Title: Role of Nitric Oxide in The Antipruritic Effect of WIN 55,212-2, A Cannabinoid Agonist

Running title: Antipruritic Mechanism of Cannabinoids

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ABSTRACT

Introduction: For centuries, cannabinoids are known to be effective in pain conditions. Itch is an unpleasant sensation that provokes a desire to scratch. Since itch and pain are two sensations sharing a lot in common, we aimed to confirm whether the cannabinoid agonist WIN 55,212-2 reduce serotonin-induced scratching behavior and observe whether modulation of nitric oxide (NO) production mediates the antipruritic effect of WIN 55,212-2.

Methods: Scratching behavior is induced by intradermal injection of serotonin (50 µg/50 µl/mouse) to Balb/c mice. The cannabinoid agonist WIN 55,212-2 (1, 3, 10 mg/kg, i.p.) was given 30 min before serotonin injection. To observe the effect of NO modulation on the antipruritic effect of cannabinoids, the endothelial nitric oxide synthase (NOS) inhibitor L-NAME (3 mg/kg, i.p.), the neuronal NOS inhibitor 7-nitroindazole (3 mg/kg, i.p.), and the NO precursor L-arginine (100 mg/kg, i.p.) were administered together with WIN 55,212-2.

Results: WIN 55,212-2 reduced serotonin-induced scratches at higher doses (3, 10 mg/kg; P < 0.0001). The endothelial NOS inhibitor L-NAME, the neuronal NOS inhibitor 7-nitroindazole, and the nitric oxide precursor L-arginine, had no influence on the antipruritic action of WIN 55,212-2. When NO modulators are used alone, only the neuronal NOS inhibitor 7-nitroindazole attenuated serotonin-induced scratches (P < 0.0001).

Conclusion: Our findings indicate that exogenous cannabinoids may attenuate serotonin-induced scratches and NO does not mediate the antipruritic effect of WIN 55,212-2. On the other hand, neuronal NOS inhibition may play role in production of serotonin-induced scratches.

Keywords: cannabinoid system, nitric oxide, pruritus, serotonin, WIN 55,212-2
1. Introduction

Cannabinoids are chemicals that produce their effects mostly via activating cannabinoid receptors (CB1, CB2); they include phytocannabinoids, synthetic cannabinoids, and endocannabinoids (Maccarrone et al., 2015; Olah, Szekanecz, & Biro, 2017; A. Ulugol, 2014). The analgesic activity of cannabinoids has been known for centuries; however, these drugs could not be used efficiently in the clinics due to the potential of drug abuse and their unwanted central side effects, including development of drug tolerance and addiction. Following many successful clinical trials, cannabinoids have been approved for indications such as neuropathic pain, multiple sclerosis, etc. first in USA and Canada, and then in many European countries in recent years (Grotenhermen & Muller-Vahl, 2012; Lucas, 2012; A. Ulugol, 2014). The number of approved indications and countries cannabinoids are used therapeutically is expected to increase in the years ahead.

Pruritus (itch), a common unpleasant symptom, is seen not only in skin diseases, but also in systemic disorders. Pain and itch share a lot in common in terms of pathophysiology, which suggests why pain mechanisms should also be evaluated when trying to reveal the mechanisms of itch (Ross, 2011; Schmelz, 2010). Recently, similar to pain, not only peripheral mechanisms but also central mechanisms have been suggested to play important roles in itch, which has started to change the approaches for the therapy of pruritus (Cevikbas, Steinhoff, & Ikoma, 2011). Understanding the mechanisms of itch in detail will contribute to the development of more efficient antipruritic medications with fewer side effects.

The number of researches regarding the analgesic effects of cannabinoids is quite remarkable. Nevertheless, the effects of cannabinoids on itch have not been investigated sufficiently despite the similarities between pain and itch mechanisms. Cannabinoid receptor agonists have been indicated to reduce itch behavior, whereas cannabinoid receptor antagonists like rimonabant increased itch behavior dose-dependently in mice (Darmani &
Pandya, 2000). Other researches also pointed out that the brain penetrating CB1 inverse agonist rimonabant creates itch sensation, and this effect is reduced by cannabinoid receptor agonists (Janoyan, Crim, & Darmani, 2002; Schlosburg, O'Neal, Conrad, & Lichtman, 2011). Furthermore, it is determined that peripheral application of the synthetic cannabinoid HU210 suppressed histamine-induced responses in human skin (Dvorak, Watkinson, McGlone, & Rukwied, 2003). In addition to these studies, there are few researches carried out regarding the role of endocannabinoid system in itch behavior (Schlosburg, Boger, Cravatt, & Lichtman, 2009; Spradley, Davoodi, Gee, Carstens, & Carstens, 2012; Tosun, Gunduz, & Ulugol, 2015).

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS) and regarded as a neuronal messenger and a modulator in the central nervous system (Snyder & Bredt, 1991). There are at least three isoforms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isoforms (Boucher, Moali, & Tenu, 1999). NO is shown to take part in many physiological and pathological phenomena; elevated NO levels are observed not only in experimental models of inflammation and hyperalgesia (Nakamura, Fujita, & Shiomi, 1996), but also in several clinical neurological disorders, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, etc. (Duncan & Heales, 2005; Zhang et al., 2017). Taking into account the similarities between pain and itch, contribution of NO into pruritic states can also be expected. There are a few studies indicating that NO production is increased in both experimental itch models and clinical pruritic diseases such as psoriasis and atopic dermatitis (Andoh & Kuraishi, 2003; Ormerod et al., 1998; Taniuchi et al., 2001), but its involvement in cannabinoid-induced antipruritic action is unknown.

In our study we aimed to confirm whether the non-selective cannabinoid CB1 and CB2 agonist WIN 55,212-2 diminish serotonin-induced scratches, which we have shown in our previous work (Todurga, Gunduz, Karadag, & Ulugol, 2016), and determine whether NO
is involved in the antipruritic effect of WIN 55,212-2. Revealing the mechanisms of these effects of cannabinoids may contribute to their utility as novel antipruritic agents.

2. Methods

2.1. Animals & Ethics

A total of 80 male Balb-c mice (Center of the Laboratory Animals, Trakya University), 3-4 months old and weighing 20-30 g, were used in the experiments (n=8 for each group). Mice were housed under controlled 12-12h light-dark cycles at the temperature of 21±2 °C with water and food available ad libitum. Adequate measures were taken to minimize pain or discomfort, and “Animal Care Ethics Committee” of Trakya University approved all experimental protocols of this study.

2.2. Serotonin-induced scratching behavior

To provoke scratching behavior, serotonin (5 µg) was administered intradermally in a volume of 50 µl into the pre-shaved rostral part of the back of the mice. Instantly after serotonin injection, the animals were put into a transparent plexiglass cylinder individually, and animal behavior was videotaped for 30 min under quiet circumstances. In general, the mice produced several scratches per second, and such manner was considered as one bout of scratching. The video was re-played to calculate the number of bouts of scratching, similar to our earlier studies (Gunduz et al., 2015; Saglam, Gunduz, & Ulugol, 2014; Todurga et al., 2016; Tosun et al., 2015).

2.3. Rotarod assessment

Since it is known that cannabinoids may have adverse effects on locomotor activity, the effect of WIN 55,212-2 on motor function was evaluated by the Rotarod test; the same
test was also applied to NO modulators. Time course to fall of the mice were documented. Before assessments, a cut-off time of 180s and a speed of 16rpm were adjusted. Rotarod tests were performed immediately before serotonin injections.

2.4. Experimental protocol

While Group 1 was selected as the vehicle group, Groups 2, 3 and 4 received respective doses of the cannabinoid receptor agonist WIN 55,212-2 (1, 3, 10 mg/kg, i.p.) 30 min before serotonin injection.

In Groups 5-7, in order to evaluate the effect of NO modulation on the antipruritic effect of cannabinoids, the non-selective NOS inhibitor L-NAME (3 mg/kg, i.p.), the nNOS inhibitor 7-nitroindazole (3 mg/kg, i.p.), and the NO precursor L-arginine (100 mg/kg, i.p.) were administered 10 min before WIN 55,212-2, respectively.

In Groups 8-10, NOS inhibitors L-NAME (3 mg/kg, i.p.) and 7-nitroindazole (3 mg/kg, i.p.) and the NO precursor L-arginine (100 mg/kg, i.p.) were given on their own, respectively. Thus, we attempted to confirm the cannabinoid action on serotonin-induced scratching behavior and investigate the mediatary effect of NO in this antipruritic effect.

2.5. Drugs

Serotonin hydrochloride was dissolved in 0.9% saline, while WIN 55,212-2, L-NAME, 7-nitroindazole and L-arginine were dissolved in 20% DMSO, 1% Tween 80, 1% ethanol and 78% saline; this composition is also used in the vehicle group. All of the drugs were purchased from Sigma-Aldrich. Drug doses and treatment times were selected from our previous researches (Gunduz, Topuz, Karadag, & Ulugol, 2016; A. Ulugol et al., 2002; Ahmet Ulugol, Karadag, Ipci, Tamer, & Dokmeci, 2004; A. Ulugol, Topuz, Gunduz, Kizilay, & Karadag, 2016).
2.6. Statistical analysis

Bartlett’s test is used to test Gaussian distribution. To assess the significance of any difference, one-way ANOVA test followed by the Bonferroni post hoc test was performed. Results are expressed as mean ± standard error of the mean. A P value <0.05 was considered to be statistically significant. GrapPad Prism 6.0 is used for statistical analysis and to plot the graphs.

3. Results

3.1. Effect of WIN 55,212-2 on serotonin-induced scratching behavior

Although WIN 55,212-2 reduced the number of scratches at its lowest dose (1 mg/kg), this effect was not statistically significant. WIN 55,212-2 elicited significant antipruritic activity at higher doses (3, 10 mg/kg, P < 0.0001, Figure 1).

3.2. Effect of NO modulators on the antipruritic activity of WIN 55,212-2

NOS inhibitors L-NAME (3 mg/kg, i.p.) and 7-nitroindazole (3 mg/kg, i.p.) and the NO precursor L-arginine (100 mg/kg, i.p.) did not change the antipruritic activity of WIN 55,212-2 (Figure 2). When NO modulators are used alone, only the nNOS inhibitor 7-nitroindazole (3 mg/kg, i.p.) significantly reduced the number of scratches produced by serotonin (P < 0.0001, Figure 2).

3.3. Effects of WIN 55,212-2 and NO modulators on locomotor performance

WIN 55,212-2 disrupted motor function at its highest dose (10 mg/kg; P <0.0001, compared to control, Figure 3). NOS inhibitors L-NAME (3 mg/kg, i.p.) and 7-nitroindazole (3 mg/kg, i.p.) and the NO precursor L-arginine (100 mg/kg, i.p.) did not exert any effect on locomotor activity (Figure 3).
4. Discussion

As mentioned earlier in the Introduction, researches focusing on the analgesic effects of cannabinoids have been carried out many times. That being said, despite many established similarities between the mechanisms of pain and itch (Cevikbas et al., 2011; Ross, 2011; Schmelz, 2010), the effects of cannabinoids on itch remain under-researched. The few existing researches on the matter demonstrated that cannabinoid receptor agonists decrease scratching behavior; and that cannabinoid receptor antagonists like rimonabant, also a brain penetrating CB1 inverse agonist, dose-dependently increase drug-induced scratches (Darmani & Pandya, 2000; Janoyan et al., 2002; Schlosburg et al., 2011; Todurga et al., 2016). Peripheral administration of the potent synthetic cannabinoid HU210 has also been reported to suppress histamine-induced scratches (Dvorak et al., 2003). In accordance with these researches and our previous report (Todurga et al., 2016), this research indicate that the cannabinoid receptor agonist WIN 55,212-2 diminishes serotonin-induced scratches dose-dependently. WIN 55,212-2 caused an insignificant decrease in the number of scratches at the dose of 1 mg/kg, whereas it almost ceased scratching behavior at higher doses (3 and 10 mg/kg).

NO modulates neuronal function and takes part in various biological tasks in the central nervous system. Moreover, the influence of NOS system on cannabinoid activity has been shown; activation of cannabinoid receptors by anandamide led to NO production (Prevot et al., 1998), and CB1 receptors stimulated cyclic GMP production in neuronal cells (Carney et al., 2009; Jones, Carney, Vrana, Norford, & Howlett, 2008). NO has also been suggested to play important roles in development of tolerance to some effects of cannabinoids; it has been indicated that NO is involved in the development of tolerance to the hypothermic and catalepsy effects of cannabinoids (Azad et al., 2001; Spina, Trovati, Parolaro, & Giagnoni,
whereas a negligible contribution to cannabinoid analgesic action have been proposed (Azad et al., 2001; Spina et al., 1998; Thorat & Bhargava, 1994). In contrast, it has been suggested that L-arginine/NO pathway is involved in the development of tolerance to the analgesic action of the cannabinoid agonist WIN 55,212-2 (Banafshe, Ghazi-Khansari, & Dehpour, 2005). Basing our research in which the starting point is the similarities between the mechanisms of pain and itch, we observed that NO modulation did not influence the antipruritic effect of cannabinoids. Neither endothelial and neuronal NOS inhibitors nor the NO precursor had any effect on the antipruritic action of the cannabinoid receptor agonist WIN 55,212-2. It can be concluded that NO does not play role in the antipruritic effect of cannabinoids.

Administration of NOS inhibitors and the NO precursor L-arginine on their own revealed different results on serotonin-induced scratches. Neither the non-selective NOS inhibitor L-NAME nor the NO precursor L-arginine had any effect on serotonin-induced scratches. However, the nNOS inhibitor 7-nitroindazole significantly reduced serotonin-induced itch behavior. In another research, in line with our findings, L-NAME was found to have no effect serotonin-induced scratches, whereas the iNOS inhibitor aminoguanidine prevented itching behavior. Researchers pointed to the involvement of NO in serotonin-induced itching and highlighted the importance of iNOS in the process, yet they had not used nNOS inhibitors (Ostadhadi, Haj-Mirzaian, Azimi, Mansouri, & Dehpour, 2015). Another report concluded that NO/cGMP pathway mediated chloroquine-induced itching and that specifically nNOS inhibition had a role in the process (Foroutan, Haddadi, Ostadhadi, Sistany, & Dehpour, 2015). Conclusions from these researches are consistent with our results, all suggesting the importance of nNOS inhibition in experimental itch models. It should also be known that involvement of eNOS cannot be excluded, since 7-nitroindazole also inhibits eNOS at higher doses (Ayajiki, Fujioka, Okamura, & Toda, 2001).
Our findings show that intraperitoneal injection of the cannabinoid receptor agonist dose-dependently decreased serotonin-induced scratching. Mechanism of action of WIN 55,212-2 may include both cannabinoid CB1 and/or CB2 receptors, since it is a non-selective CB1/CB2 agonist. On the basis of the currently presented data, it cannot be decided if the actions are realized at the level of the central nervous system or already at the periphery; peripherally restricted CB1 agonists could have been used to minimize central side effects, but in this case peripheral side effects should be taken into consideration and this was not the purpose of this study. Another possible mechanism for cannabinoids is that they may also inhibit transient receptor potential (TRP) channels, especially TRPV1, which plays an important role in sensing itch as well as pain (Morita et al., 2015; Ross, 2011; Schmelz, 2010). In our study, neither the endothelial and the neuronal NOS inhibitors nor the NO precursor influenced the antipruritic action of cannabinoids; on the other hand, the neuronal NOS inhibitor decreased scratching when used alone. We concluded that NO does not mediate the antipruritic action of cannabinoids and that neuronal NOS inhibition plays role in serotonin-induced itching. Whatever the mechanism of action may be, we suggest that cannabinoids have the potential to be used as antipruritic drugs, if their side effects can be reduced.

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Conflict of interest

The authors declare no conflict of interest.
References


Figure Legends

Figure 1. Effects of systemic administration of the cannabinoid agonist WIN 55,212-2 (1, 3, 10 mg/kg, i.p.) on serotonin-induced scratches. * P < 0.0001, compared to control.

Figure 2. Effects of the non-selective NOS inhibitor L-NAME (3 mg/kg, i.p.), selective nNOS inhibitor 7-nitroindazole (3 mg/kg, i.p.) and the NO precursor L-arginine (100 mg/kg, i.p.) on serotonin-induced scratches and on the antipruritic effect of WIN 55,212-2 (3 mg/kg, i.p.). * P < 0.0001, compared to control.
Figure 3. Effects of WIN 55,212-2 (1, 3, 10 mg/kg, i.p.), L-NAME (3 mg/kg, i.p.), 7-nitroindazole (3 mg/kg, i.p.) and L-arginine (100 mg/kg, i.p.) on locomotor performances. * $P < 0.0001$, compared to control.