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Bee Venom Decreases LPS-Induced Inflammatory Responses in Bovine Mammary Epithelial Cells

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The world dairy industry has long been challenged by bovine mastitis, an inflammatory disease, which causes economic loss due to decreased milk production and quality. Attempts have been made to prevent or treat this disease with multiple approaches, primarily through increased abuse of antibiotics, but effective natural solutions remain elusive. Bee venom (BV) contains a variety of peptides (*e.g.*, melittin) and shows multiple bioactivities, including prevention of inflammation. Thus, in the current study, it was hypothesized that BV can reduce inflammation in bovine mammary epithelial cells (MAC-T). To examine the hypothesis, cells were treated with LPS (1 μg/ml) to induce an inflammatory response and the anti-inflammatory effects of BV (2.5 and 5 μg/ml) were investigated. The cellular mechanisms of BV against LPS-induced inflammation were also investigated. Results showed that BV can attenuate expression of an inflammatory protein, COX2, and pro-inflammatory cytokines such as IL-6 and TNF-α. Activation of NF-κB, an inflammatory transcription factor, was significantly downregulated by BV in cells treated with LPS, through dephosphorylation of ERK1/2. Moreover, pretreatment of cells with BV attenuated LPS-induced production of intracellular reactive oxygen species (*e.g.*, superoxide anion). These results support our hypothesis that BV can decrease LPS-induced inflammatory responses in bovine mammary epithelial cells through inhibition of oxidative stress, NF-κB, ERK1/2, and COX-2 signaling.

Keywords: Bee venom, inflammation, mammary epithelial cells, bovine mastitis, lipopolysaccharide

Introduction

Bovine mastitis is a persistent inflammatory disease in the udder of lactating dairy cows. This common bovine infectious disease causes enormous economic loss because of decreased milk production and poor quality [1]. Bovine mastitis is caused by infection from a diverse group of microorganisms, including pathogenic bacteria. In particular, the gram-negative bacterium *Escherichia coli* is one of the primary causes of bovine mastitis [2].

Alveoli are sack-like structures in which milk is synthesized and secreted. Mammary epithelial cells that line the alveoli are not only essential to milk production but also act as the first line of defense against pathogen infection [3]. Pathogens entering the mammary glands are recognized by Toll-like-receptors (TLRs) [4]. Lipopolysaccharide (LPS), derived from the outer membrane of *E. coli*, activates TLR4 [5]. The TLR signaling pathways trigger a series of downstream signaling cascades, including mitogen-activated protein kinases (MAPKs; *e.g.*, extracellular signal receptor-activated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, and transcription factor nuclear factor-kappa B (NF-κB)) [6]. In addition, proinflammatory cyclooxygenase-2 (COX-2) expression is increased by activation and nuclear trans-

location of NF-κB [7]. Moreover, NF-κB signaling pathways result in the release of various proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), which are associated with upregulating the inflammatory reaction [8]. Therefore, it is important to investigate the activity of MAPKs and NF-κB as biomarkers of inflammatory signaling in bovine mammary epithelial cells (MAC-T) [9].

For decades, the dairy industry has utilized antibiotics to treat bovine mastitis [10, 11]; however, production of milk contaminated with antibiotics has been a major concern to consumers. Vaccination is another way to protect dairy cattle from mastitis, but since a variety of pathogens are involved in bovine mastitis, vaccination is ineffective [12]. In consequence, new non-synthetic therapeutic alternatives that are effective in treatment of mastitis are necessary.

Bee venom (BV) contains a variety of peptides (*e.g.*, melittin) that show multiple biological functions, including reduction of inflammation [13]. In particular, melittin, a small protein that contains 26 amino acid residues (NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$), is the major alkaloid of BV of *Apis mellifera* [14]. Melittin is known to possess anticancer, anti-inflammatory, and antibacterial effects in cells [15–17]. For example, melittin inhibited LPS-induced COX-2 expression via decreased activation of NF-κB in murine macrophage cells [18]. However, the anti-inflammatory effect of BV has not been studied in MAC-T cells. Therefore, it was hypothesized that BV can reduce inflammation in MAC-T cells. To examine this hypothesis, the cells were treated with LPS (1 μg/ml) to induce inflammatory responses and the anti-inflammatory effects of BV were investigated. In particular, the intracellular signaling cascades such as COX-2, NF-κB, and oxidative stress were investigated in MAC-T cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM/high glucose) was obtained from Lonza (Switzerland). Fetal bovine serum (FBS) was obtained from PAN Biotech (Germany). Penicillin/streptomycin was purchased from Welgene (Korea). Phosphate-buffered saline (PBS) and trypsin were obtained from Gibco (USA). Progesterone, insulin, and LPS from *E. coli* O111:B4 were obtained from Sigma Aldrich (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (USA). Primary antibodies against COX-2, NF-κB p65, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lamin B, and secondary antibodies against goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP, and specific pharmacological inhibitors (PD98059 and BAY11-7082) were purchased from Santa Cruz Biotechnology (USA). Primary antibodies against phospho-Erk1/2 and Erk1/2 were purchased from Cell Signaling Technology (USA). Dihydroethidium (DHE) was purchased from Invitrogen (USA). ProLong Gold Antifade reagent containing 4,6-diamidino-2-phenylindole (DAPI) was obtained from Life Technologies (USA). BV was obtained from experimental colonies of honeybees at the National Academy of Agricultural Science in Korea as described previously [19]. Briefly, BV was collected with a special collector (Chungjin Biotech Co., Ltd, Korea). To remove supernatant impurities, the collected BV was diluted in cold water and then centrifuged at 10,000 ×*g* for 5 min at 4°C. The final collected BV was lyophilized and stored at 4°C until use.

Cell Culture and Treatments

MAC-T cells were cultured in DMEM containing 10% FBS, penicillin/streptomycin, 5 μg/ml insulin, and 1 μg/ml progesterone at 37°C and 5% CO_2 in a humidified atmosphere. The cells were grown until 90–100% confluency and then pretreated with BV at concentrations of 2.5 and 5 μg/ml for 12 h. Then, the cells were stimulated with LPS (1 μg/ml) for 12 h by adding it directly into BV-treated cell culture medium in order to investigate the anti-inflammatory effect of BV. Concentrations of BV were chosen on the basis of cell viability data. LPS concentration was based on previous studies using bovine mammary epithelial cells [20]. For the antagonist study, cells were incubated for 1 h with the inhibitors (PD98059 and BAY11-7082), followed by stimulation with LPS (1 μg/ml, 12 h).

Cytotoxicity Test

The cytotoxicity of BV was determined by MTT assay. Cells were seeded in a 96-well plate and then treated with BV (0–40 μg/ml), followed by incubation for 24 h. The optical density was measured at 570 nm with a spectrophotometer (Biotek Instrument, USA). To reconfirm the cytotoxicity of BV, the trypan blue dye exclusion test was performed. Cells were grown in 6-well plates and then treated with BV (0–10 μg/ml) for 24 h. Viable cells were identified by trypan blue dye exclusion and counted using a hemocytometer.

Preparation of Cell Lysate, SDS-PAGE, and Western Blot Analysis

To collect total proteins, cells were lysed on ice using RIPA-buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor mixture (2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, and 1 mM Na_3VO_4). All lysates were collected by scrapping the culture dishes with cell scrappers and centrifuged at 23,500 ×*g* for 20 min at 4°C to remove cell debris. Protein concentrations were analyzed using a Pierce BCA protein assay kit (Sigma-Aldrich). The cell lysates were stored at −80°C until use. For Western blot analysis, protein samples were separated using 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. The membranes were

incubated with 3% skim milk buffer for 1 h 30 min at room temperature to block the nonspecific binding sites. Then, the blots were incubated overnight at 4°C with a primary antibody. After washing, the membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature and visualized using ECL detection reagents (Thermo Scientific, USA).

Assessment of Superoxide Levels

The intracellular superoxide level was measured as described previously [21]. MAC-T cells were grown to 90% confluency on a cover glass in 6-well plates. The cells were treated with BV for 12 h, followed by LPS (1 μg/ml) for 4 h. The cells were then incubated with DHE (1 μM) for 30 min followed by PBS washes. The cells on coverslips were then fixed with 4% formaldehyde and incubated for another 10 min. After rinses in PBS, the nuclei of cells were stained with ProLong Gold Antifade reagent containing DAPI. Finally, the cells were visualized with an Olympus IX71 fluorescence microscope at 200× magnification and images were taken with an Olympus DP71 camera and DP controller software (Olympus Optical Co. Ltd, Japan).

Real-Time PCR Analysis

Cells were grown in 6-well plates and total RNA was extracted from the cells using TRIzol reagent (Ambion, USA) according to the manufacturer's protocol. Reverse transcription was performed using the TOPscript RT DryMIX kit (Enzynomics, Korea). The levels of mRNA expression were assessed by real-time PCR using the PikoReal Real-Time PCR System (Thermo Fisher Scientific) and 2× Real-Time PCR mix (SolGent, Korea). The thermal conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 20 sec and 58°C for 40 sec, followed by 60°C for 30 sec and a hold at 4°C. Data analysis was performed using the relative quantification method (ΔΔCq), in which relative mRNA expression for target mRNAs (*i.e.*, IL-6 and TNF-α) was compared to that of a constitutively expressed gene (*i.e.*, GAPDH) in the mRNA samples from untreated and treated cells. The primer sequences used were IL-6, 5′-AGC GCA TGG TCG ACA AAA TCT C-3′ and 5′-AAC CCA GAT TGG AAG CAT CCG T-3′; TNF-α, 5′-ACG GGC TTT ACC TCA TCT ACT CAC-3′ and 5′-TTG ACC TTG GTC TGG TAG GAG ACT-3′; and GAPDH, 5′-ATG ATT CCA CCC ACG GCA AGT T-3′ and 5′-ACC ACA TAC TCA GCA CCA GCA T-3′.

Nuclear Fractionation

Nuclear translocation of NF-κB p65 was determined as described previously [22]. Cells were grown in a 10-cm cell culture dish and then treated with BV (2.5 and 5 μg/mL) for 12 h, followed by LPS (1 μg/ml) for 12 h. For the cytosolic fraction, cells were lysed in hypotonic buffer solution (20 mM Tris (pH 7.4), 10 mM NaCl and 3 mM $MgCl_2$) containing a protease inhibitor mixture. After addition of 10% Triton-X 100, cell lysates were centrifuged at 650 ×*g* for 10 min at 4°C and the supernatants were collected as cytosolic fractions. For the nuclear fraction, the remaining pellets were resuspended in cell extraction buffer (100 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, and 0.1% SDS) containing the protease inhibitor mixture. The homogenates were then centrifuged at 14,000 ×*g* for 20 min at 4°C and the supernatants were collected as the nuclear fractions. The nuclear fraction was analyzed by SDS-PAGE and western blot assay for the NF-κB p65. Lamin B, a nucleus-specific housekeeping protein, was used as the loading control.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined with SPSS-PASW statistics software ver. 18.0 for Windows (SPSS, USA) by one-way ANOVA and the groups were compared using Tukey's posthoc test. A probability value of $p < 0.05$ was considered statistically significant.

Results

Cytotoxicity of BV in MAC-T Cells

To investigate the cytotoxicity of BV, MAC-T cells were treated with BV (0–40 μg/ml) for 24 h. Results from MTT and trypan blue dye exclusion tests showed that BV had no

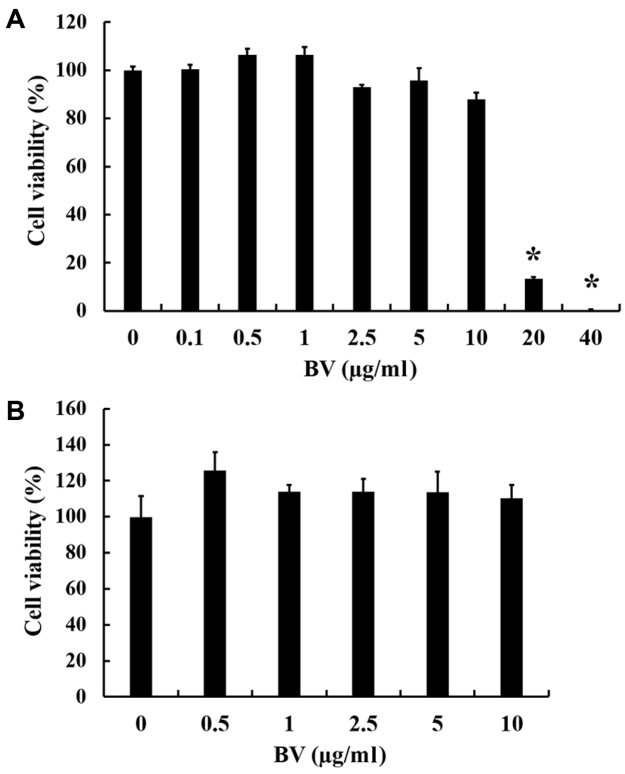

Fig. 1. Cytotoxicity of bee venom (BV) in MAC-T cells.
(**A**) MTT assay and (**B**) trypan blue dye exclusion assay were used to determine the viability of MAC-T cells. Cells were treated with various concentration of BV for 24 h. Values represent the mean ± SEM ($n = 4$); * indicates a significant difference vs. the control ($p < 0.05$).

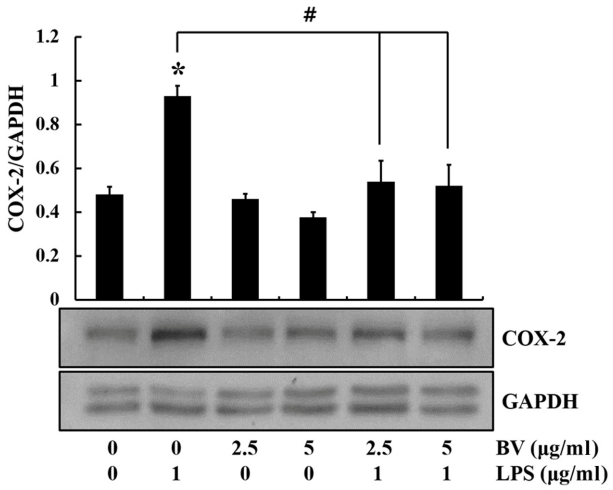

Fig. 2. Bee venom (BV) decreases LPS-induced COX-2 protein expression in MAC-T cells.

Cells were pretreated with BV (2.5 and 5 μg/ml) for 12 h, followed by LPS treatment (1 μg/ml) for 12 h. Western blot analysis was performed to measure COX-2 protein expression in whole-cell lysates. GAPDH was used as the loading control. The Western blots shown are representative images of three independent experiments. * indicates a significant difference vs. the control ($p < 0.05$). # indicates a significant difference vs. LPS only ($p < 0.05$).

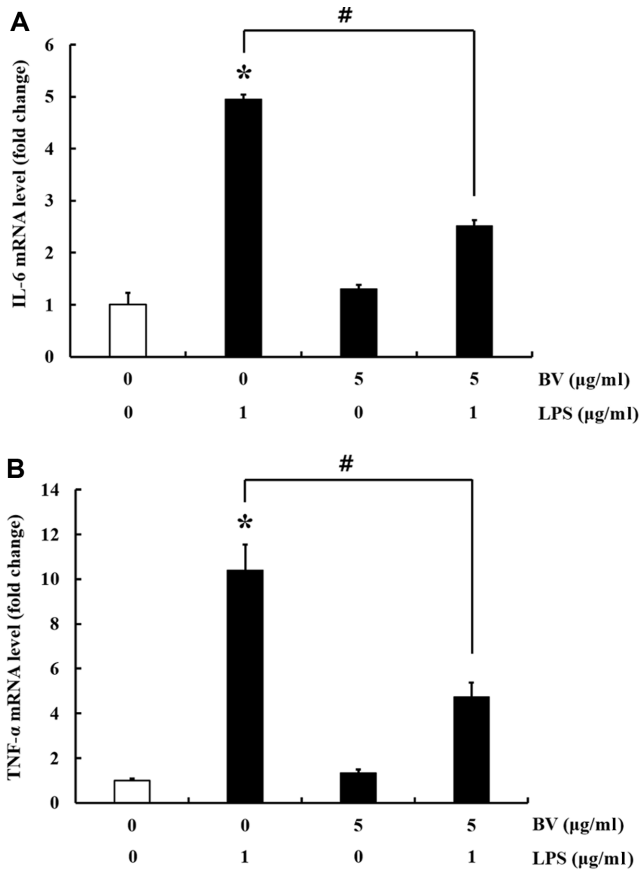

Fig. 3. Bee venom (BV) decreases LPS-induced mRNA expression of (**A**) IL-6 and (**B**) TNF-α in MAC-T cells.

Cells were pretreated with BV (5 μg/ml) for 12 h, followed by LPS treatment (1 μg/ml) for 12 h. The mRNA expression levels were measured using real-time PCR. Values represent the mean ± SEM ($n = 3$). Experiments were repeated a minimum of three times. * indicates a significant difference vs. the control ($p < 0.05$). # indicates a significant difference vs. LPS only ($p < 0.05$).

cytotoxic effects on cells up to 10 μg/ml, as compared with the control (Figs. 1A and 1B). Thus, the BV concentrations of 2.5 and 5.0 μg/ml were used in the subsequent experiments.

Effects of BV on LPS-Induced COX-2 Expression

COX-2 is an enzyme responsible for causing inflammation and is also a marker of inflammation [23]. In particular, COX-2 is used as a target for therapeutic intervention of mastitis [24]. Thus, the expression of COX-2 is a reliable indicator for inflammatory reactions in cells. To determine whether BV can attenuate LPS-induced COX-2 expression, cells were pretreated with BV (2.5 and 5 μg/ml, 12 h), followed by LPS treatment (1 μg/ml, 12 h). Western blot analysis demonstrated that BV significantly attenuated LPS-induced COX-2 expression (Fig. 2), suggesting anti-inflammatory effects of BV in MAC-T cells.

Effects of BV on LPS-Induced Inflammatory Gene Expression

To examine the effect of BV on the production of proinflammatory cytokines, cells were pretreated with BV (5 μg/ml, 12 h) and then stimulated with LPS (1 μg/ml, 12 h). Expression of inflammation-associated genes (IL-6 and TNF-α) was determined using the real-time PCR technique. LPS significantly increased the expression of these proinflammatory genes, compared with the control (Figs. 3A and 3B). In contrast, pretreatment of cells with BV markedly blocked the LPS-induced gene expression (Figs. 3A and 3B).

Effects of BV on LPS-Induced Oxidative Stress

Cellular oxidative stress is a critical event in inflammatory diseases such as bovine mastitis [25]. To examine intracellular reactive oxygen species (ROS) generation, cells were treated with a fluorescent dye (DHE), which is visualized in the presence of superoxide anion inside cells. The intensity of fluorescent light indicates the amount of superoxide produced in the cells. Cells treated with LPS (1 μg/ml, 4 h) showed significantly increased cellular superoxide production

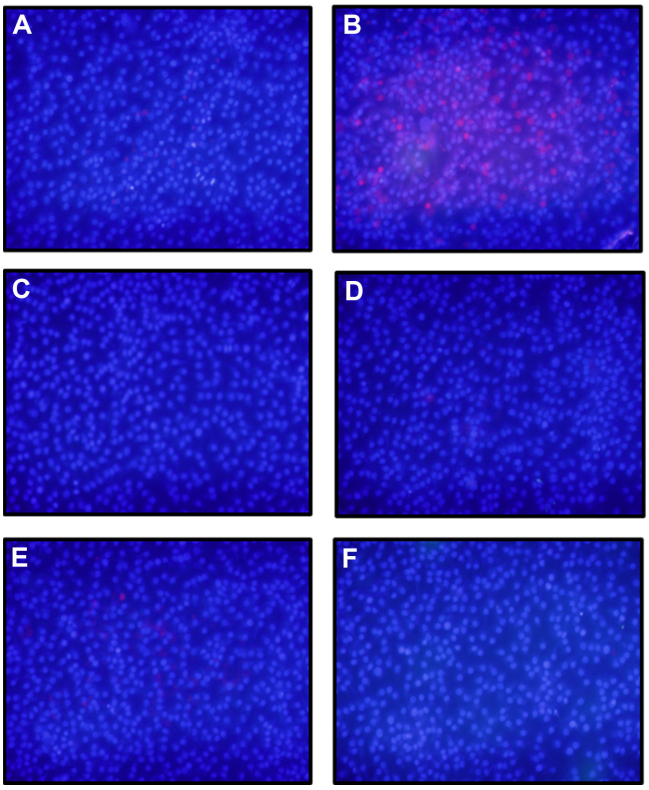

Fig. 4. Bee venom (BV) attenuated production of the superoxide anion in MAC-T cells.
Cells were treated with BV for 12 h and then stimulated with LPS for 4 h. The cells were stained with DHE to detect superoxide anion production. The intensity of red fluorescence (superoxide anion) was assessed using a fluorescence microscope at 200× magnification. The nuclei were stained with DAPI (blue fluorescence). The images shown are representatives of three independent experiments. (**A**) Control, (**B**) LPS 1 μg/ml, (**C**) BV 2.5 μg/ml, (**D**) BV 2.5 μg/ml and LPS 1 μg/ml, (**E**) BV 5 μg/ml, and (**F**) BV 5 μg/ml and LPS 1 μg/ml.

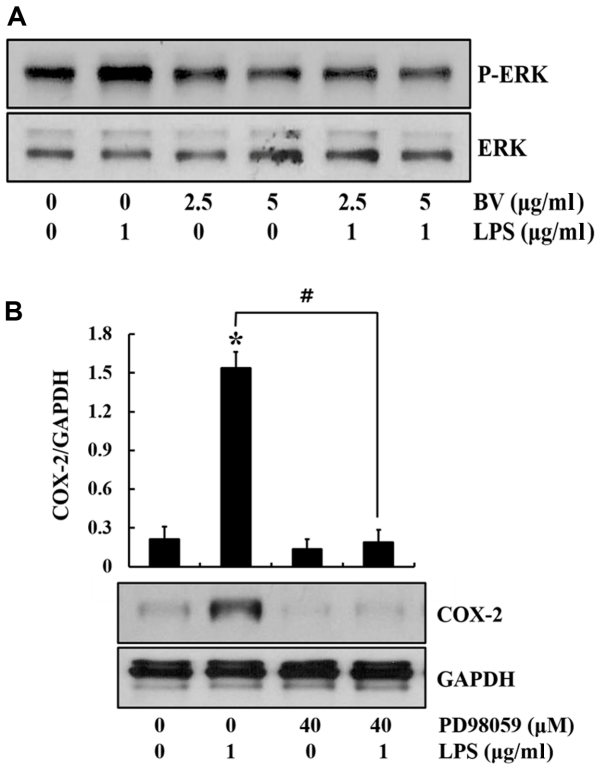

Fig. 5. Bee venom (BV) decreased phosphorylation of ERK1/2 in MAC-T cells.
(**A**) Cells were pretreated with BV (2.5 and 5 μg/ml) for 12 h, followed by LPS treatment (1 μg/ml) for 30 min. (**B**) MAC-T cells were pretreated with PD98059 (inhibitor of ERK) at 40 μM for 1 h, followed by stimulation with LPS (1 μg/ml) for 12 h. Western blot analysis was used to measure protein expression in whole-cell lysates. ERK or GAPDH was used as the loading control. The western blots shown are representative images of three independent experiments. * indicates a significant difference vs. the control ($p < 0.05$). # indicates a significant difference vs. LPS only ($p < 0.05$).

(Fig. 4). Pretreatment of cells with BV (2.5 and 5.0 μg/ml, 12 h) dramatically prevented the observed LPS-induced superoxide production (Fig. 4). These results indicate that BV has an antioxidant effect against LPS-induced oxidative stress in MAC-T cells.

Effects of BV on LPS-Induced Activation of ERK1/2 and NF-κB

LPS is known to activate several intracellular signaling molecules, including ERK1/2 and NF-κB. Previous studies have reported that activation of these signaling molecules can increase inflammatory responses such as COX-2 expression [26]. To determine the involvement of inflammatory signaling molecules and the role of BV, ERK1/2 and NF-κB activation was examined in MAC-T cells. Cells were pretreated with BV for 12 h, followed by LPS challenge for 30 min, which is an optimal time-point for observing LPS-induced ERK1/2 activation. Results showed that LPS increased phosphorylation of ERK1/2, whereas BV markedly attenuated this cellular event (Fig. 5A). To confirm the association of ERK1/2 in LPS-induced COX-2 expression, cells were treated with pharmacological inhibitors for ERK1/2 (PD98059). Results showed that LPS-induced COX-2 expression was abolished when cells were treated with PD98059, suggesting ERK1/2 is a mediator of LPS-induced inflammation (Fig. 5B). Activation of NF-κB, a redox-sensitive transcription factor, was identified by nuclear translocation of NF-κB p65 subunit. NF-κB was markedly activated in cells treated with LPS (1 μg/ml, 12 h), but BV decreased these inflammatory events at both 2.5 and 5.0 μg/ml (Fig. 6A).

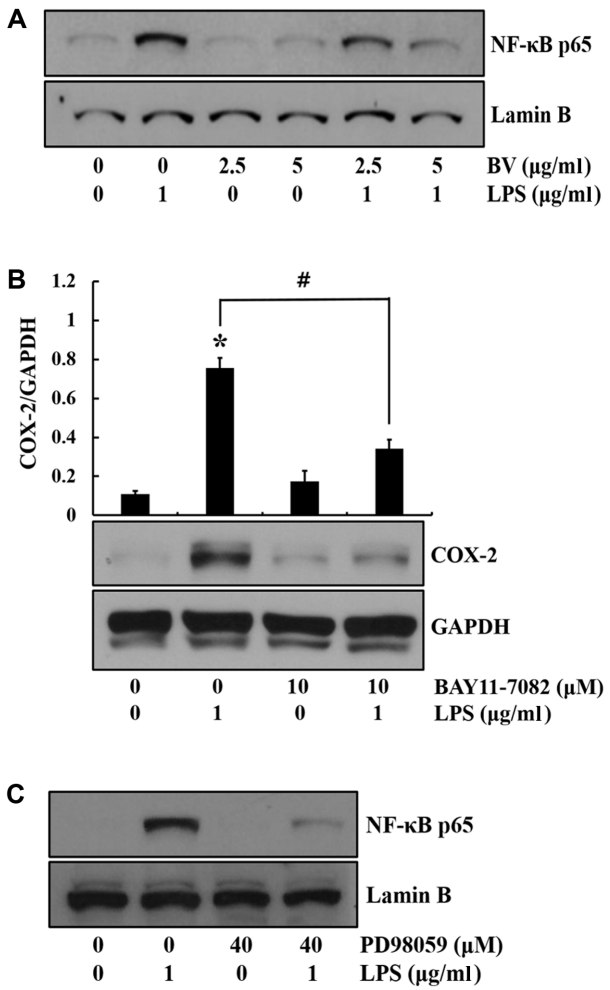

Fig. 6. Bee venom (BV) decreased the nuclear translocation of the NF-κB p65 subunit in MAC-T cells.

(**A**) Cells were pretreated with BV (2.5 and 5 μg/ml) for 12 h, followed by LPS treatment (1 μg/ml) for 12 h. (**B**) MAC-T cells were pretreated with BAY11-7082 (an inhibitor of NF-κB) at 10 μM for 1 h, followed by stimulation with LPS (1 μg/ml) for 12 h. (**C**) Cells were pretreated with PD98059 (a pharmacological inhibitor of ERK1/2) at 40 μM for 1 h, followed by LPS treatment (1 μg/ml) for 12 h. In nuclear translocation experiments, the level of nuclear NF-κB p65 was determined in the nuclear fraction using Western blot analysis, and lamin B was used as the loading control. In Western blot analysis of COX-2, protein expression was measured in whole-cell lysates, and GAPDH was used as the loading control. The Western blots shown are representative images of three independent experiments. * indicates a significant difference vs. the control ($p < 0.05$). # indicates a significant difference vs. LPS only ($p < 0.05$).

Furthermore, to investigate the role of NF-κB in LPS-induced COX-2 expression, cells were treated with an NF-κB inhibitor (BAY11-7082) at 10 μM for 1 h, followed by challenge with LPS (1 μg/ml, 12 h). Pharmacological inhibition of NF-κB decreased the LPS-induced COX-2 expression (Fig. 6B). These data suggest that COX-2 expression is regulated by a transcription factor, NF-κB. In addition, the regulatory role of ERK1/2 on NF-κB was investigated in MAC-T cells. Results showed that nuclear translocation of NF-κB p65 was mediated by ERK1/2 signaling molecules (Fig. 6C). Our results demonstrated that BV decreases LPS-induced inflammatory responses through deactivation of ERK1/2 and NF-κB in MAC-T cells.

Discussion

Bovine mastitis is one of the most common diseases of dairy cattle. Since industrial loss is large, attempts have been made to prevent this economically important disease. Microorganisms associated with bovine mastitis are frequently controlled by antibiotics. However, overuse of antibiotics in dairy cows has caused concerns about negative health effects to humans. Thus, the challenge for the dairy industry is to search for strategies to reduce the use of synthetic antibiotics in dairy cows to minimize human intake of antibiotics. In this context, research about seeking natural substances that prevent bovine mastitis has drawn more attention in recent years.

BV from *Apis mellifera* and its major component, melittin, are known to show antimicrobial effects [27]. For example, both whole BV and melittin have been shown to possess similar *E. coli* killing activities [19]. Previous studies have also demonstrated that melittin has anti-inflammatory effects [15]. Pathologically, bovine mastitis is defined as an inflammatory response of the mammary gland most commonly toward pathogenic infections [28]. One of the most common pathogens is gram-negative *E. coli*, which infects the mammary gland during parturition and early lactation [29]. Alveolar epithelial secretory cells are found in the udder and these cells synthesize milk as well as serve as the first line of defense against the invasion of pathogens [3]. Thus, mammary epithelial cells are considered an important subject to investigate inflammatory responses. Consequently, an established bovine mammary epithelial cell line (MAC-T) is widely used for in vitro experimental settings because biological responses are similar to primary cultures of bovine mammary epithelial cells [30]. Therefore, in the current study, MAC-T cells were employed to study the inflammatory responses and potential preventive effects of BV. Bovine mastitis is caused by a diverse group of pathogenic gram-positive and -negative bacteria. In the case of gram-positive bacterial infections such as *Staphylococcus aureus*, a bacterial cell wall component, lipoteichoic acid

(LTA), is responsible for the inflammatory responses [31]. LTA is associated with biofilm formation that allows bacterial adherence to the host. Moreover, LTA moderately induces proinflammatory cytokines such as TNF-α [32]. Thus, gram-positive *S. aureus* is known to cause chronic and subclinical mastitis [32]. In contrast, gram-negative *E. coli* is known to cause acute and severe mastitis [32]. An endotoxin, LPS, is found in the outer membrane of gram-negative *E. coli* and thus was used in the present study to induce inflammatory responses in MAC-T cells. LPS has often been employed in the induction of inflammatory response in MAC-T cells [33]. Our experimental setting also mimics the inflammatory responses observed during bovine mastitis [34, 35].

Before observing the anti-inflammatory effects, the cytotoxicity of BV was tested in MAC-T cells. Both MTT and trypan blue dye exclusion assays were performed to collect reproducible and reliable concentration data. In both tests, cell viability was not significantly decreased at BV concentrations up to 10 μg/ml. Significant cell death was observed at 20−40 μg/ml BV in the MTT assay and thus concentrations up to 5 μg/ml BV were used in the subsequent experiments. COX-2 is an indicator of inflammatory responses because expression of this protein is upregulated during inflammatory stimuli in cells. For example, overexpression of COX-2 was observed in cells stimulated by LPS and mastitis-inducing bacteria [36]. Like these previous reports, our data demonstrated that COX-2 expression was increased in cells treated with LPS (1 μg/ml). In contrast, LPS-induced COX-2 expression was significantly decreased by pretreating cells with BV. Since COX-2 is a well-known marker for cell inflammation, our data suggest that inflammatory responses due to LPS exposure can be decreased by BV in MAC-T. Similarly, a previous report demonstrated that LPS-induced COX-2 expression was markedly decreased by BV in microglial cells [37]. Moreover, in rat C6 glioma cells, LPS-induced inflammatory molecule expression (*i.e.*, COX-2 and IL-1β) was downregulated by BV [38]. Like these previous reports using other cell types, our data demonstrated that BV is an effective anti-inflammatory substance in bovine mammary epithelial cells.

Furthermore, cellular mechanisms responsible for LPS-induced inflammatory responses were investigated (*i.e.*, COX-2 expression). Exposure of cells to LPS was previously reported to produce inflammation in mammary epithelial cells by producing oxidative stress such as superoxide anion [39]. Since BV showed anti-inflammatory properties in MAC-T cells, the antioxidant properties of BV against LPS-induced oxidative stress were examined. LPS stimulated the production of superoxide in MAC-T cells but this was prevented by treatment of cells with BV, indicating that BV has potential antioxidant properties. According to recently published data, BV has strong antioxidant activities such as free radical scavenging activity and inhibition of lipid peroxidation [27]. In addition, BV showed significant anti-inflammatory effects in the same study. Like these previous studies, our data suggest that the antioxidant properties of BV may contribute to the anti-inflammatory effects, at least in part, in LPS-challenged cells.

Numerous studies have shown that the MAPKs such as ERK1/2 are activated upon stimulation of cells, including pathogenic infection [40−42]. In this context, to further investigate the possible mechanisms underlying the anti-inflammation effects of BV, phosphorylation of ERK1/2 was determined in cells exposed to LPS. In fact, ERK1/2 has been known to play important roles in the activation of NF-κB and COX-2 expression [43]. Our data indicate that LPS increased phosphorylation of ERK1/2, whereas BV attenuated this inflammatory signaling cascade in MAC-T cells. This observation was confirmed in the subsequent experiments because cells with blocked ERK1/2 phosphorylation by PD98059 showed significantly lower COX-2 expression. These data suggest that ERK1/2 is a major signaling cascade in delivering the LPS-induced inflammatory signaling cascade in MAC-T cells. Similar data about the importance of ERK1/2 in the inflammatory signaling pathway have been reported. For example, BV markedly decreased mRNA expression of COX-2 by attenuating the ERK1/2 phosphorylation in BV2 microglial cells [37]. A redox-sensitive transcription factor, NF-κB is known to be activated during inflammatory responses such as LPS exposure in cells [44]. Our data demonstrated that NF-κB is regulated by the phosphorylation status of ERK1/2. Treatment of cells with an ERK inhibitor (PD98059) blocked LPS-induced NF-κB activation through blocking of NF-κB p65 nuclear translocation. Treatment of cells with BV showed such cellular anti-inflammatory responses in cells. Our data also showed that blocking of NF-κB activation attenuated COX-2 expression. Similar finding was reported in a previous study using BV2 microglia and RAW 264.7 cells, where BV reduced COX-2 expression via blocking of NF-κB activation during LPS stimulation [45]. The nuclear translocation of NF-κB regulates the transcription of inflammation-related genes, such as IL-6, TNF-α, and IL-1β [46]. These cytokines play an important role in promoting and regulating the cellular inflammation in response to bacterial pathogens [47]. Therefore, NF-κB has been considered as a target to inhibit the inflammatory response

in numerous cells [48]. Since BV blocked activation of NF-κB, the transcriptional expression of inflammatory cytokines (*e.g.*, IL-6 and TNF-α) was observed in cells. As expected, these inflammatory cytokines were significantly downregulated in cells treated with BV. These findings imply that expression of multiple NF-κB-regulated inflammatory genes can be reduced when cells are in the presence of protective substances, such as BV. Other than MAC-T cells, the reduced expression of inflammatory cytokines due to treatment of cells with BV has been observed in other types of cells, such as the U937 human myeloid cell line [49], murine RAW 264.7 macrophage cell line [50], and HaCaT human keratinocyte cell line [51].

In conclusion, BV showed antioxidant and anti-inflammatory properties in bovine mammary epithelial cells (MAC-T) against LPS challenge. Such protective effects of BV were achieved by scavenging superoxide, attenuating activation of ERK1/2 and NF-κB, and downregulation of COX-2 and proinflammatory cytokines (IL-6 and TNF-α). This work highlights the protective and anti-inflammatory effects of BV against LPS-induced inflammatory responses, but more work needs to be completed to determine if the addition of BV in bovine feed or intramammary administration may protect the mammary gland against inflammatory diseases.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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