPS- and 0.1 ㎍/㎖ bee venom-treated group, (D) LPS- and 1 ㎍/㎖ bee venom-treated group, (E) LPS- and 100 ㎍/㎖ ASA-treated group.

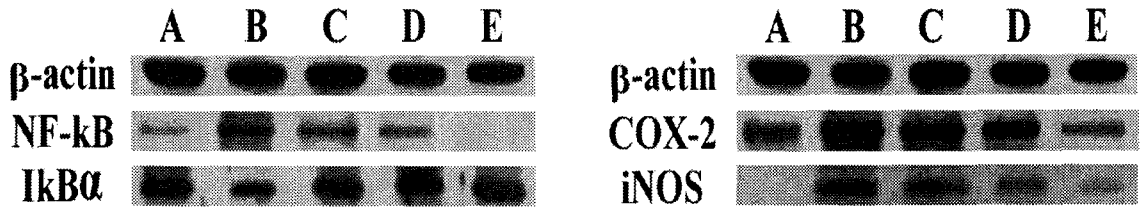

Fig. 2. Western blot analysis. Cells were pre-treated with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ ASA for 1 h followed by 5 ㎍/㎖ lipopolysaccharide (LPS) treatment for 24 h. (A) Control, (B) LPS-treated group, (C) LPS- and 0.1 ㎍/㎖ bee venom-treated group, (D) LPS- and 1 ㎍/㎖ bee venom-treated group, (E) LPS- and 100 ㎍/㎖ acetylsalicylic acid (ASA)-treated group. β -actin was used as the internal control and it was detected at the position corresponding to a molecular weight of 46 kDa. Right: Protein expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Left: Protein expression of nuclear NF-κ B (p65) and cytosolic Iκ Bα .

expressions of COX-2 protein (70 kDa) and iNOS protein (130 kDa) were detected in those cells treated with 5 ㎍/㎖ LPS. In those cells treated with LPS and bee venom (0.1 ㎍/㎖ and 1 ㎍/㎖), however, the expressions of iNOS and COX-2 were decreased (Fig. 2, right).

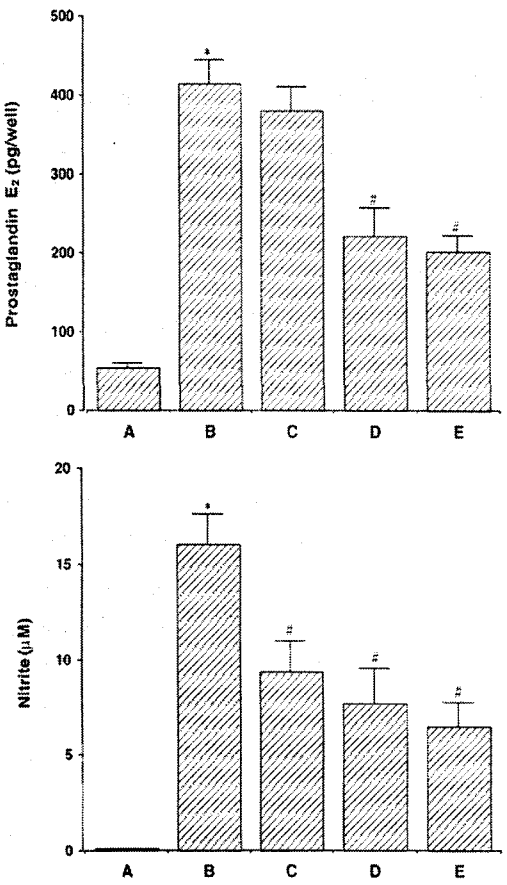

Fig. 3. Measurement of prostaglandin E_2 (PGE_2) and nitric oxide (NO) production in microglial BV2 cells. Cells were pre-treated with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ acetylsalicylic acid (ASA) for 1 h followed by 5 ㎍/㎖ lipopolysaccharide (LPS) treatment for 24 h. * represents $P < 0.05$ compared to the control. # represents $P < 0.05$ compared to the LPS-treated group. (A) Control, (B) LPS-treated group, (C) LPS- and 0.1 ㎍/㎖ bee venom-treated group, (D) LPS- and 1 ㎍/㎖ bee venom-treated group, (E) LPS- and 100 ㎍/㎖ ASA-treated group.

To visualize the nuclear NF-κB translocation, LPS was treated onto the cells at a concentration of 5 ㎍/㎖ for 24 h, as based on previous study by D'Acquisto et al.[11]. From the results of the present study, cytosolic IκB α (IκB subunit) was detected as a protein of approximately 37 kDa in the control group. After stimulation with 5 ㎍/㎖ LPS for 24 h, the expression of IκBα protein in the cytoplasm was decreased, suggesting a degradation of IκBα, while a 1 h pre-treatment with bee venom at concentrations of 0.1 ㎍/㎖ and 1 ㎍/㎖ inhibited the IκBα degradation induced by 5 ㎍/㎖ LPS for 2 h in mouse BV2 microglial cells. In addition, treatment of the cells with 5 ㎍/㎖ LPS for 2 h promptly increased the NF-κB (p65 subunit) level in the nucleus, while a 1 h pre-treatment with bee venom at concentrations of 0.1 ㎍/㎖ and 1 ㎍/㎖ inhibited the NF-κB translocation induced by 5 ㎍/㎖ LPS for 24 h in mouse BV2 microglial cells (Fig. 2, left).

3. Effect of bee venom on the PGE_2 and NO synthesis

From the PGE_2 immunoassay, after 24 h of exposure to LPS, the amount of PGE_2 from the culture medium was increased from 54.00 ± 6.42 pg/㎖ to 414.00 ± 30.27 pg/㎖, and it was decreased to 380.00 ± 29.44 pg/㎖, 221.00 ± 35.51 pg/㎖, and 201.48 ± 19.54 by the treatment with 0.1 ㎍/㎖ bee venom, 1 ㎍/㎖ bee venom, and 100 ㎍/㎖ ASA, respectively (Fig. 3, upper).

From the NO detection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 0.11 ± 0.01 μM to 16.02 ± 1.59

μ M, and it was decreased to 9.37 ± 1.62 μ M, 7.73 ± 1.79 μ M, and 6.51 ± 1.28 μ M by treatment with 0.1 μg/mℓ bee venom, 1 μg/mℓ bee venom, and 100 μg/mℓ ASA, respectively (Fig. 3, lower).

Ⅳ. Discussion

Microglia are macrophage-like cells of the central nervous system (CNS), and they generally considered being immunologically quiescent under normal conditions. The activation of microglia that is induced by CNS injury or infection is associated with neurodegenerative disorders[3]. Inflammation is a complex process involving numerous mediators of a cellular and plasma origin, and these mediators have elaborate and interrelated biological effects.

PGE_2 and NO are involved in various pathophysiological processes including inflammation and carcinogenesis, and iNOS and COX-2 are known as the main enzymes for the production of these mediators[12]. Elevation of COX-2 activity is closely associated with the occurrence of cancer, arthritis, and several types of neurodegenerative disorders. Specific COX-2 inhibitors can attenuate the symptoms of inflammation[5,13]. NO exerts diverse and multifunctional effects in the host cells. After an exposure to endogenous and exogenous stimulators such as LPS and viral infection, iNOS is induced quantitatively in the various cells, and it triggers several deleterious cellular responses inducing inflammation, sepsis, and stroke[6,7,14]. In addition, COX activity and the subsequent production of PGE_2 are closely related to the generation of NO radicals. Salvemini et al.[15] reported that NO modulates the activity of COX-2 in a cGMP-independent manner, and NO plays a critical role in the release of PGE_2 by the direct activation of COX-2. Inhibition on the iNOS expression in murine macrophages has been suggested as another possible mechanism for the effect of non-steroidal anti-inflammatory drugs[16].

NF-κ B regulates the expression of various genes including iNOS, COX-2, and cytokines, and these genes and their products that play critical roles in apoptosis, autoimmune diseases, and inflammation. Because of its ubiquitous role in the pathogenesis of inflammatory gene expression, NF-κ B is a current therapeutic target for treating a variety of diseases[8]. NF-κ B is activated in response to various inflammatory stimuli including bacterial LPS, cytokines, and viral proteins[9]. Under normal conditions, NF-κ B is present in the cytoplasm as an inactive heterotrimer consisting of p50, p65, and Iκ Bα subunits. On activation, the degradation of Iκ Bα exposes a localization signal on the p50/p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in several promoters, which in turn results in gene transcription of proinflammatory genes. In particular, the p65 subunit of NF-κ B is required for binding of the inhibitory Iκ B, and so it activates transcription[17].

Bee venom therapy has been used to relieve pain and to treat inflammatory dise-

ases in human[18] and experimental animals[1,2]. Bee venom has an antinociceptive effect on formalin-induced pain behavior, and it was also reported that bee venom is useful for the treatment of the pain and edema associated with chronic inflammatory diseases[19]. In addition, the pharmacological activities of bee venom on the anti-inflammatory process include the inhibition of COX-2 expression and the blocking of pro-inflammatory cytokines production such as TNF-α and interlukin-1β [20]. However, the molecular mechanisms for the anti-inflammatory effect of bee venom have not yet been clarified.

In the present study, bee venom was shown to suppress PGE_2 and NO production by inhibiting LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression through the NF-κB transcriptional pathway in mouse BV2 microglia cells. The present results suggest that bee venom can exert its anti-inflammatory and analgesic effects probably by suppressing COX-2 and iNOS expressions, resulting in the inhibition of PGE_2 and NO synthesis. It is very possible that bee venom can offer a valuable mode of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE_2 and NO synthesis.

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