Nicotinic Acetylcholine Receptors Mediate the Suppressive Effect of an Injection of Diluted Bee Venom into the GV3 Acupoint on Oxaliplatin-Induced Neuropathic Cold Alloodynia in Rats

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Oxaliplatin, a platinum-based chemotherapy drug, often induces acute neuropathic pain, especially cold allodynia, even after a single administration. Subcutaneous injection of diluted bee venom (BV) into acupoints has been used to treat various pain symptoms in traditional oriental medicine. Although we previously demonstrated the suppressive effect of BV injection on oxaliplatin-induced cold allodynia in rats, its neurochemical mechanism remained unclear. This study investigates whether and how the cholinergic system mediates the relieving effect of BV injection on cold allodynia in oxaliplatin-administered rats. The behavioral signs of cold allodynia induced by an oxaliplatin administration (6 mg/kg, intraperitoneally (i.p.)) were evaluated by a tail immersion test in cold water (4°C). BV (0.25 mg/kg, subcutaneously (s.c.)) injection into the Yaoyangguan acupoint, located between the spinous processes of the fourth and fifth lumbar vertebrae, significantly alleviated the cold allodynia. This relieving effect of BV injection on oxaliplatin-induced cold allodynia was blocked by a pretreatment with mecamylamine (a non-selective nicotinic receptor antagonist, 2 mg/kg, i.p.), but not by atropine (a non-selective muscarinic receptor antagonist, 1 mg/kg, i.p.). Further, dihydro-β-erythroidinehydrobromide (DHβE, an α4β2 nicotinic antagonist, 5 mg/kg, i.p.) prevented the anti-allodynic effect of BV, whereas methyllycaconitine (an α7 nicotinic antagonist, 6 mg/kg, i.p.) did not. Finally, intrathecal administration of DHβE (10nm) blocked the BV-induced anti-allodynic effect. These results suggest that nicotinic acetylcholine receptors, especially spinal α4β2 receptors, but not muscarinic receptors, mediate the suppressive effect of BV injection on oxaliplatin-induced acute cold allodynia in rats.

Key words oxaliplatin; bee venom; cholinergic; cold allodynia; nicotinic receptor; rat

Oxaliplatin is a third-generation platinum-based chemotherapy drug for advanced colorectal cancer. Unlike the other platinum compounds (e.g. cisplatin and carboplatin), oxaliplatin does not induce nephrotoxicity and hepatotoxicity, but often induces a very acute painful neuropathy soon after an administration. This acute neuropathy is the only major dose-limiting toxicity associated with oxaliplatin use and is characterized by the rapid onset of cold-induced peripheral dysesthesia, paresthesia, or hypoesthesia of the hands, feet, or throat. Previous animal studies also have shown that a single administration of oxaliplatin (6 mg/kg, intraperitoneally (i.p.)) reproduces the neurotoxic profile, especially cold allodynia. However, its mechanism is still unclear and an effective treatment of established neuropathic cold allodynia remains to be found.

A subcutaneous injection of diluted bee venom (BV) into acupoints, so called ‘bee venom acupuncture,’ has been used as a part of traditional oriental medicine to relieve pain and treat various diseases, such as arthritis, rheumatism, cancer, asthma and skin diseases. It is a kind of peripheral nerve stimulation as similar to electroacupuncture (EA), but with the different method of stimulus (chemical vs. electrical). Thus, the analgesic mechanisms of BV injection are somewhat different from those of EA, although both therapeutic largely share the neurochemical pathway for the activation of the endogenous analgesic system. EA-induced analgesia is mediated by the endogenous opioid and/or the non-opioid analgesic system, such as the central noradrenergic, cholinergic, serotonergic and γ-aminobutyrate (GABAergic) pain inhibitory pathways. In contrast, the analgesic effects of BV injection are reported to be mainly mediated by the central noradrenergic system, but not by the endogenous opioids. In a rat model of oxaliplatin-induced neuropathic pain, we recently demonstrated that EA significantly relieves acute cold alldynia via the release of the endogenous opioids, but not via the activation of the noradrenergic system. In the same rat model, interestingly, the anti-allodynic effect of BV injection is only partially mediated by the noradrenergic system, not by the opioids, suggesting that the other non-opioid analgesic mechanism might play an important role in mediating the BV effect on oxaliplatin-induced cold allodynia.

Cholinergic modulation is one of the pharmacological methods to the treatment of pain. Acetylcholine receptors are present in the spinal cord dorsal horn, in which nociceptive processing occurs. They are classified into two distinct receptors, nicotinic and muscarinic acetylcholine receptors, and the activation of spinal nicotinic or muscarinic receptors is known to produce analgesia. Although our previous study showed that spinal muscarinic, but not nicotinic, receptors mediate the anti-allodynic effect of EA in nerve-injured rats, a cholinergic mechanism for BV-induced analgesia has not yet been studied. In the present study, we examined if and how the cholinergic system plays a role in the relieving effects of BV injection on acute cold alldynia in oxaliplatin-adminis-
Allodynia in Rats

MATERIALS AND METHODS

Animals Young adult male Sprague-Dawley rats (average age 200 g, 7 weeks old) were housed in cages (3–4 rats per cage) with water and food available ad libitum. The room was maintained with a 12 h light/dark cycle (a light cycle; 08:00–20:00, a dark cycle; 20:00–08:00) and kept at 23±2°C. All animals were acclimated in their cages for 1 week prior to any experiments. All of the procedures were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-14-010) and were conducted in accordance with the guidelines of the International Association for the Study of Pain.21

Oxaliplatin Administration Oxaliplatin was obtained from Sigma Chemical Co., U.S.A. As described previously,22 oxaliplatin was dissolved in a 5% glucose solution at a concentration of 2 mg/mL and was intraperitoneally administered at 6 mg/kg.

Behavioral Test Cold allodynia induced by an oxaliplatin administration was assessed in rats by using the tail immersion test in a water bath maintained at 4°C. Each rat was lightly immobilized in a plastic holder and its tail was drooped for proper application of cold water stimuli. The rats were adapted to the holder for 2 d before starting behavioral tests. After the tail was immersed in 4°C water, the tail withdrawal latency (TWL) to an abrupt tail movement was measured with a cut-off time of 15 s. The tail immersion test was repeated five times at 5 min intervals. When calculating the average TWL, the cutoff time was assigned to normal responses. The average TWL was taken as a measure of the severity of cold allodynia; a shorter TWL was interpreted as more severe allodynia.

Treatment of Antagonists and BV Injection BV from Apis mellifera was purchased from Sigma (V3375) and was dissolved in normal saline (N.S) to a concentration of 0.025 mg/mL. BV is known to contain many active components, including peptides (e.g. melittin and apamine), enzymes (e.g. phospholipase A2) and small molecules (e.g. histamine).

To investigate the cholinergic mechanism of BV-induced analgesia, oxaliplatin-administered rats were randomly divided into three groups: N.S+BV, atropine (ATR)+BV, and mecamylamine hydrochloride (MEC)+BV. After a baseline cold sensitivity (TWL) was measured, ATR+BV and MEC+BV groups were treated intraperitoneally with ATR (non-selective muscarinic receptor antagonist, 1 mg/kg, Sigma) and MEC (non-selective nicotinic receptor antagonist, 2 mg/kg, Sigma), respectively. N.S+BV group was treated intraperitoneally with normal saline. Thirty minutes later, BV (0.25 mg/kg) dissolved in N.S was injected subcutaneously into the Yaoyangguan (GV3) acupoint. This acupoint is located between the spinous processes of the fourth and the fifth lumbar vertebrae23 and BV injection (1 mg/kg, subcutaneously (s.c.)) into the Yaoyangguan showed a greater anti-allodynic effect in oxaliplatin-administered rats than BV injection into the Quchi (LI11, located on forelimb) or Zusanli (ST36, located on hindlimb) acupoint.24 We confirmed such acupoint-specific effects at a low dose of BV (0.25 mg/kg, s.c.) used in this study (Supplementary Fig. 1). The tail immersion test was performed again 30 min and 1 h after BV injection.

To examine the nicotinic acetylcholine receptor mechanism of BV-induced anti-allodynia, methyllycaconitine citrate (MLA, selective α7 nicotinic receptor antagonist, 6 mg/kg, Tocris) or dihydro-β-erythroidinehydrobromide (DHβE, selective α4β2 nicotinic receptor antagonist, 5 mg/kg, Tocris) was administered intraperitoneally. To further confirm whether spinal α4β2 nicotinic receptors mediate the analgesic effect of BV injection, DHβE (50 µL, dissolved in N.S at a concentration of 10 nm) was administered intrathecally under isoflurane anesthesia as described previously.25 The doses of antagonists were selected based on previously published studies that show selectivity for the individual receptor subtypes.

Statistical Analysis All data are presented as mean±standard error of the mean (S.E.M.). For statistical analysis, paired t-test or one-way repeated measures ANOVA followed by Dunnett’s multiple comparison test was used. In all cases, p<0.05 was considered significant.

RESULTS

Effects of Non-selective Muscarinic and Nicotinic Receptor Antagonists on BV-Induced Anti-allodynia As shown in our previous study,26 a significant sign of cold allodynia was induced from 3 d after a single oxaliplatin (6 mg/kg, i.p.) administration was assessed in rats by using the tail immersion test in a water bath maintained at 4°C. Each rat was lightly immobilized in a plastic holder and its tail was drooped for proper application of cold water stimuli. The rats were adapted to the holder for 2 d before starting behavioral tests. After the tail was immersed in 4°C water, the tail withdrawal latency (TWL) to an abrupt tail movement was measured with a cut-off time of 15 s. The tail immersion test was repeated five times at 5 min intervals. When calculating the average TWL, the cutoff time was assigned to normal responses. The average TWL was taken as a measure of the severity of cold allodynia; a shorter TWL was interpreted as more severe allodynia.

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Statistical Analysis All data are presented as mean±standard error of the mean (S.E.M.). For statistical analysis, paired t-test or one-way repeated measures ANOVA followed by Dunnett’s multiple comparison test was used. In all cases, p<0.05 was considered significant.
injection and lasted up to 7 d after an injection. Thus, we tested whether and how the cholinergic system mediates the relieving effect of BV injection on cold allodynia from 3 to 7 d after an oxaliplatin administration. Note that the TWLs measured prior to an oxaliplatin injection (“pre-Oxa”) were nearly cut-off value (15 s) and the TWLs measured 3 to 7 d after an oxaliplatin injection (before an administration of antagonists, “Before”) were markedly decreased to about 5 s (Figs. 1–3).

To see which acetylcholine receptors mediate the suppressive effect of BV injection on cold allodynia, we examined the effect of a non-selective muscarinic receptor antagonist ATR or a non-selective nicotinic antagonist MEC on BVA-induced anti-allodynia. Each of the antagonists alone had no significant effect on cold allodynia (Supplemental Fig. 2). In addition, no significant difference in TWL before and after N.S injection (p > 0.05) was observed (Supplemental Fig. 1). In the N.S + BV group, there was a significant increase in TWL after BV treatment (p < 0.05, Fig. 1A). A systemic injection of ATR (1 mg/kg, i.p.) did not prevent the anti-allodynic effect of BV injection (p > 0.05, Fig. 1B), whereas an injection of MEC (2 mg/kg, i.p.) markedly blocked the BV effect (p > 0.05, Fig. 1C). This indicates that nicotinic acetylcholine receptors, but not muscarinic receptors, are involved in the relieving effect of BV injection on oxaliplatin-induced cold allodynia.

**Effects of Nicotinic Receptor Subtype Antagonists on BV-Induced Anti-allodynia**

To determine which nicotinic receptor subtype plays a major role in mediating the anti-allodynic action of BV injection, selective antagonists against α7 and α4β2 nicotinic receptors were used. An i.p. pretreatment with N.S (Fig. 2A) or α7 nicotinic antagonist MLA (Fig. 2B) did not prevent the anti-allodynic effect of BV treatment (p > 0.05). In contrast, the relieving effect of BV injection on cold allodynia was blocked by an i.p. pretreatment with α4β2 nicotinic antagonist DHβE (p > 0.05, Fig. 2C). We further showed that an intrathecal (i.t.) injection of DHβE blocked the suppressive effect of BV injection on cold allodynia (Fig. 3B, p > 0.05), but an i.t. injection of N.S did not (Fig. 3A, p > 0.001). This suggests that BV treatment alleviates cold allodynia in oxaliplatin-injected rats via the activation of spinal α4β2 nicotinic acetylcholine receptors.

**DISCUSSION**

About 85% to 95% of oxaliplatin-treated patients rapidly develop pain without motor dysfunction during the oxaliplatin administration period, peaking at the first 24–48 h.3,5 There have been only a small number of reports showing the effective treatment of oxaliplatin-induced neuropathic pain. Thus,
new therapeutic options for this pain are critically needed. In this regard, we recently showed that a subcutaneous injection of diluted BV into the GV3 acupoint markedly attenuates oxaliplatin-induced cold allodynia in rats with its effect lasting at least 2 h, which is longer than the duration of morphine (2 mg/kg, i.p.) analgesia (ca. 1 h). This result suggests that BV injection could be a potential therapeutic option. However, the mechanisms of the anti-allodynic effect of BV injection is still unclear, because the BV effect was not blocked by an opioid antagonist, naloxone, and was just partially blocked by the α-adrenergic antagonist, phentolamine. In the present study, we demonstrated that another non-opioid pain inhibitory pathway, i.e., the cholinergic system, plays a crucial role in mediating the analgesic effect of BV injection on oxaliplatin-induced acute cold allodynia.

Both the nicotinic and muscarinic acetylcholine receptors are located in the deep and superficial dorsal horn of the spinal cord, in which nociceptive information is transmitted and regulated. In this study, we clearly showed that an i.p. administration of non-selective nicotinic receptor antagonist MEC blocked the anti-allodynic effect of BV treatment in oxaliplatin-injected rats, whereas an injection of muscarinic receptor antagonist ATR did not (Fig. 1). This suggests that BV-induced anti-allodynia in a rat model of oxaliplatin-induced neuropathic pain is mediated by the activation of inhibitory receptors, including nicotinic acetylcholine receptors. Among the pentameric ligand-gated ion channel proteins, two nicotinic receptor subtypes are known to be mainly involved in pain modulation, i.e., the heteromeric α4β2 and the homomeric α7 receptor. The α7 nicotinic receptor agonist alleviated an inflammatory pain. The α4β2 nicotinic receptors have been implicated in thermal acute pain and the α4β2 receptors are known to be involved in inhibitory synaptic transmission in the spinal dorsal horn. The present data indicate that BV treatment activates spinal α4β2 nicotinic receptors to relieve cold allodynia signs. An i.p. or i.t. injection of selective α4β2 nicotinic receptor antagonist DHβE prevented the anti-allodynic effect of BV injection while an i.p. injection of α7 nicotinic antagonist MLA did not change the BV effect (Figs. 2, 3). These findings are in agreement with prior studies showing that α4β2 nicotinic acetylcholine receptors mediate spinal antinoceptive and anti-allodynia.

It has been reported that acupuncture or EA analgesia is centrally mediated by activation of the descending pain inhibitory system that finally involves spinal opioidergic, noradrenergic and cholinergic receptors. Our previous studies indicate that each of these endogenous analgesic systems is centrally mediated by activation of the descending pain inhibitory system that finally involves spinal opioidergic, noradrenergic and cholinergic receptors, but not nicotinic receptors, were involved in such EA effect. We recently showed that EA significantly reduced acute cold allodynia in oxaliplatin-injected rats via the stimulation of the endogenous opioids, not via the activation of the noradrenergic system. In contrast, the present results, together with our previous report, indicate that the relieving effect of BV injection on oxaliplatin-induced cold allodynia does not involve the activation of muscarinic acetylcholine receptors and opioid receptors, but involves the activation of nicotinic acetylcholine receptors and/or the partial activation of noradrenergic receptors. We also confirmed that BV injection into a Yaoyangguan acupoint that is proximal to the tail showed a greater analgesic effect than BV injection into Zusanli or Quchi acupoint that is distal to the tail (Supplementary Fig. 1), whereas EA stimulation showed the opposite result (unpublished data). These findings strongly suggest that BV injection and EA stimulation might have distinct mechanisms for their anti-allodynic effects in oxaliplatin-administered rats. However, we cannot completely rule out the other possibility that the expression levels of nicotinic and muscarinic receptors might be different between the oxaliplatin-injected and nerve injured rats, which affects the distinct cholinergic mechanisms of BV- and EA-induced anti-allodynia. Further quantitative studies are needed to address this issue.

In conclusion, we showed that the relieving effect of BV injection on oxaliplatin-induced cold allodynia was blocked by a systemic administration of non-selective nicotinic receptor antagonist, but not by an administration of muscarinic receptor antagonist. Further, the anti-allodynic effect of BV injection was blocked by a systemic or i.t. administration of α4β2 nicotinic receptor antagonist, but not by an administration of α7 nicotinic antagonist. These results suggest that BV treatment alleviates oxaliplatin-induced neuropathic cold allodynia in rats via the activation of nicotinic acetylcholine receptors, especially spinal α4β2 receptors. Thus, our findings may provide a useful scientific evidence for the application of subcutaneous injection of diluted BV for the management of neuropathic cold allodynia, a dose-limiting side effect by an oxaliplatin administration in cancer patients.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


