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The cannabinoid receptor agonist, WIN 55, 212-2, attenuates tumor-evoked hyperalgesia through peripheral mechanisms

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Abstract

Several lines of evidence suggest that cannabinoids can attenuate various types of pain and hyperalgesia through peripheral mechanisms. The development of rodent cancer pain models has provided the opportunity to investigate novel approaches to treat this common form of pain. In the present study, we examined the ability of peripherally administered cannabinoids to attenuate tumor-evoked mechanical hyperalgesia in a murine model of cancer pain. Unilateral injection of osteolytic fibrosarcoma cells into and around the calcaneus bone resulted in tumor formation and mechanical hyperalgesia in the injected hindpaw. Mechanical hyperalgesia was defined as an increase in the frequency of paw withdrawals to a suprathreshold von Frey filament (3.4mN) applied to the plantar surface of the hindpaw. WIN 55, 212-2 (1.5 to 10μg) injected subcutaneously into the tumor-bearing hindpaw produced a dose-dependent decrease in paw withdrawal frequencies to suprathreshold von Frey filament stimulation. Injection of WIN 55,212-2 (10μg) into the contralateral hindpaw did not decrease paw withdrawal frequencies in the tumor-bearing hindpaw. Injection of the highest antihyperalgesic dose of WIN 55,212-2 (10μg) did not produce catalepsy as determined by the bar test. Co-administration of WIN 55,212-2 with either cannabinoid 1 (AM251) or cannabinoid 2 (AM630) receptor antagonists attenuated the antihyperalgesic effects of WIN 55, 212-2. In conclusion, peripherally administered WIN 55,212-2 attenuated tumor-evoked mechanical hyperalgesia by activation of both peripheral cannabinoid 1 and cannabinoid 2 receptors. These results suggest that peripherally-administered cannabinoids may be effective in attenuating cancer pain.

Keywords

cancer pain; cannabinoids; hyperalgesia; WIN 55; 212-2

1. Introduction

Pain resulting from cancer still presents a major therapeutic challenge. Current estimates indicate that over half of patients with cancer experience pain, and nearly two-thirds in advanced disease stages experience pain (van den Beuken-van Everdingen et al., 2007). Of

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those reporting cancer pain, it is estimated that one-third of all cancer patients rate their pain as moderate to severe (van den Beuken-van Everdingen et al., 2007). Although the prevalence of cancer pain differs among the various types of cancer, it is often reported by individuals with both primary and metastatic bone cancer, with greater than 60% of individuals with metastatic bone cancer suffering from severe pain (Coleman, 2006).

Cancer pain is most often treated with opioid drugs; however, these drugs produce dose-limiting side effects and sometimes provide inadequate analgesia (Hanks and Forbes, 1997; Cherny, 2004). Current estimates suggest that adequate analgesia is not achieved in all cancer patients using the World Health Organization's (WHO's) analgesic ladder (Azevedo et al., 2006). Understanding the mechanisms related to the generation and maintenance of cancer pain is needed for the development of novel and more efficacious therapies to treat this condition.

The use of cannabinoids to treat cancer pain may provide a novel therapeutic approach. Two endogenous receptors for cannabinoids have been isolated and cloned so far, cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors (Matsuda, et al., 1990; Munro, et al., 1993), and both have been localized to various neuronal and non-neuronal tissues. Previous studies have shown that systemic administration of cannabinoids produces antinociception and attenuates hyperalgesia and allodynia in animal models of acute and chronic pain (for reviews see Walker et al., 1999; Hohmann, 2002; Walker and Huang, 2002; Mbvundula et al., 2004). The efficacy of systemically delivered cannabinoids is limited by adverse cannabimimetic effects (catalepsy, hypolocomotion, and disruptions in memory) which are mediated through CB_1 receptor activation in the central nervous system (for review see Iversen, 2003). One way to avoid these centrally-mediated cannabimimetic effects, while still providing analgesia, is through the selective targeting of peripheral CB_1 receptors expressed on nociceptive primary afferent dorsal root ganglion (DRG) neurons (Hohman and Hekenham, 1999; Ahluwalia et al., 2000) and cutaneous nerve terminals (Ständer et al., 2005; Amaya et al., 2006; Agarwal et al., 2007).

A recent study from our lab demonstrated that systemic administration of the non-selective cannabinoid receptor agonist CP 55,940 attenuated tumor-evoked mechanical hyperalgesia in an established murine model of cancer pain through activation of CB_1 receptors (Hamamoto et al., 2007). In this cancer pain model, osteolytic fibrosarcoma cells were injected into and around the calcaneus bone which produces a tumor mass and eventually leads to hyperalgesia and sensitization of C nociceptors overlying the tumor on the plantar surface of the hindpaw (Cain et al., 2001; Wacknik et al., 2001). Since systemically administered cannabinoids can produce adverse cannabimimetic effects, the present study was designed to determine if local injection of the non-selective cannabinoid receptor agonist WIN 55,212-2 can attenuate tumor-evoked mechanical hyperalgesia and whether this effect occurred through activation of CB_1 or CB_2 receptors.

2. Results

2.1. Tumor-evoked mechanical hyperalgesia

Prior to implantation of fibrosarcoma cells, the paw withdrawal frequency evoked by a 3.4mN von Frey filament was 0–20% (data not shown). Mechanical hyperalgesia was fully developed by 6 days post-implantation at which time paw withdrawal frequencies increased in the tumor-bearing hindpaw to 70–100% (data not shown). No changes in paw withdrawal frequencies were observed in the contralateral hindpaw where paw withdrawal frequencies remained 0–20% (data not shown). These data are consistent with prior studies in our lab demonstrating the time-course and magnitude of mechanical hyperalgesia following implantation of

fibrosarcoma cells into and around the calcaneus bone (Cain et al., 2001; Hamamoto et al., 2007).

2.2. WIN 55,212-2 attenuation of tumor-evoked mechanical hyperalgesia

Ten to fourteen days after fibrosarcoma cell implantation, tumor-bearing mice were randomly divided into separate groups and received an intraplantar injection of vehicle or WIN 55,212-2 at doses of 1.5, 2.5, 5, or 10μg(n=6–8 per group). Paw withdrawal response frequencies were determined before and at 10, 30, 60, 90, and 120 min after drug administration. Injection of vehicle into the tumor-bearing hindpaw did not alter paw withdrawal frequencies which remained approximately 85% throughout the time course. Injection of WIN 55,212-2 into the tumor-bearing hindpaw dose-dependently decreased mean paw withdrawal frequencies (figure 1). WIN 55,212-2 at doses of 2.5, 5, and 10μg significantly decreased the paw withdrawal frequencies compared to vehicle ($p<0.05$). These data indicate that peripherally administered WIN 55,212-2 attenuated tumor-evoked mechanical hyperalgesia dose-dependently.

To ensure that the antihyperalgesic effects were due to peripherally-mediated actions and not systemic effects, WIN 55,212-2 was injected into the contralateral hindpaw and paw withdrawal frequencies were determined in the tumor-bearing hindpaw. Intraplantar injection of WIN 55,212-2 (10μg) or vehicle into the contralateral hindpaw did not alter paw withdrawal frequencies in the tumor-bearing hindpaw which remained approximately 70% throughout the time course (figure 2). These data indicate that the antihyperalgesic effects of locally administered WIN 55,212-2 were not due to systemic effects.

2.3. Cataleptic effects of WIN 55,212-2

The bar test was used to determine whether intraplantar administration of WIN 55,212-2 produced catalepsy defined as an increase in the time spent with front paws on an elevated bar. Prior to injection of WIN 55,212-2, naive mice only kept their front paws on the bar for approximately 2 seconds (s). Intraplantar injection of vehicle or the highest dose of WIN 55,212-2 that produced antihyperalgesia (10μg) did not increase the duration of time mice spent with their front paws on the bar (Figure 3). In contrast, intraplantar injection of 25μg of WIN 55,212-2 increased time spent on the bar at 30 minutes post administration ($p<0.05$). These data indicate that intraplantar injections of higher doses of WIN 55,212-2 are capable of producing catalepsy, but the decrease in paw withdrawal frequencies produced by the antihyperalgesic doses of WIN 55,212-2 used in the present study are not due to catalepsy.

2.4. Contribution of CB_1 and CB_2 receptors to the effects of WIN 55,212-2

To determine the contribution of CB_1 and CB_2 receptors to the decrease in paw withdrawal frequencies produced by WIN 55,212-2, selective cannabinoid receptor antagonists were co-administered with WIN 55,212-2. Co-administration of the CB_1 receptor antagonist AM251 (1μg) blocked the decrease in paw withdrawal frequencies produced by 10μg of WIN 55,212-2 ($p<0.05$, figure 4A). Co-administration of the CB_2 receptor antagonist AM630 (1μg) also blocked the decrease in paw withdrawal frequencies produced by 10μg of WIN 55,212-2 ($p<0.05$, figure 4B). These data suggest that the decrease in tumor-evoked mechanical hyperalgesia following intraplantar injection of WIN 55,212-2 into the tumor-bearing hindpaw was mediated by CB_1 and CB_2 receptors.

3. Discussion

In summary, local intraplantar injection of WIN 55,212-2 into the tumor-bearing hindpaw dose-dependently attenuated tumor-evoked mechanical hyperalgesia. The antihyperalgesic effects of WIN 55,212-2 were not the result of catalepsy and were blocked by co-administration of either CB_1 or CB_2 receptor antagonists. Injection of WIN 55,212-2 into the contralateral

hindpaw did not alter mechanical hyperalgesia in the tumor-bearing hindpaw. Overall, these results show that local injection of WIN 55,212-2 attenuated tumor-evoked mechanical hyperalgesia through activation of both peripheral CB_1 and CB_2 receptors.

3.1. Cannabinoid attenuation tumor-evoked hyperalgesia

Results from the present study agree with earlier studies where systemic administration of cannabinoids attenuated tumor-evoked hyperalgesia following implantation of osteolytic fibrosarcoma cells into the humerus (Kehl et al., 2003) or into the calcaneus bone (Hamamoto et al., 2007). However, in the present study, cannabinoids were administered directly to the site of the tumor and decreased tumor-evoked mechanical hyperalgesia exclusively through peripheral mechanisms rather than through systemic administration which presumably has both central and peripheral components (Kehl et al., 2003; Hamamoto et al., 2007). The present study also found that WIN 55,212-2 attenuated tumor-evoked hyperalgesia through both CB_1 and CB_2 receptors while prior studies (Kehl et al., 2003; Hamamoto et al., 2007) found that systemic administration of cannabinoids only attenuated tumor-evoked hyperalgesia through activation of CB_1 receptors. Our results also agree with a recent report that showed local administration of WIN 55,212-2 or the CB_2 receptor agonist AM1241 attenuated tumor-evoked mechanical hyperalgesia in a murine model of cancer pain using human oral squamous cell carcinoma cells (Guerrero et al., 2008). Thus, in two separate murine cancer pain models that utilize different cancer cell lines, fibrosarcoma and squamous cell carcinoma, activation of both peripheral CB_1 and CB_2 receptors produced antihyperalgesia. Further studies should determine if changes in endocannabinoid tone occur in the tumor microenvironment and possibly affect tumor-evoked hyperalgesia.

3.2. Peripheral mechanisms of cannabinoid antihyperalgesia

A great deal of evidence has accumulated in recent years showing that local, site-directed administration of cannabinoids can attenuate hyperalgesia in a variety of animal pain models through peripheral mechanisms. Peripheral administration of cannabinoids produced antihyperalgesic effects in animal models of inflammatory pain (Richardson et al., 1998; Amaya et al., 2006; Gutierrez et al., 2007), neuropathic pain (Fox et al., 2001; Guidon and Beaulieu, 2006), heat injury (Johanek and Simone, 2004), and capsaicin-evoked hyperalgesia (Johanek at al., 2001) through activation of CB_1 receptors. Interestingly, we also found that the antihyperalgesic effects of WIN 55,212-2 on tumor-evoked mechanical hyperalgesia were due to activation of CB_2 receptors. This observation is also consistent with earlier studies in which activation of CB_2 receptors produced both antinociception and antihyperalgesia in a variety of pain models. Peripheral or systemic administration of selective CB_2 receptor agonists produced antinociception to heat (Malan Jr. et al., 2001; Ibrahim et al., 2005; Ibrahim et al., 2006) and attenuated hyperalgesia produced by carrageenan (Nackley et al., 2003;Quartiho et al., 2003; Elmes et al., 2005; Gutierrez et al., 2007), capsaicin (Hohmann et al., 2004), and neuropathic injury (Ibrahim et al., 2003). Peripheral injection of CB_2 receptor agonists also decreased mechanically-evoked responses of nociceptive spinal cord neurons following carrageenan-evoked inflammation and spinal nerve ligation (Elmes et al., 2004).

The underlying peripheral antihyperalgesic mechanisms responsible for the decrease of tumor-evoked mechanical hyperalgesia by WIN 55, 212-2 are not yet known. A recent study using mice with a conditional knockdown of CB_1 receptors in Nav1.8-expressing nociceptive sensory neurons found a loss of peripherally-mediated cannabinoid analgesia in models of neuropathic and inflammatory pain (Agarwal et al., 2007). Results from that study suggest the underlying antihyperalgesic effects of peripherally administered cannabinoids may result from activation of CB_1 receptors located on nociceptive primary afferent fibers (Agarwal et al., 2007). Additionally, intraplantar administration of the selective CB_1 agonist, arachidonyl-2-chloroethylamide (ACEA), attenuated mechanically-evoked responses of nociceptive spinal

cord neurons following carrageenan inflammation, which was also likely due to activation of CB_1 receptors on nociceptors (Kelly et al., 2003). Further support for that mechanism of action is suggested by previous studies showing that activation of CB_1 receptors can decrease high-voltage activated calcium currents (Ross et al., 2001; Khasabova et al., 2002, 2004) and reduce capsaicin-evoked calcium transients (Millns et al., 2001; Sagar et al., 2005) in nociceptive dorsal root ganglion neurons. It is likely that activation of CB_1 receptors on nociceptive primary afferent fibers is responsible for the decrease in tumor-evoked mechanical hyperalgesia in our study. Although the type of nociceptors affected by cannabinoids has not yet been determined, it is possible that local administration of WIN 55,212-2 into the tumor-bearing hindpaw decreases sensitization of C nociceptors. We have shown that a proportion of C nociceptors in the skin overlying the tumor had ongoing activity and lowered response thresholds for heat (Cain et al., 2001). Future studies should address the effects of cannabinoids on changes in the response properties of nociceptors during tumor-evoked hyperalgesia.

WIN 55, 212-2 also decreased tumor-evoked mechanical hyperalgesia through activation of CB_2 receptors possibly on non-neuronal cells in the tumor microenvironment. Activation of CB_2 receptors on keratinocytes results in the release of endogenous opioids which mediates the antinociception produced by CB_2 receptor agonists (Ibrahim et al., 2005). However, there is currently no evidence of opioid mobilization by WIN 55, 212-2. Previous studies have demonstrated that peripherally administered opioid receptor agonists attenuate tumor-evoked hyperalgesia through peripheral mechanisms (Menéndez et al., 2003; Menéndez et al., 2005; Baamonde et al., 2005). It is likely that peripherally administered opioids have direct actions on nociceptors during tumor-evoked hyperalgesia since both the response properties of cutaneous C nociceptors and hyperalgesia are attenuated by local administration of morphine in a rat model of inflammatory pain (Stein et al., 1993; Wenk et al., 2006). Future studies should address the relationship between activation of CB_2 receptors on the release of endogenous opioids in attenuating tumor-evoked hyperalgesia and nociceptor activity.

3.3. Analgesic effects of cannabinoids in humans

Although numerous studies in animals demonstrate the antinociceptive and antihyperalgesic properties of cannabinoids, very few studies have examined quantitatively their analgesic efficacy in humans. A study using Δ-9- tetrahydrocannabinol, the main psychoactive component of cannabis, found its analgesic efficacy was similar to codeine in patients with cancer pain (Noyes Jr. et al., 1975). CT-3 (Karst et al., 2003) and ajulemic acid (Salim et al., 2005), both analogs of Δ-9- tetrahydrocannabinol, were reported to have analgesic efficacy in patients with chronic neuropathic pain. Additional controlled, clinical studies are needed to address the therapeutic potential of cannabinoids in chronic pain states including cancer pain.

3.4. Conclusions

In conclusion, tumor-evoked mechanical hyperalgesia was dose-dependently attenuated by local administration of WIN 55, 212-2 into the tumor-bearing hindpaw. The antihyperalgesia produced by WIN 55,212-2 was mediated by both CB_1 and CB_2 receptors and not due to systemic effects or catalepsy. Based on results from the present study, peripherally acting cannabinoid receptor agonists may be useful to treat chronic cancer pain in humans either alone or in combination with other therapies.

4. Experimental Procedures

4.1 Subjects

Adult (> 6 weeks old) male C3H/He mice weighing 25–29 grams were used for this study. Animals were obtained from the National Institutes of Health (Frederick, MD), housed on a 12-hour light/dark schedule, and allowed *ad libitum* access to food and water. Each animal was

used in only one experiment. All animal procedures and protocols were approved by the Animal Care Committee at the University of Minnesota, and experiments were conducted according to the guidelines established by the International Association for the Study of Pain.

4.2 Cancer cell implantation

NCTC clone 2472 fibrosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained and implanted as described previously (Wacnik et al 2001). Briefly, fibrosarcoma cells were grown to confluency in 75 cm^2 in NCTC 135 medium (Sigma Chemical, St. Louis, MO) with a pH of 7.4 and containing 10% horse serum (ATCC, Manassas, VA). These fibrosarcoma cells were chosen because they are syngenic with C3H/He mice. Fibrosarcoma cells were trypsinized, pelleted, and resuspended in phosphate buffered saline (PBS) prior to implantation. Mice were placed in an enclosed chamber and anesthetized with 2% halothane, and fibrosarcoma cells (2×10^5/10 μl PBS) were injected unilaterally into and around the calcaneus bone in each animal's left hind paw using a 0.3ml insulin syringe. None of the mice used in this study displayed any motor dysfunction following implantation of fibrosarcoma cells. Prior studies from our lab and others have demonstrated that implantation of NCTC 2472 fibrosarcoma cells into and around the calcaneus bone reliably results in tumor formation in the injected hindpaw that progressively degrades the calcaneus bone beginning around post-implantation day (PID) six while also infiltrating overlaying hypodermal tissue (Cain et al., 2001; Wacnick et al., 2001). Moreover, prior studies from our lab and others have shown that mechanical hyperalgesia develops and remains consistent between PID 6–16 (Cain et al., 2001; Wacnick et al., 2001).

4.3 Drug preparation and administration

(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)py rrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmeth anone mesylate (WIN 55,212-2), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophen yl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), and 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone (AM630) were all purchased from Tocris Bioscience (Ellisville, MO). All drugs were prepared in a stock solution of 5% Tween 80, 5% DMSO, and 90% saline, and then further diluted in sterile physiological saline. All drugs were administered via subcutaneous intraplantar injection in a volume of 10μl.

4.4 Measurement of mechanical hyperalgesia

Paw withdrawal response frequency evoked by mechanical stimulation was assayed using a Semmes-Weinstein von Frey monofilament (3.4 mN bending force) (Stoelting, Wood Dale, IL). Animals were placed on an elevated wire mesh platform under individual glass dishes and allowed to acclimate to the testing environment for 30 minutes prior to testing. The filament was applied to the plantar surface of each hindpaw ten times for 1–2 seconds with an interstimulus interval of 5–6 seconds. Paw withdrawal response frequency was calculated as the number of paw withdrawals elicited by von Frey filament stimulation divided by 10. Baseline measures were determined for each animal for three days prior to fibrosarcoma cell implantation, and animals were tested daily following implantation of fibrosarcoma to monitor the development of mechanical hyperalgesia. Previous work from our lab and others has shown that both sham-injected and naive mice do not differ in their paw withdrawal response frequency which typically ranges between 10–20% (Cain et al., 2001; Wacnick et al., 2001). Mechanical hyperalgesia was defined as a paw withdrawal frequency $\geq$ 70%. The experimenter was blinded to the identity of drugs used.

4.5 Measurement of Catalepsy

Catalepsy was assayed using the bar test (Kuschinsky and Hornykiewicz, 1972). Briefly, each mouse was placed with forelimbs on a metal bar (1cm diameter) positioned 5cm above and parallel to the counter top. The time (seconds) the animal spent with forelimbs on the metal bar was recorded over a 60 second time period. Catalepsy was defined as a statistically significant increase in the time spent on the metal bar. The experimenter was blinded to the identity of drugs used.

4.6 Data Analysis

All data are presented as mean ± S.E.M. Within group comparisons at each dose of WIN 55, 212-2 (or vehicle) were made using one-way repeated measures ANOVA followed by paired t-tests with the Bonferroni correction for multiple comparisons. Between groups comparisons at each time point were made using one-way ANOVA followed by un-paired t-tests with the Bonferroni correction. For all statistical analyses, a probability value <0.05 was considered significant.

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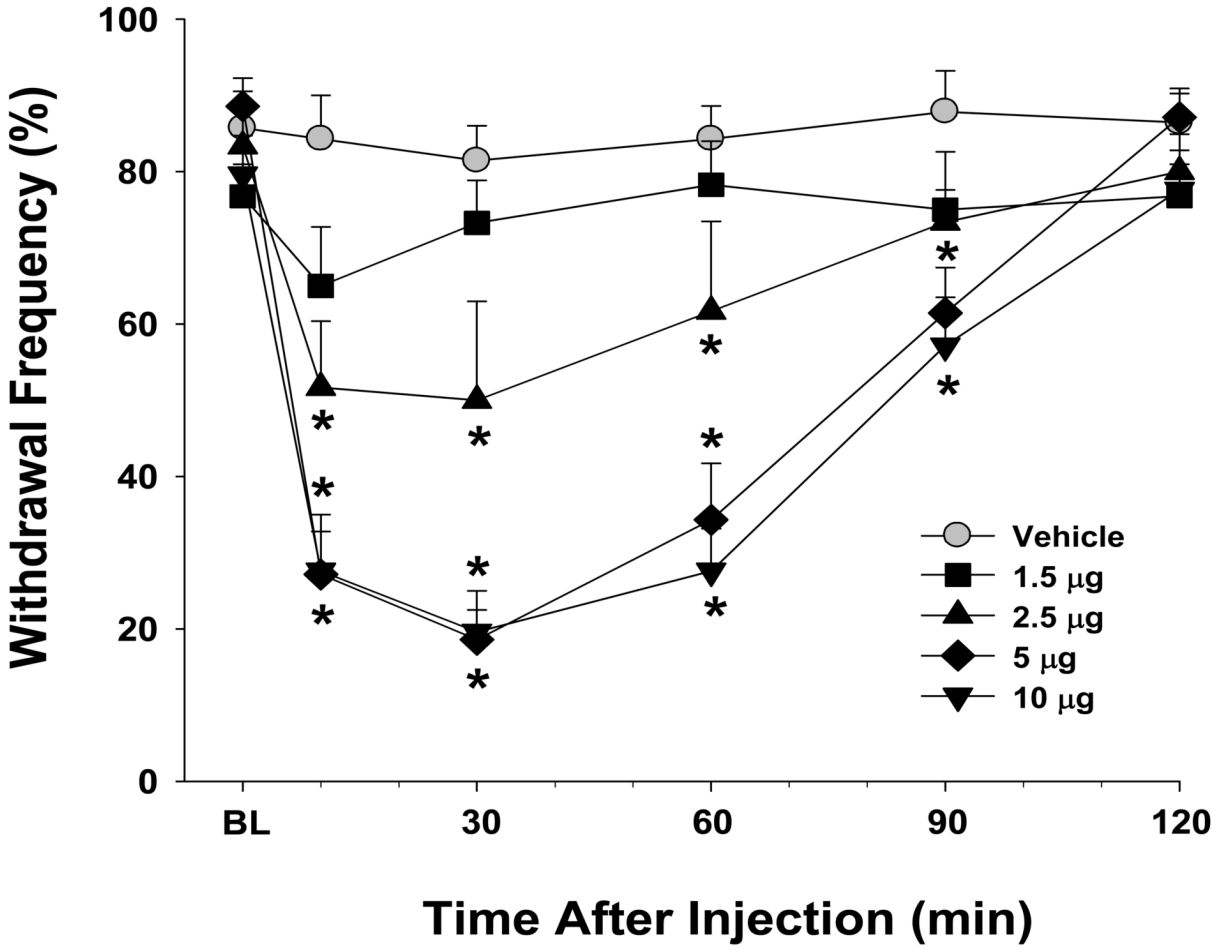

Figure 1.
WIN 55,212-2 dose-dependently attenuates mechanical hyperalgesia in a murine model of cancer pain. Local injection of 2.5, 5, or 10μg of WIN 55,212-2 into the tumor-bearing hindpaw reduced the mean paw withdrawal frequency evoked by a suprathreshold von Frey monofilament (3.4mN). * indicates a significant difference from vehicle ($p<0.05$).

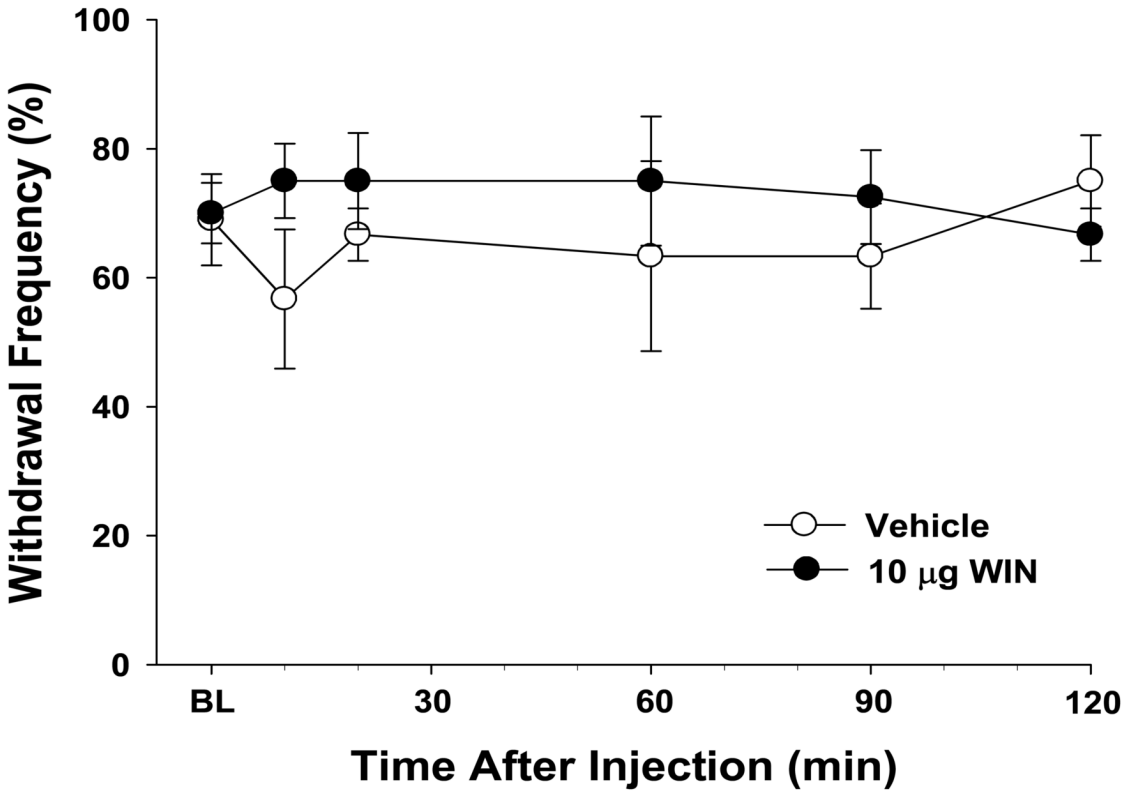

Figure 2.

Antihyperalgesia produced by intraplantar injection of WIN 55,212-2 occurred through peripheral mechanisms. Administration of WIN 55,212-2 (10μg) into the contralateral hindpaw did not decrease paw withdrawal frequencies in the tumor-bearing paw. This indicates that the antihyperalgesia produced by injection of WIN 55,212-2 into the paw did not occur via systemic uptake of the drug

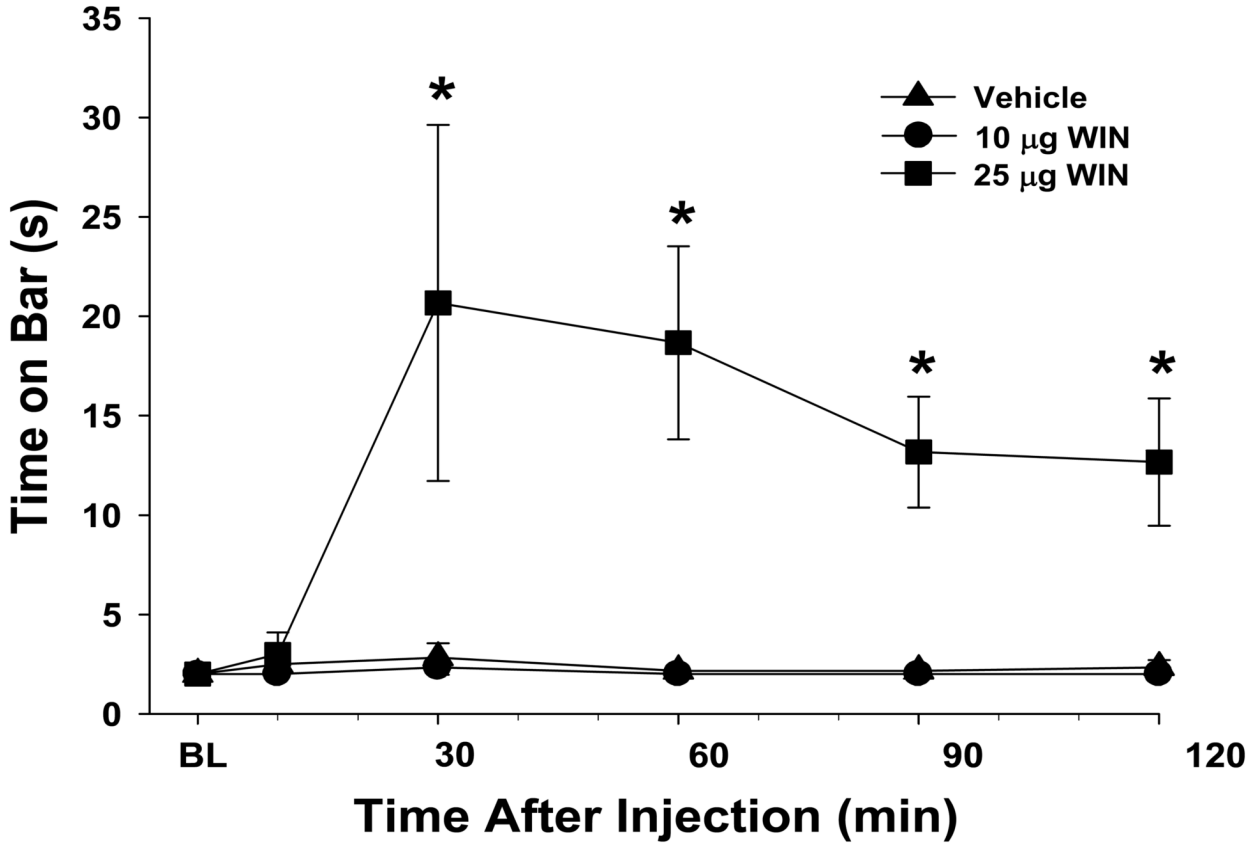

Figure 3.

Attenuation of tumor-evoked mechanical hyperalgesia following intraplantar injection of WIN 55,212-2 is not due to catalepsy. Neither vehicle nor 10μg of WIN 55,212-2 increased the amount of time mice spent on the bar. However, intraplantar injection of the 25μg dose of WIN 55,212-2 produced catalepsy. *indicates a significant difference from vehicle ($p<0.05$).

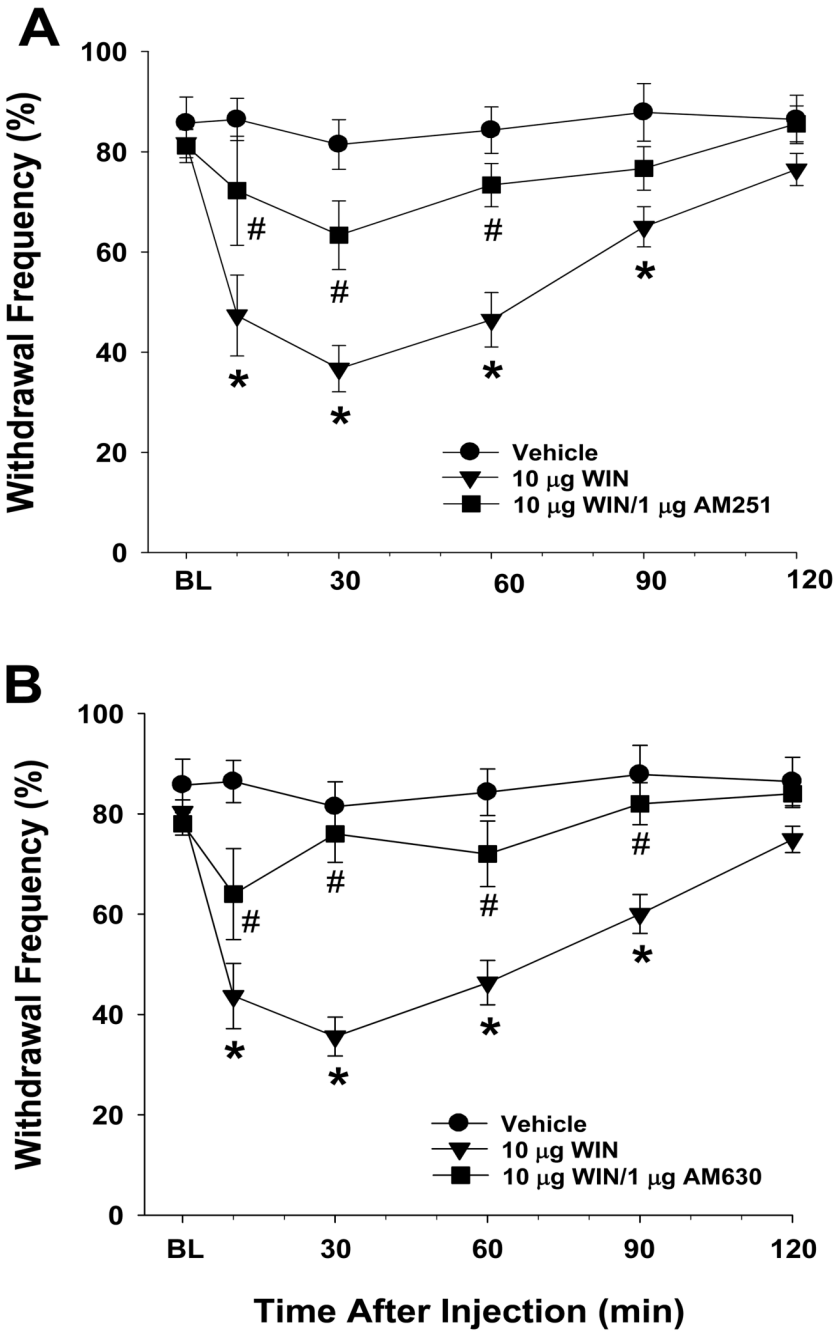

Figure 4.

CB_1 and CB_2 receptors contribute to the antihyperalgesia produced by WIN 55,212-2. Co-administration of 1 μg of either the CB_1 receptor antagonist, AM251 (A), or the CB_2 receptor antagonist, AM630 (B) with 10μg of WIN 55,212-2 attenuated the antihyperalgesia produced by WIN 55,212-2. * indicates a significant difference from vehicle ($p<0.05$); # indicates a significant difference from WIN 55,212-2 (10μg).