

Local Peripheral Effects of β -Caryophyllene through CB₂ Receptors in Neuropathic Pain in Mice

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ABSTRACT

β -Caryophyllene (BCP) is known as a common constitute of the essential oils of numerous food plants and primary component in Cannabis. In this study, we investigated the effect of local intraplantar (i.pl.) injection of BCP on mechanical hypersensitivity induced by partial sciatic nerve ligation (PSNL) in mice. Relative to sham operation controls, mice with the PSNL displayed a maximum level of hyperresponsiveness to von Frey metallic filament on post-operative day 7. PSNL-induced allodynia was seen in the ipsilateral side of nerve ligation, but not in the contralateral side. The i.pl. injection of BCP into the ipsilateral hindpaw to PSNL attenuated mechanical allodynia in a dose-dependent manner. BCP injection into the contralateral hindpaw did not produce anti-allodynic effects, suggesting a local peripheral anti-allodynic effect of BCP. Anti-allodynic effects induced by i.pl. injection of BCP were prevented by pretreatment with the cannabinoid (CB)₂ receptor antagonist AM630, but not by the CB₁ receptor antagonist AM251. These data suggest that i.pl. injection of BCP could produce anti-allodynia by activating peripheral CB₂ receptors, but not CB₁ receptors in a mouse model of neuropathic pain. Taken together, these results suggest that peripheral CB₂ receptors may contribute to the effectiveness of BCP in the treatment of neuropathic pain disorders.

Keywords: β -Caryophyllene (BCP); Neuropathic Pain; Partial Sciatic Nerve Ligation (PSNL); Peripheral Cannabinoid (CB) Receptor

1. Introduction

Plants are used for various purposes including their cosmetic, nutritive, and biomedical properties. Plant essential oils are typically composed of volatile aromatic terpenes and phenylpropanoids. The natural sesquiterpene β -caryophyllene (BCP) is found in many essential oils of different spice and food plants, such as clove, oregano, thyme, black pepper and cinnamon [1,2], all of which have been used as natural remedies and also as fragrances. This compound is also known to be anti-microbial [3], anti-oxidant [3,4], and anti-carcinogenic [5] and to possess skin penetration-enhancing properties [6]. Moreover, BCP is also a major component in the essential oil of *Cannabis sativa* L [7]. BCP showed marked anti-inflammatory activity against carrageenan- and pro-

staglandin E₁-induced edema in rats as well as anti-arthritis activity [8-10]. Oral administration of BCP significantly reduced the inflammation of colon [11], the carrageenan-induced inflammatory response in wild-type mice but not in mice lacking cannabinoid (CB)₂ receptors [12]. However, the antinociceptive efficacy of intraplantar (i.pl.) BCP on partial sciatic nerve ligation (PSNL)-induced mechanical allodynia in mice is unknown. Cannabinoid CB receptors, CB₁ and CB₂ receptors, at the peripheral and central sites have been proposed to mediate the CB-induced antinociceptive effects [13-17]. CB₂ receptors are not found in the central nervous system (CNS), but are predominantly expressed in immune cells, their roles including the modulation of cytokine release and immune cell migration [18,19]. CB₂ receptor selective agonists produced peripheral antinociception [15, 20,21], but do not cause the effects of CNS [15,22], sug-

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gesting that selective activation of CB₂ receptors may achieve the goal of peripheral pain relief without CNS effects. Therefore, in our study we used local injections to the injured paw in order to exclude the role of central effects, and validate the role of peripheral CB receptors in neuropathic pain. Peripheral nerve damage can result in long-lasting anomalous pain conditions referred to as neuropathic pain. These abnormal pain states are often manifested by an increased sensitivity to nociceptive stimuli, termed hyperalgesia, as well as the perception of typical innocuous stimuli being painful, a state referred to as allodynia [23]. CB₂ receptor selective agonists have been also known to produce antinociception without overt behavioral effects in neuropathic pain [20,22,24, 25]. The aims of this work was 1) to investigate whether i.pl. injection of BCP would produce antinociception in PSNL-induced mechanical allodynia model in mice; and 2) to determine a possible role of peripheral CB receptors in BCP-induced anti-allodynic effects.

2. Materials and Methods

2.1. Animals and Neuropathic Surgery

Male mice of ddY strain weighing 22 - 24 g were purchased from Kyudo Industries, Kumamoto, Japan. They were housed in cages of 15 - 20 animals matched for weight and placed in a colony room. Animals were allowed free access to standard food (Clea Japan, Inc., Osaka, Japan) and tap water in an air-conditioned room under a constant 12:12 h light/dark cycle (light on 08:00 h) at a room temperature of 22°C - 24°C and 50% - 60% relative humidity. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals [26]. Additionally, the study was approved by the Committee of Animal Experiments in Daiichi College of Pharmaceutical Sciences.

Partial ligation of the sciatic nerve of mice was performed under pentobarbital anesthesia (50 mg/kg, i.p.) following the methods Malmberg and Basbaum [27]. Briefly, the common sciatic nerve of the right hand leg of mice was exposed at high thigh level through a small incision and dorsal 1/3 to 1/2 of the nerve thickness was tightly ligated with a silk suture. The wound was closed with a single muscle suture, and antibiotic powder was dusted over the wound area following surgery. For sham surgery, the sciatic nerve was exposed as described above, but no contact was made with the nerve. To minimize differences in technique, all operations were done by the same person. Immediately following surgery, the animals were kept in a soft bag cage with some food inside so that they could feed themselves without having difficulty in standing. The wound healed within 1 to 2 days, and the mice behaved normally. The behavior of the mice was monitored carefully for any visual indica-

tion of motor disorders or change in weight. Testing procedures were conducted on day 7 after PSNL, except for the time-course experiment of PSNL-induced allodynia.

2.2. Mechanical Threshold

Behavioral testing was conducted from 10:00 to 16:00 in a quiet room. Each animal received drugs only once and was used in only one experiment. The mice were weighed and placed individually in a Plexiglas chamber (11.0 × 17.0 × 14.0 cm, wire mesh floor) and allowed to acclimatize for at least 1 hour. The threshold for nociceptive responsiveness to mechanical stimuli applied to the hindpaw was assessed using an electronic version of the von Frey test (dynamic plantar anesthesiometer, model 37400; Ugo Basile, Milan, Italy). The servo-controlled mechanical stimulus (a pointed metallic filament) was applied to the plantar surface, which exerted a progressively increasing punctate pressure, reaching up to 3.0 g within 5.0 s. The pressure evoking a clear voluntary hindpaw withdrawal response (usually close to 3.0 g) was recorded automatically and taken as the mechanical nociceptive threshold index.

2.3. Drugs

β -Caryophyllene (*trans*-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo [7.2.0] undec-4-ene; BCP) (Sigma, St. Louis, MO, USA) diluted in jojoba wax (*Simmondsia chinensis*) (KSA International, Co. Ltd., Kanagawa, Japan), was injected to the plantar surface of the hindpaw in mice. *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) and 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl] (4-methoxyphenyl) methanone (AM630) (Tocris Cookson, Bristol, UK), dissolved in physiological saline was administered i.pl. or subcutaneous (s.c.) 30 min before BCP. I.pl. and s.c. injections were given in a volume of 20 μ L/site and 0.1 mL/10 g of body weight, respectively.

2.4. Analyses of Data

All data are expressed as means \pm SEM. Statistical differences between groups were assessed with a two-way ANOVA followed by Bonferroni's test. The 5% ($P < 0.05$) level of statistical significance was set in all experiments.

3. Results

3.1. The Effects of BCP on Mechanical Allodynia

The responsiveness to mechanical stimuli was determined on days 1 - 35 after PSNL. Compared with the sham-operated mice, PSNL resulted in mechanical allo-

dynia demonstrated by significantly lower threshold to von Frey filaments from day 7 to day 28 post-PSNL (Figure 1). The maximal decrease in withdrawal threshold after PSNL was observed on day 7. On day 7 post-PSNL, i.pl. injection of BCP (4.5, 9.0 and 18.0 $\mu\text{g}/\text{paw}$) dose-dependently increased the mechanical response threshold on the nerve injured side. The effect of BCP reached a maximum at 5 min and lasted for 15 min on nerve-ligated side. Jojoba wax control, injected into the nerve-ligated side, did not affect the mechanical thresholds. To ensure that the effects of i.pl. injections of BCP were local and not due to systemic diffusion, BCP (18.0 $\mu\text{g}/\text{paw}$) was injected into the hindpaw contralateral. Mechanical threshold following i.pl. injection of BCP in the contralateral hindpaw was not statistically different when compared with the jojoba wax control group in PSNL mice (Figure 2).

3.2. Peripheral CB₂ Receptor Mediates the Effects of BCP

To determine if anti-allodynic effect of BCP was mediated by peripheral CB systems, animals were pretreated systemically with AM251 (3.0 mg/kg, s.c.), a selective CB₁ receptor antagonist and AM630 (1.0 mg/kg, s.c.), a selective CB₂ receptor antagonist, 30 min before i.pl. injection of BCP (18.0 $\mu\text{g}/\text{paw}$). The selective CB₂ receptor antagonist, AM630 significantly reversed the inhibitory effects of BCP on the PSNL-induced mechanical allodynia, whereas the selective CB₁ receptor antagonist, AM251 gave no effect (Figure 3). In further experiments, AM251 and AM630 were pretreated directly into the same site on the hindpaw in prior to i.pl. injection of BCP. I.pl. pretreatment with AM630 (4.0 $\mu\text{g}/\text{paw}$) could also antagonize significantly anti-allodynic effects of

BCP, whereas AM251 (12.0 $\mu\text{g}/\text{paw}$) gave no effect (Figure 4). AM251 and AM630 used in this experiment alone did not induce a significant effect as compared to saline controls (data not shown).

4. Discussion

The present study demonstrates that i.pl. injection of BCP reversed mechanical allodynia in a mouse PSNL neuropathic pain model. The anti-allodynia induced by BCP was inhibited by pretreatment with AM630, a selective CB₂ receptor antagonist, while pretreatment with AM251, a selective CB₁ receptor antagonist, did not change the effects of BCP. These results suggest that BCP, injected into the plantar surface of the hindpaw,

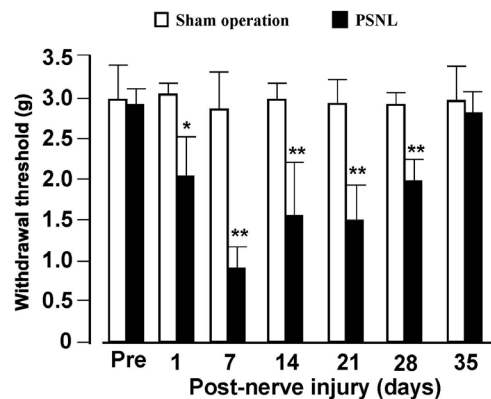


Figure 1. The time course of mechanical withdrawal thresholds after partial sciatic nerve ligation in the von Frey test. Mechanical withdrawal thresholds of the injured and sham-operated right hindpaws are expressed as grams and data represent the mean \pm SEM of 10 mice in each group. Asterisks indicate significant decreases in thresholds compared to the sham-operated mice. * $P < 0.05$, ** $P < 0.01$.

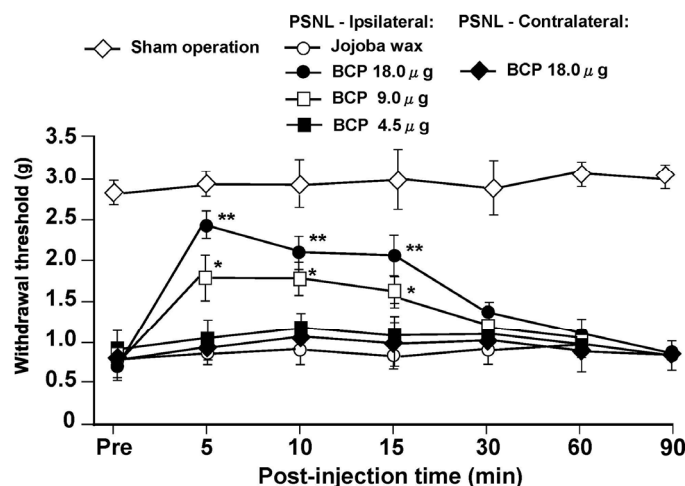


Figure 2. Intraplantar injection of BCP reduced partial sciatic nerve ligation (PSNL)-induced mechanical allodynia in mice. The withdrawal threshold was determined in PSNL and sham mice (hindpaw ipsilateral side and contralateral side) before and 5, 10, 15, 30, 60 and 90 min after administration. Each point represents the mean \pm SEM for groups of 10 mice. ** $P < 0.01$, * $P < 0.05$ when compared to jojoba wax-control.

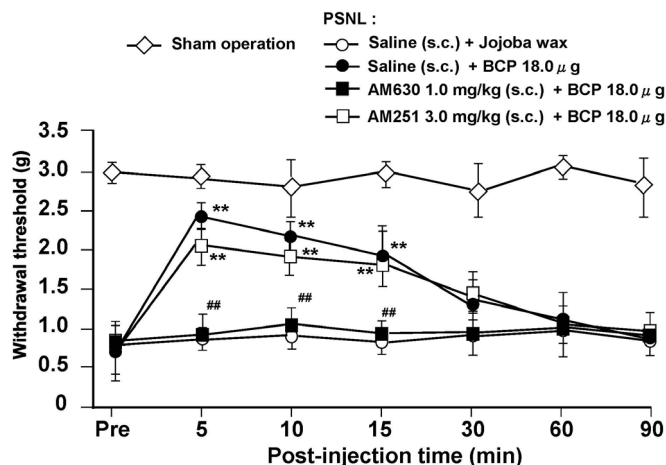


Figure 3. Antagonism induced by s.c. injection of the selective CB₂ receptor antagonist, AM630 and the selective CB₁ receptor antagonist, AM251 on the anti-allodynia produced by i.pl. injection of BCP in the PSNL model. Each point represents the mean \pm SEM for groups of 10 mice. ** $P < 0.01$ when compared to saline (s.c.) + jojoba wax-control. ## $P < 0.01$ when compared to s.c. saline plus BCP (18.0 μ g/paw).

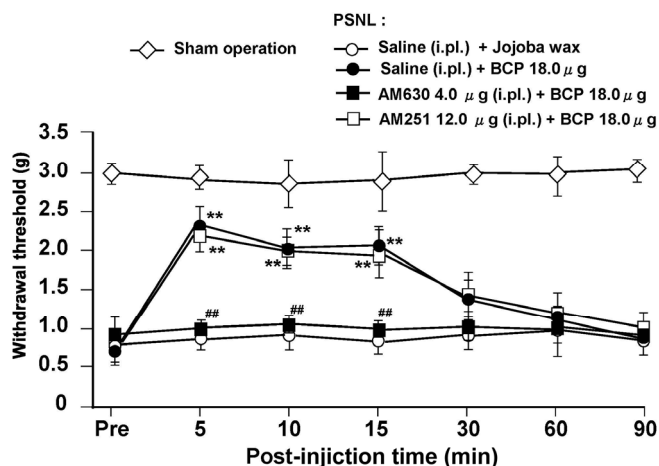


Figure 4. Antagonism induced by local injection of the selective CB₂ receptor antagonist, AM630 and the selective CB₁ receptor antagonist, AM251 on the anti-allodynia produced by i.pl. injection of BCP in the PSNL model. Each point represents the mean \pm SEM for groups of 10 mice. ** $P < 0.01$ when compared to saline (s.c.) jojoba wax-control. ## $P < 0.01$ when compared to i.pl. saline + BCP (18.0 μ g/paw).

produces its anti-allodynic effects by activating local peripheral CB₂ receptors.

Injury to the sciatic nerve in mice as well as rats produces a prolonged mechanical allodynia and thermal hyperalgesia [27,28]. This experimental model has established as a reliable and objective method to access neuropathic pain. We confirmed that PSNL produced a rapid onset and prolonged mechanical allodynia in mice. The mechanical allodynia developed in 1 day and reached a maximum in 7 days in mice with PSNL. PSNL-induced mechanical allodynia was dose-dependently reversed by local (plantar surface of the hindpaw) injection of BCP on day 7 post-PSNL. This result suggests that i.pl. injection of BCP is an effective post-injury treatment in neuropathic pain conditions.

In this study, injection of BCP into the hindpaw ipilat-

eral to the injured side could protect the mice from mechanical allodynia induced by PSNL. Local injection of BCP into the contralateral hindpaw gave no effects on mechanical allodynia, strongly supporting a local effect of BCP on peripheral cutaneous nociceptors. It seems that i.pl. treatment with BCP is beneficial as BCP is anti-allodynic in neuropathic pain and have limited CNS penetration to produce the unwanted CNS effects. It should be noted that locally applied BCP failed to produce anti-allodynic effects in sham-operated mice at a dose of 18.0 μ g/paw. At this dose, BCP attenuated significantly mechanical hypersensitivity in neuropathic mice. The employment of CB receptor-selective antagonists allows for the involvement of CB receptor subtypes in BCP-induced anti-allodynia. Locally applied BCP (9.0 and 18.0 μ g/paw) significantly reduced the PSNL-in-

duced neuropathic pain in mice. The effects of BCP were inhibited by pretreatment with the selective CB₂ receptor antagonist, AM630 (1.0 mg/kg, s.c. and 4.0 μ g, i.pl.) but not by pretreatment with the selective CB₁ receptor antagonist, AM251 (3.0 mg/kg, s.c. and 12.0 μ g, i.pl.). The doses AM630 (1 mg/kg) and AM251 (3 mg/kg) used in the present experiment have been previously shown to inhibit the effect of WIN 55,212-2, a CB receptor agonist *in vivo* [18,29]. In binding assays, AM630 is CB₂ selective with 165-fold lower *Ki* value in CB₂ transfected CHO cell membranes than in membranes of CB₁ transfected cells [30], and AM251 is a high degree selectivity (306-fold) for CB₁ receptors [31]. Therefore, the data suggests that the anti-hyperalgesic effects of BCP in neuropathic mice are mediated through activation of peripheral CB₂ receptors. In line with our study, systemic or local administration of CB₂ receptor selective agonists have been shown to produce antinociception without overt behavioral effects in several pain models [15,20,32,33].

Besides peripherally mediated anti-allodynic effect, peripheral administration of BCP or its containing essential oil also indicates anti-inflammatory and anti-arthritis potential [8-11,34]. Moreover, oral administration of BCP inhibits potent anti-inflammatory effects in wild-type mice but not in mice lacking CB₂ receptors [12]. This is consistent with our present study, suggesting the involvement of specific CB receptor subtype, a CB₂ receptor, in BCP-induced peripheral anti-allodynia. Interestingly, the essential oil component (E)-BCP selectively binds to the CB₂ receptor, leading to the cellular activation as a CB₂ agonist and anti-inflammatory effects [12]. Thus, the anti-allodynic effect of BCP points to the peripheral CB₂ receptor as an interesting target in searching for new peripherally active analgesics for chronic pain therapy. The action site of BCP is of particular interest since neuropathy observed in patients is often coupled with not only neuropathic pain but also inflammatory symptoms [35,36].

The peripheral mechanism by which BCP produces anti-allodynic effect in the present findings are unclear. However, there are various lines of evidence that selective activation of peripheral CB₂ receptors is sufficient to display antinociception in models of acute, inflammatory and nerve injury-induced nociception [15,21,22,32,37-39]. Indeed, CB₂ receptors are expressed primarily on mast cells and immune system such as B cells, T cells and macrophages [19,40,41]. Activation of CB₂ receptors on mast or immune cells could inhibit the release of molecules that sensitize the peripheral nociceptor (e.g. histamine, serotonin, prostaglandins, interleukin-1 β , tumor necrosis factor- α , and nerve growth factor). A recent study have demonstrated that the oral treatment of BCP causes a significant reduction of prostaglandin E₂ and tumor necrosis factor- α generation in carrageenan injected

paw [34]. Therefore, it seems that activation of peripheral CB₂ receptors might decrease the sensitivity of primary afferent neurons by inhibiting the release of sensitizing substances from neighboring mast and immune cells. Moreover, there is evidence for the presence of CB₂ receptors on peripheral nerve terminals [42,43]. Indeed, Walczak *et al.* (2005) have shown that the saphenous partial ligation-induced neuropathic pain model increases the expression of CB₂ receptors in the paw skin [44]. Another action mechanism of BCP is that i.pl. BCP may stimulate CB₂ receptors and inhibit the responsiveness of primary afferent neurons by stimulating local release of β -endorphin, an endogenous opioid peptide, from keratinocytes, which are very abundant in skin and have been reported to express CB₂ receptors [35]. However, further studies are required to explain the specific mechanisms underlying the observed BCP effect.

5. Conclusion

In conclusion, i.pl. injection of BCP reduced the mechanical allodynia by the PSNL model in mice. The anti-allodynic effects of BCP was antagonized by s.c. and i.pl. pretreatment with the selective CB₂ receptor antagonist, AM630, but not by the selective CB₁ receptor antagonist, AM251. The results suggest that a local effect of BCP on cutaneous nociceptors is mediated through CB₂ receptors. BCP is predicted to be effective in treating allodynia without the central side effects of cannabinoids-based drugs retaining activity at the CB₁ receptor.

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