Bee Venom Pretreatment Has Both an Antinociceptive and Anti-Inflammatory Effect on Carrageenan-Induced Inflammation

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ABSTRACT. Although the injection of bee venom (BV) has been reported to evoke tonic pain and hyperalgesia, there is conflicting evidence in the literature indicating that BV can also exert an anti-inflammatory and antinociceptive effects on inflammation. In this regard, BV has been traditionally used in Oriental medicine to relieve pain and to treat chronic inflammatory diseases such as rheumatoid arthritis. The present study was designed to test the hypothesis that BV induces acute nociception under normal conditions, but that it can serve as a potent anti-inflammatory and antinociceptive agent in a localized inflammatory state. The experiments were designed to evaluate the effect of BV pretreatment on carrageenan (CR)-induced acute paw edema and thermal hyperalgesia. In addition, spinal cord Fos expression induced by peripheral inflammation was quantitatively analyzed. In normal animals subcutaneous BV injection into the hindlimb was found to slightly increase Fos expression in the spinal cord without producing detectable nociceptive behaviors or hyperalgesia. In contrast, pretreatment with BV (0.8 mg/kg) 30 min prior to CR injection suppressed both the paw edema and thermal hyperalgesia evoked by CR. In addition, there was a positive correlation between the percent change in paw volume and the expression of Fos positive neurons in the spinal cord. These results indicate that BV pretreatment has both antiinflammatory and anti-nociceptive effects in CR-induced inflammatory pain. These data also suggest that BV administration may be useful in the treatment of the pain and edema associated with chronic inflammatory diseases.

KEY WORDS: anti-inflammation, antinociception, bee venom, carrageenan, Fos, immunohistochemistry.

It has been recently reported that unilateral, subcutaneous injection of bee venom (BV) into the plantar surface of the hindpaw evokes prolonged and tonic behavioral responses resembling clinical persistent pain [6, 23]. Moreover, intraplantar BV injection induces Fos expression in the ipsilateral dorsal horn of the spinal cord and the spinal Fos expression induced by BV is attenuated by morphine treatment [25]. These data suggest that BV can be a useful agent not only for assessing the mechanisms of pain transmission, but also for evaluating the effectiveness of novel analgesic drugs in the treatment of tonic pain. Although intraplantar BV injection can produce persistent pain under normal conditions, there are conflicting data indicating that BV can be both anti-inflammatory and antinociceptive under conditions of inflammation. In this regard it is interesting to note that BV therapy has been utilized as a traditional alternative medical approach to relieve pain and to treat inflammatory diseases such as rheumatoid arthritis in humans [1, 3]. In experimental animals, adjuvant induced arthritis has been shown to be successfully suppressed by long-term BV treatment [8, 12]. BV or its constituents have also been reported to be effective in the treatment of rheumatoid arthritis in humans [37]. Recently, we also demonstrated that BV treatment produced antinociceptive and anti-inflammatory effects on adjuvant induced arthritis model [21]. Furthermore, it is notable that BV injection into acupoint induced greater anti-arthritis potency as compared with non-acupoint injection in our experiment [21].

The exact mechanisms underlying these reported anti-inflammatory and antinociceptive effects of BV remain to be determined. One postulated mechanism involves the inhibition of Phospholipase A2 (PLA2). PLA2 is one of the major inflammatory components of BV (12% of dry wt) and has been shown to evoke paw edema after subplantar injection [14, 22]. However, Saini and co-workers have reported that melittin, a major component of BV (50% of dry wt), binds to secretory PLA2 and inhibits its enzymatic activity which serves to suppress inflammation [33]. Based on the literature and on preliminary data in our laboratory, we hypothesize that BV is nociceptive under normal conditions but further postulate that it may serve as an anti-inflammatory and/or antinociceptive agent under conditions of inflammation. If this is true, BV may be useful in treating certain types of inflammatory diseases. To test this hypothesis we evaluated the potential antinociceptive and anti-inflammatory effects of BV pretreatment on carrageenan (CR) induced inflammation in the rat.

Subcutaneous injection of CR into the hindpaw produces an acute inflammation associated with ipsilateral edema. Furthermore, behavioral studies have shown that peripheral CR injection evokes thermal hyperalgesia in a dose depen-
dent manner, peaking at 3–4 hr after CR injection, which correlates with the time of maximal edema formation [13, 31]. Electrophysiological studies have also shown that subcutaneous injection of CR increases neuronal responses in the ventrobasal thalamic nuclei [11]. In addition, CR injection is associated with alterations in the magnitude of the C-fiber evoked response of multi-receptive dorsal horn neurons in the spinal cord [38]. Finally, it has been shown that CR-induced hyperalgesia not only parallels the development of peripheral inflammation, but also the expression of Fos protein in the spinal cord [2, 16]. Numerous studies have utilized spinal cord Fos expression to evaluate the effectiveness of anti-inflammatory drugs and analgesics in CR-induced hyperalgesia/inflammation [16, 31]. Both CR-induced hyperalgesia and spinal Fos expression have been shown to be reduced by systemic administration of opioid [17], NMDA antagonists [4] and nonsteroidal anti-inflammatory drugs (NSAIDs) [16]. Based on these data, CR-induced hyperalgesia/inflammation in rodents has been widely accepted as an appropriate model in which to test the efficacy of anti-inflammatory and/or analgesic drugs. Quantitative analysis of spinal cord Fos expression has also been used extensively in this model both to measure the degree of inflammation-induced nociception and to ascertain the potency of antinociceptive drugs.

This study was designed to investigate the effect of BV pretreatment in both normal animals and in the CR-induced acute inflammatory animal model. The influence of BV pretreatment on both thermal hyperalgesia and paw edema produced by CR injection into the hindpaw was evaluated. In addition, CR-induced spinal cord Fos expression was quantitatively analyzed in order to determine if a correlation exists between the volume of peripheral paw edema and the number of spinal cord neurons expressing the Fos protein.

**MATERIALS AND METHODS**

**Animals:** Experiments were performed on male Sprague-Dawley rats, 230–250 g, obtained from the Laboratory Animal Center of Seoul National University (SNU). The rats were kept in a colony room with an ambient temperature of 22°C and a 12-hr alternating light-dark cycle (8 am 7:00 onset). Food and water were available *ad libitum*. Animals were allowed to adapt to the new environment in the experimental room at least 1 hr prior to the start of each experiment and were anesthetized and humanely sacrificed immediately following the experiment. The experiments were carried out on independent groups of animals during the light phase and all testing was conducted in a quiet room by the same experimenter who was blind to the experimental condition of the animal. All of the methods used in the present study were approved by the Animal Care and Use Committee at SNU and conformed to NIH guidelines (NIH publication No. 86-23, revised 1985). In addition, the ethical guidelines set forth by the International Association for the Study of Pain (IASP) for investigating experimental pain in conscious animals were followed [43].

**Experimental groupings:** Two major sets of experiments were performed. In the first series of experiments, the time course of hyperalgesia induced by BV was observed in normal animals. BV (*Apis mellifera*, Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.9% saline and administered subcutaneously into the right hindlimb (especially Zusanli acupoint) at a dose of 0.8 mg/kg (n=10). The Zusanli acupoint (ST36) was located 5 mm lower and lateral to the anterior tubercle of the tibia. In addition, the same dosage of BV was administered directly into the plantar surface of the right hindpaw (n=10) in order to compare our results with those described previously by Chen and coworkers [6].

In the second series of experiments, we evaluated the effect of BV pretreatment on CR induced inflammation and nociception. Peripheral inflammation was induced by intraplantar injection of CR (6 mg/150 μl of saline; CR obtained from Sigma) into the right hindpaw. Intraplantar injection of CR was performed under light isoflurane (3% with 70% N2O/30% O2 mixture) anesthesia. Animals recovered from anesthesia within a few minutes after CR injection. Thirty minutes prior to CR injection BV (0.8 mg/kg or 0.08 mg/kg) or saline was injected subcutaneously into Zusanli acupoint on the right hindlimb. Five groups of animals (2 experimental and 3 control groups) were used to study the anti-inflammatory effect of BV on paw edema and to quantify spinal cord Fos expression. Animals in the experimental groups (BV-CR) received either 0.08 mg/kg of BV (n=6) or 0.8 mg/kg of BV (n=8). The three groups of controls were as follows: (1) a saline control group (SAL-CR, n=4), (2) a BV (0.8 mg/kg) control group (BV-CR, n=4), and (3) a saline pretreatment and CR injected control group (SAL-SAL, n=6). In a separate set of experiments, we also evaluated the potential antinociceptive effect of BV on CR-induced inflammatory thermal hyperalgesia. Twenty-five animals were used for these experiments and were divided into five groups (n=5 rats per group) identical to those described above (two BV-CR groups; SAL-SAL; BV-SAL; and SAL-CR).

**Measurement of paw edema:** Both the ipsilateral and contralateral paw volumes were measured at the tibio-tarsal joint using a plethysmometer (Ugo Basile, Italy) before (VOLpre) and at 3 hr after (VOLpost) CR injection. VOLpre was used as the basal volume for statistical analysis comparison. Data were then converted to percentage change of paw volume using the following equation: % change of paw volume = 100 X (VOLpost - VOLpre)/VOLpre. Each value represents the mean of three repeated measurements.

**Measurement of thermal hyperalgesia:** Thermal hyperalgesia was tested in order to evaluate the effect of BV in both normal animals and in CR-induced inflammatory animals. The threshold response to a noxious thermal stimulus was determined using a commercially available thermal plantar tester (Dae-jong, Korea) according to the protocol first described [13]. Briefly, animals were acclimatized to the testing room for at least 2 hr prior to the start of behavioral testing. Following acclimation, radiant heat (Osram 75W,
12V) was applied to the plantar surface of the hindpaw until the rat lifted its paw. A photoelectric cell automatically turned the heat source off when the reflected light beam was interrupted (i.e., when the animal withdrew its paw) and the time at which this occurred was recorded as the paw withdrawal latency (PWL). Heating was terminated at a 15-second cutoff to prevent tissue damage if an animal failed to withdraw its paw prior to the cutoff. Results are expressed as the difference (ΔPWL) in the PWL score before (PWLPRE) and after (PWLPST) intraplantar injection of CR and statistical comparisons between groups assessed on this difference.

**Immunohistochemistry:** Three hours after the CR injection, the animals were deeply anesthetized with 5% isoflurane and perfused transcardially with calcium-free Tyrode’s solution followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffered saline (PBS, pH 6.9). The spinal cord was removed immediately after perfusion, post-fixed in the same fixative for 4 hr and then cryoprotected in PBS containing 30% sucrose (pH 7.4).

A series of frozen sections (40 μm thickness) was cut through the lumbar L4-L5 spinal cord segments using a cryostat (Microm, Germany). After elimination of endogenous peroxidase activity with 3% hydrogen peroxide in PBS and preblocking with 1% normal goat serum and 0.3% triton X-100 in PBS, the sections were incubated in polyclonal rabbit anti-Fos antisera (Calbiochem, CA, U.S.A., 1:10,000) at 4°C overnight. The sections were rinsed in PBS and processed with the avidin-biotin-peroxidase technique as previously described [21]. Briefly, the sections were incubated in biotinylated goat anti-rabbit IgG (Vector Labs, CA, U.S.A., 1:200) for 1 hr at room temperature and washed. The tissue was then incubated in peroxidase-conjugated streptavidin (1:200, Vector Labs) for 1 hr at room temperature. Finally, Fos-like immunoreactive (FLI) neurons were visualized using 3,3-diaminobenzidine (DAB, Sigma) intensified with 0.2% nickel chloride (Sigma). The sections were mounted on gelatin-subbed slides. The slides were then dried, dehydrated in graduated ethanols (70%–100%), cleared in xylene, and coverslipped with mountant (Permount, Fisher Scientific, IL, U.S.A.). Immunohistochemical controls consisted of sections incubated without primary or secondary antibodies.

**Cell counting and image analysis:** Tissue sections were first examined using darkfield microscopy (Zeiss Axioscope, Germany) to determine the segmental level of the spinal cord and spinal laminae according to Kwon et al. [21]. The sections were then examined under brightfield microscopy at X100 to localize FLI neurons.

L4-5 spinal cord segments were initially identified in situ using an accurate dissection method and by counting individual spinal roots. We then compared the identified L4-5 lumbar segments to those identified purely by histological analysis based on previous reports [16, 21, 28]. We found that we obtained the same results using either of the two methods. As a result, we utilized microscopic examination to determine the L4-5 segments for the majority of animals in this study.

For quantitative analysis of segmental and regional FLI neurons, lumbar spinal cord segments were scanned and a total of 5 sections from the L4-5 segments showing the maximal level of Fos expression were subsequently quantified for each animal. The selected sections were digitized with 4096 gray levels using a cooled CCD (Micromax Kodak 1317, Princeton Instruments, AZ, U.S.A.) equipped computer-assisted imaging analysis system (Metamorph, Universal Imaging Co., PA, U.S.A.). In order to maintain a constant threshold for each image and to compensate for subtle variability of immunostaining, we only counted neurons that were at least 30% darker than the average gray level of each image after shading correction were performed. The microscope illumination and data acquisition settings were fixed throughout the entire analysis procedure. To assess the effect of BV treatment on spinal cord Fos expression, the following four gray matter regions were selected for analysis based on cytoarchitectonic criteria as previously described [21]: 1) superficial dorsal horn (SDH, laminae I and II), 2) nucleus proprius (NP, laminae III and IV), 3) neck (NECK, laminae V and VI), and 4) the ventral horn (VENT, laminae VII-IX).

**Statistical analysis:** One way ANOVA was performed to determine the overall effect of BV pretreatment on CR-induced paw edema and on spinal Fos expression. In the paw withdrawal test, repeated measures of a two-way ANOVA was performed to determine the overall effect of BV pretreatment on CR-induced thermal hyperalgesia. Paired and unpaired t-tests were then used to determine the P value where appropriate, when ANOVA indicated a significant group difference. Correlation was made between the percent increase of ipsilateral paw edema and the total number of FLI neurons counted in 5 sections from the L4-5 spinal cord segments. A P value < 0.05 was considered statistically significant. All values are expressed as the mean ± SEM.

**RESULTS**

**Anti-inflammatory effect of BV pretreatment on CR-induced inflammation:** Three hours after intraplantar injection of CR, ipsilateral paw volume was significantly increased in the SAL-CR group as compared with the paw volume before CR-injection (Fig. 1, 108.9 ± 6.5% increase, p<0.001). Although BV injection into the hindlimb produced minor inflammatory signs, such as redness at the injection site, inflammatory paw edema was not observed in the BV-SAL group (Fig. 1). Statistical analysis showed a significant inhibitory effect of BV pretreatment on CR-induced edematous paw volume in the BV (0.8 mg/kg)-CR group compared to the SAL-CR group (F (2,17) = 10.08, p<0.001). The suppressive effect of BV pretreatment on edematous paw volume was also found to be dose-dependent (Fig. 1, a 6.1% and 26.0% reduction of paw volume, using 0.08 mg/kg and 0.8 mg/kg BV respectively, as com-
Antinociceptive effect of BV pretreatment on CR-induced thermal hyperalgesia: The overall mean paw withdrawal latency (PWL) was 10.2 ± 0.44 sec (n=45) prior to injection of BV or CR. Thermal pain threshold was measured at 2, 4, 8 and 24 hr after BV injection into either the hind limb or the plantar surface of the right hindpaw in normal animals. When BV (0.8 mg/kg) was injected into the hind limb, no significant changes in PWL were detected at any of the timepoints examined (Fig. 2, closed circle). In contrast, intraplantar injection of BV (0.8 mg/kg) was found to dramatically decrease PWL at the 2 hr timepoint, but the PWL returned to normal by 24 hr after BV injection (Fig. 2, open circle).

The PWL of the CR-injected paw was significantly reduced from 10.44 ± 0.43 to 4.8 ± 0.66 sec (p<0.001) in SAL-CR treated animals at 3 hr after the CR-injection (Fig. 3). Pretreatment with BV prior to CR injection resulted in dose-related increases in the nociceptive thresholds of the PWL test (Fig. 3). The effect of BV (0.8 mg/kg) pretreatment on CR-induced inflammatory hyperalgesia was statistically significant [F(2,24)=5.61, p<0.01, Fig. 3].

The effect of BV pretreatment on spinal cord Fos expression: Very few FLI neurons were observed in the lumbar spinal cord of the SAL-SAL control group (Figs. 4A and 5). In the BV-SAL group, there was a significant increase in the total number of FLI neurons in the ipsilateral spinal cord as compared with SAL-SAL group (p<0.01, Figs. 4 B and 5). It is observed that the distribution of FLI neurons of the BV-SAL group was concentrated in the SDH (Fig. 4).

Intraplantar CR injection evoked a significant increase in the number of FLI neurons in the ipsilateral L4-5 segments of the spinal cord (SAL-CR group) as compared with the SAL-SAL group (p<0.001, Figs. 4 C and 5), although the number of FLI neurons in the contralateral lumbar spinal cord of all groups receiving CR was negligible (data not shown). In the SAL-CR group, the FLI neurons were predominantly located in the SDH (95.3 ± 4.2 neurons per segment) and in the NECK (61.6 ± 4.5 neurons per segment) of the spinal cord dorsal horn (Figs. 4 C and 5). A small number of FLI neurons were also present in the NP and the VENT regions of the spinal L4-5 segments (21.4 ± 3.1 and 17.5 ± 3.1 per segment, respectively). In addition, there was a positive correlation between the percent change in paw volume and the total number of FLI neurons per spinal cord segment in the CR injected animals (r = 0.73, p<0.05).
The effects of bee venom on inflammation

Subcutaneous pretreatment with BV (0.08 or 0.8 mg/kg) reduced the total number of FLI neurons in the L4-L5 spinal cord segments at 3 hr post-CR injection in a dose dependent manner (5.4% and 28.9%, respectively) in comparison to the SAL-CR group. Regional analysis showed that the number of FLI neurons in both the SDH and the deep dorsal horn (NP and NECK) was dose-dependently reduced (r=0.99, p<0.05; r=0.99, p<0.05 respectively) by BV pretreatment. However, only the higher dose (0.8 mg/kg) of BV pretreatment produced a statistically significant decrease in the number of FLI neurons in the spinal cord (p<0.01, Figs. 4 D and 5).

Discussion

Although it has been shown that BV injection evokes persistent pain and hyperalgesia [6, 23], BV has traditionally been used in Oriental medicine to relieve pain and to treat chronic inflammatory diseases such as rheumatoid arthritis [3, 8, 12, 37]. Recently, we clearly demonstrate that BV has anti-inflammatory effect on adjuvant induced arthritis model [21]. While the basis for the anti-inflammatory effect of BV is presently unknown, it has been reported that synthetic melittin (one of the major components of BV) binds to secretory phospholipase A2 (PLA2) and inhibits its enzymatic activity in vitro [33]. PLA2 is a group IIA enzyme that is massively over-expressed in a variety of severe inflammatory diseases. Since the enzyme degrades membrane phospholipids and releases arachidonic acid, it has been hypothesized that this activity can lead to a loss of tissue and organ integrity and function [27]. Since melittin decreases the activity of PLA2, it is feasible to postulate that this could reduce certain aspects of the inflammatory process. On the other hand, direct injection of BV or its constituents has been shown to produce an inflammatory response. In this regard, intraplantar injection of melittin (10 μg/paw) in normal mice elicits 70–80% of maximal paw edema responses at 60 min after injection [14]. Thus based on this and other
effects, whether feasible based on pain and inflammation under normal conditions. However, based on its use in traditional Chinese medicine, it is also feasible that BV might serve as an anti-inflammatory and/or antinociceptive agent under conditions of inflammation. This latter hypothesis served as the basis for the present investigation. The first portion of this study evaluated whether the injection of BV evoked thermal hyperalgesia and paw edema in normal animals as previously reported [5, 6, 23]. In the second phase of this study we examined the effects of BV pretreatment on both CR induced thermal hyperalgesia and on the development of paw edema (a measure of peripheral inflammation). Finally, the antinociceptive effect of BV was further evaluated in the present study by quantification of Fos expression in the spinal cord.

The effect of BV injection in normal animals: Although the subcutaneous injection of BV into the hindlimb (Zusani acupoint) produced a mild redness in the injected area, it did not evoke any detectable nociceptive behaviors or inflammatory signs (edema, redness, etc.) in the planter surface of the hindpaw. On the other hand, direct intraplantar injection of BV produced thermal hyperalgesia and minor inflammatory signs, including redness and swelling (Figs. 2 and 3). These results indicate that BV produces localized inflammation and nociception that is restricted to the area of injection.

Luo and his colleagues have reported that intraplantar injection of BV produced Fos expression in the L4-5 segments of the spinal cord [25]. These investigators indicated that maximal Fos expression occurred at 2 hr post-BV injection and that Fos expression lasted for 96 hr. In the present study, subcutaneous injection of BV into hindlimb also produced spinal cord Fos expression 3 hr after BV injection (Figs. 4B and 5). However, we observed far fewer Fos neurons as compared to the numbers reported by Luo et al. [25] following intraplantar BV injection. This discrepancy is probably due to differences in the site of injection (subcutaneous versus intraplantar) and to the perfusion/fixed time. Nonetheless it is important to note that these data suggest that subcutaneous injection of BV into the hindlimb of normal animals induce less noxious stimulation than intraplantar injection of BV.

It has been reported that intraplantar injection of BV produces inflammatory signs, such as redness and slight edema, even at doses as low as 0.01 mg and that it evokes persistent thermal hyperalgesia and pain behaviors [6, 23]. PLA2 (30 μg/paw), a major inflammatory component of BV (12% of dry wt), transiently evokes paw edema after subplantar injection [14, 22]. As indicated above melittin has also been reported to induce paw edema after plantar injection in mice [22]. Early studies suggested that melittin could activate endogenous PLA2 and thus it was postulated that the inflammatory activity of melittin might be induced by stimulating endogenous PLA2 activity [34]. However, the paw edema induced by melittin is not effectively inhibited by a PLA2 inhibitor [14]. Currently this mismatched data might be explained by the fact that PLA2 is a common contaminant of commercially purified melittin [9] and by the fact that synthetic melittin acts as a possible scavenger of endogenous PLA2 [33]. Therefore, it is likely that some of the inflammatory pain induced by BV injection is evoked by BV PLA2 in the normal animal.

The effect of BV pretreatment on CR-induced paw edema: Edema is a characteristic sign of inflammation. Numerous inflammatory mediators, including histamine, prostaglandin, leukotrienes and bradykinin, are released at sites of inflammation [15] and many of these substances produce nociception and appear to be involved in the development of hyperalgesia [42]. The rat CR inflammation model induces edema in the hindpaw and has been shown to be a reliable model for assessing the anti-inflammatory properties of drugs since non-toxic levels of CR administration are effective and variability is relatively low as compared with other edema producing materials [41].

Three hours after intraplantar injection of CR, ipsilateral paw volume was significantly increased in the SAL-CR group as compared with the pre-injection paw volume. Although low dose of BV (0.08 mg/kg) did not produce anti-inflammatory effect, pretreatment with high dose of BV (0.8 mg/kg) significantly reduced the peripheral edema evoked by CR injection (Fig. 1). An anti-inflammatory effect of BV has been previously reported in a Freund's adjuvant induced chronic arthritis model [8, 12]. Our recent study also reproduces the anti-inflammatory effect of BV in the same model [21]. The mechanism(s) underlying the BV anti-inflammatory effect is not yet understood, and because BV contains a number of constituents, many of which may be involved, it is a difficult area to investigate. In this regard BV contains a number of different peptides including adalpin, mast cell degranulating (MCD) peptide, melittin and...
apamin [1]. Although purified MCD peptide (1 mg/kg) and adolapin (20 μg/kg) have anti-inflammatory activity [19, 26, 35], these substances are present in very small quantities (1–2%) in whole BV. Saini and his coworkers have suggested that melittin may have potential value as a therapeutic agent in various inflammatory conditions because the formation of a melittin-PLA2 complex following BV injection is able to suppress the development of inflammation [33]. Recently, it has also been reported that rat paw edema induced by PLA2 from BV is significantly inhibited by crotoptinin which is a non-toxic and non-enzymatic acid polypeptide naturally complexed with PLA2 [22].

In addition to an effect on PLA2, melittin has been reported to block neutrophil superoxide production [36]. In particular, it has been shown that human leukemic cells treated with sublytic doses of melittin become resistant to complement-mediated lysis [32]. Since CR-induced inflammation can promote the complement cascade, superoxide production and arachidonic acid metabolism, it is possible that pretreatment with BV blocks one or more of these inflammatory cascades in a relatively confined anatomical region and thus reduces the inflammatory response near the site of injection. It should be noted that melittin also possesses hemolytic activity [7, 18] and this must be considered prior to using BV as a therapeutic agent. In this regard it is interesting that the c-terminal 15 residue synthetic fragment of melittin is 300 times less hemolytic compared with melittin itself [39]. Further investigation is clearly required to understand the mechanisms underlying the anti-inflammatory effect of BV and its components, such as melittin, prior to testing BV in clinical trials.

The effect of BV pretreatment on CR-induced thermal hyperalgesia: We recently demonstrate that BV has antinociceptive activity against both thermal and mechanical hyperalgesia induced by chronic arthritic pain [21]. The present study also demonstrate that pretreatment with BV at the dose of 0.8 mg/kg increases the nociceptive thermal threshold three hours after CR-injection. This antinociceptive effect of whole BV may be explained by a direct local effect acting at near the site of CR injection or by an indirect mechanism acting centrally to stimulate the descending pain inhibitory system. The antinociceptive effect of BV can be partially reproduced by some of its individual components, such as adolapin. It has been previously reported that adolapin can exert a potent analgesic effect in both the acetic acid induced writhing assay and in the Randall-Sellito test [35]. Thus BV or its constituent, adolapin, could act on nerve fibers innervating the footpad, either directly or indirectly, via the release of local mediators, to suppress nociceptive transmission.

However, since BV was not injected directly into the same site as the CR (Zusani point versus hindlimb), it is possible that the antinociceptive effect of BV is indirect. One possible explanation for this is that it is mediated by the descending pain inhibitory system via a counter-irritation mechanism (i.e., the pain-relieving effects of pain elicited from heterotopic body areas). It has been reported that activation of descending pain inhibitory pathways, such as the bulbo-spinal antinociceptive circuitry, can be triggered by heterotopic nociceptive events [24]. Capsaicin, one of the commercially available counter-irritants, produces itching, pricking and burning sensations caused by the excitation of vanilloid receptor subtype-1 (VR1) on primary afferent nociceptors. Since repeated application of capsaicin is followed by a prolonged period of hypoalgesia, usually referred to as desensitization or nociceptor inactivation, it has been traditionally used to test certain types of pain [10, 30]. Recently, it has been reported that counter-irritation induced by capsaicin excitation of VR1 sensory receptors inhibits the development of a subsequent inflammatory reaction at distant body sites in the rat [40]. Such counter-irritation mechanisms may underlie the BV antinociceptive effects observed in the present study.

Certainly BV contains several inflammatory components that act directly or indirectly on primary afferents, like capsaicin does, to produce nociception. These include: histamine, which acts directly on primary afferent nociceptive endings; phospholipase A2, which activates arachidonic acid metabolism to produce prostanooids which in turn act directly on primary afferents; and hyaluronidase, which has been shown to cause pain following injection into the skin [23, 29]. In the present study, while subcutaneous injection of BV into the hindlimb did not produce detectable nociceptive behavior or hyperalgesia in the hindpaw region (as did direct intraplantar injection; see also ref [6]), we did observe some redness in the injection site. This suggests that a small amount of inflammation occurred in the site of BV injection. This is further supported by the finding of increased Fos expression in the lumbar spinal cord, which suggests that BV injection activated nociceptive input to the spinal cord from the Zusani point of hindlimb. Thus the BV induced inflammation in the area of the hind limb could have produced a counter-irritation mechanism that suppressed the production of inflammation and nociception at the site of CR injection. In addition, blockage of the sciatic nerve with lidocaine resulted in a complete suppression of the BV-induced neuronal firing in the spinal dorsal horn, suggesting that the central neuronal changes following subcutaneous BV are peripherally-dependent [5]. Based on these findings we speculate that the increase in the CR-induced thermal pain threshold is the result of a counter-irritation mechanism caused by the heterotopic injection of the BV.

The effect of BV pretreatment on Fos expression in the spinal cord: In the present study, pretreatment with BV suppressed spinal cord Fos expression evoked by CR injection. This suppression occurred in all regions of the ipsilateral lumbar spinal cord gray matter except the ventral horn (Fig. 5). It has been previously reported that systemic injection of anti-inflammatory drugs reduces both spinal cord Fos expression and the peripheral edema produced by CR injection [16, 31]. In these reports, there is a clear relationship between the effect of these drugs on Fos expression and their effect on the signs of CR-induced inflammation. Similarly, in the present study there was a strong positive corre-
loration between the percent change in paw volume and the total number of PLI neurons per spinal cord segment in the CR injected animals. In addition, BV pretreatment was found to suppress the inflammatory response and to cause a parallel reduction in neuronal Fos expression in the spinal cord, indicating that BV pretreatment has both an antinociceptive and anti-inflammatory effect on CR-induced inflammation.

This study was conducted to evaluate the putative antinociceptive/anti-inflammatory effect of BV on CR induced inflammation. Subcutaneous injection of BV into the Zusanli point of the hindlimb was found to produce a slight increase in Fos expression in the spinal cord without producing detectable nociceptive behavior and hyperalgesia in normal animals. On the other hand, hindlimb pretreatment with BV suppressed both the edematous paw volume and the thermal hyperalgesia evoked by intraplantar injection of CR. Although the mechanisms involved in the suppressive effects of BV pretreatment on CR induced inflammation remain to be elucidated, our results demonstrate that pretreatment with BV has both antinociceptive and anti-inflammatory effects on CR induced inflammation. These data suggest that BV may be a useful anti-inflammatory/analgesic agent for the treatment of inflammatory disease states.

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