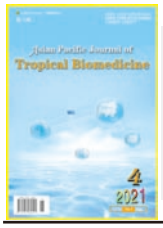

Original Article

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.310200

Impact Factor: 1.90

Cannabinoid CB_2 receptors and spinal microglia are implicated in tingenone-mediated antinociception in mice

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ABSTRACT

Objective: To investigate the antinociceptive effect of tingenone on inflammatory pain, as well as and the involvement of the cannabinoid receptors type 2 (CB_2) and spinal microglia in this process.

Methods: Male Swiss mice were subjected to inflammatory pain induced by intraplantar injection of carrageenan. The nociceptive threshold was measured by von Frey filaments test. Tingenone was administered orally 60 min before carrageenan injection. To evaluate the involvement of CB_2 receptor, endocannabinoids, and microglia, AM630 (a CB_2 receptor antagonist), MAFP (an inhibitor of an enzyme that hydrolyses endocannabinoids), and minocycline (a microglial inhibitor) were given intrathecally 20 min before tingenone administration. In addition, an immunofluorescence assay was used to evaluate CB_2 receptor and CD11B (a microglial marker) expression in the spinal cord dorsal horn.

Results: Tingenone significantly reduced carrageenan-induced hyperalgesia, which was reversed by pretreatment with AM630. MAFP and minocycline potentiated and prolonged the tingenone-induced antinociception. CD11B expression was increased in the spinal cord dorsal horn of mice with inflammatory pain pretreated with tingenone, which was reduced by AM630, MAFP, and minocycline.

Conclusions: CB_2 receptors and endocannabinoids participate in the tingenone-induced antinociception which may involve the inhibition of microglia at spinal level.

KEYWORDS: Tingenone; Antinociception; CB_2; Cannabinoid receptor; Endocannabinoids; Microglia

1. Introduction

Pain is generally defined as an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage'[1]. Natural products such as herbal medicines have been used as effective and safe alternatives for pain control[2]. Studies have demonstrated that herbal medicines produce fewer complications and fewer side effects when compared with conventional treatments. Among herbal medicines, pentacyclic triterpenoids have been reported for their analgesic and anti-inflammatory effects[3]. Pentacyclic triterpenoids such as tingenone (Tg), which is isolated from roots of *Maytenus imbricata* (*M. imbricata*), have been shown to produce antinociception in mice, in a process that involves opioidergic and nitrergic systems at peripheral level[4-6].

Cannabinoid receptors type 2 (CB_2) have also been shown to participate in the Tg-induced antinociception[7], while other studies have demonstrated the involvement of CB_2 receptors in peripheral antinociception[8,9]. Another study found that the CB_2 receptor agonist palmitoylethanolamide-an endocannabinoid-inhibited the formalin-induced nociception and mechanical hyperalgesia induced by intraplantar injection of prostaglandin E_2[10]. This effect was reversed by intraplantar pretreatment with CB_2 receptor antagonists[10-12]. Although studies have demonstrated that both Tg and CB_2 receptors are involved in antinociception, no study has investigated a possible antinociceptive effect of Tg at spinal level with participation of CB_2

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How to cite this article: Moura CCV, dos Santos RS, Duarte LP, Galdino G. Cannabinoid CB_2 receptors and spinal microglia are implicated in tingenone-mediated antinociception in mice. Asian Pac J Trop Biomed 2021; 11(4): 141-147.

Article history: Received 15 May 2020; Revision 21 July 2020; Accepted 22 September 2020; Available online 9 March 2021

receptors.

Studies have shown that glial cells are involved in the genesis of nociception, especially in chronic conditions[13,14]. This population of cells is composed of astrocytes essential for the formation and function of synapses by releasing molecular signals[15], and microglia, a specific population of macrophages found in the central nervous system (CNS), which is quiescent when inactivated but after some injuries becomes activated and hypertrophic[16]. Loggia *et al.*[17] described that this cell population is responsible for the release of pro-inflammatory proteins that are crucial in nociceptive impulse transmission[13,18,19], leading to the response of allodynia and hyperalgesia.

Given this context, the endocannabinoid system may be an important modulatory pathway of crosstalk between microglia-astrocytes-neurons. Endocannabinoids are released 'on demand' by neurons, as well as by astrocytes and microglia[20]. Thus, this study aimed to investigate the influence of CB_2 receptors on the spinal microglia during Tg-induced antinociception.

2. Materials and methods

2.1. Animals

This study used male Swiss mice (25–30 g) provided by the Central Vivarium of the Federal University of Alfenas, MG. All mice were housed in a climate-controlled room [(23 ± 2) ℃, (50 ± 10)% relative humidity] under a 12 h light/dark cycle.

2.2. Drugs

The following drugs were used: carrageenan (2%, intraplantar (i.pl.)]; 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4 methoxyphenyl) methanone [AM630, 4 μg, intrathecal (i.t.)], a CB_2 receptor antagonist; (5Z, 8Z, 11Z, 14Z)-5,8,11,14-eicosatetraenyl-methylester phosphonofluoridic acid (MAFP, 2 μg, i.t.), an irreversible fatty acid amide hydrolase (FAAH) inhibitor and minocycline hydrochloride (30 μg, i.t.), a microglial inhibitor. Carrageenan and minocycline were purchased from Sigma (MO, USA) and AM630 and MAFP were purchased from Tocris Bioscience (Ellisville, MO). Carrageenan and minocycline were dissolved in sterile saline, MAFP was dissolved in saline and ethanol (2%, Merck, NJ, USA), and AM630 was dissolved in sterile saline, DMSO (2%, Sigma, USA) and cremophor (5%, Sigma, USA).

2.3. Plant

Tg was isolated from roots of *M. imbricata* (Celastraceae) plant. The plant's roots were carefully collected, dried, and powdered in a mill. The powder (1.5 kg) was submitted to extractions in a Soxhlet extractor with the following organic solvents: hexane/ethyl ether (1:1), ethyl acetate, and methanol (2 L). Afterwards, the solvents were removed from the extracts in a rotator evaporator. During this phase, the following quantities of each filtrate were obtained: 16.1 g from the hexane/ethyl ether extract, 21.2 g from the ethyl acetate extract, and 176.7 g from the methanol extract. From the hexane/ethyl ether extract, 1.5 g of Tg was isolated and characterized following a previous study[21]. The hexane/ethyl ether extract (3.0 g) was subjected to silica gel (300.8 g) column chromatography and eluted with hexane-ethyl acetate[22]. Three hundred and fifty fractions of 100 mL each were obtained, and the fractions 66 to 85 produced an orange solid (314.0 mg), yielding 15.7% and 145.0–147.9 ℃ m.p., being identified as Tg.

2.4. Injections

2.4.1. I.pl. injection

For i.pl. injections, the animals were lightly anaesthetised with 2% isoflurane and O_2 (2 L/min) by an inhalation analgesia system using a calibrated vaporiser. The mice were positioned flat to allow an unobstructed view of the paws, and the right hind paws of the animals were cleaned with ethyl alcohol (70%) and carefully positioned for the subcutaneous injection of the test agents using a hypodermic syringe with a 24-gauge needle[23]. I.pl. injections of carrageenan were given 60 min after Tg administration.

2.4.2. I.t. injection

Before i.t. injections, the dorsal (lumbar) regions of the animals were shaved, and then they were lightly anaesthetised with 2% isoflurane and O_2 (2 L/min) *via* an inhalation analgesia system using a calibrated vaporiser and positioned to facilitate the palpation of the L5–L6 intervertebral spaces in the lumbar spine. The i.t. injections were performed in this intervertebral space using a 10-μL Hamilton syringe connected to a 28-gauge needle[24]. A flick of the tail was considered as an indicator of successful i.t. administration. I.t. injections of each drug were given 20 min before Tg administration.

2.4.3. Gavage administration

The administration of Tg was by oral gavage. The animals were gently restrained by the experimenter and maintained in an upright (vertical) position and the gavage needle (18 gauge) passed went through the roof of the mouth until reaching the oesophagus. The volume administered was equivalent to 1% of the weight of each animal. Tg was given 60 min before carrageenan injection.

2.5. Nociceptive test

To measure the mechanical nociceptive threshold (g), the von Frey filament test was used (Stoeling, Wood Dale, IL, USA). For this test, the mice were allocated in small boxes on a metal mesh floor, on an elevated wire mesh platform in individual glass compartments and acclimated for at least 30 min[25]. A mechanical stimulus was applied to the middle of the plantar surface using a series of von

Frey filaments with increasing bending forces (0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g) and the pressure value recorded at right paw withdrawal. Mean values of three consecutive tests applied at 3 min intervals were recorded.

A total of 102 mice were divided into the following groups (n=6): Cg + Veh, animals that received i.pl. injection of the carrageenan and oral pretreatment with Tg vehicle; Cg + Tg, animals that received i.pl. injection of the carrageenan and oral pretreatment with Tg at the 5.3, 15.9 and 53.0 mg/kg doses; and Veh + Veh, animals that received i.pl. injection of the carrageenan vehicle and oral pretreatment of Tg vehicle. In addition, to investigate the participation of the endocannabinoid system in the Tg-induced antinociception, similar groups were used to those previously described with the pre-administration of AM630 (a CB_2 receptor antagonist) and MAFP (a FAAH inhibitor). To investigate the involvement of microglia in the Tg-induced antinociception, similar groups were used with intrathecal pre-administration of minocycline. The doses of Tg used in this study were chosen following a previous study[4].

2.6. Immunoreactivity of cannabinoid CB_2 receptor and microglia

To evaluate the expression and co-localisation of CB_2 receptors and microglia in the spinal cord, double stain immunofluorescence was performed in sections of the spinal cord (L4–L6 segments). For that, the animals were euthanised and transcardially perfused with 0.01 M phosphate buffer solution, pH 7.4, followed by paraformaldehyde (4%) and 0.1 M phosphate buffer solution (pH 7.4). After that, the samples were fixed and stored in sucrose for cryoprotection. Afterwards, spinal cord sections (20 μm) were washed (0.1 M PBS) and incubated in 0.5% triton X glycine (0.1 M), then washed and incubated again for 1 h in 3% bovine serum albumin (Sigma-Aldrich). The sections were incubated overnight at 4 ℃ in anti-CB_2 receptor antibody (1:100; Santa Cruz, USA) and anti-CD11B (1:500; Millipore, USA). After incubation in the primary antibody, sections of tissue were washed and incubated in a 488 anti-rabbit goat IgG (1:200, Santa Cruz, USA) and Alexa Fluor 594 anti-rat goat IgG (1:400; ThermoFisher, USA) for 2 h. After that, the sections were assembled using fluoromount and analysed by epifluorescence acquired using a confocal microscope (K2, Nikon, Japan). Sections were visually chosen using a 10× objective. Images were analysed using ImageJ software (NIH, Bethesda, USA).

2.7. Experimental protocol

2.7.1. Protocol 1

To evaluate the antinociceptive effect of Tg, the animals orally received three doses of Tg (5.3, 15.9, and 53.0 mg/kg), 1 h before carrageenan injection (Supplementary Figure 1). The mechanical nociceptive threshold was measured before (baseline) and at 1, 3, 4, 5, 6, and 7 h after carrageenan injection (Supplementary Figure 1).

2.7.2. Protocol 2

To investigate the involvement of the endocannabinoid system in this Tg-induced antinociception, the baseline latency of nociceptive threshold was firstly measured and AM630 (4 μg/5 μL) and MAFP (2 μg/5 μL) were i.t. injected 20 min before administration of Tg (Supplementary Figure 1). Then, measurements of nociceptive mechanical threshold were performed 20, 60, 120, 180, 240, 300, 360, 420, 480 and 1440 min after carrageenan injection (Supplementary Figure 1).

2.7.3. Protocol 3

To investigate microglial involvement in Tg-induced antinociception, minocycline (30 μg/5 μL i.t.) was administered 20 min before Tg (Supplementary Figure 1). The nociceptive mechanical threshold was evaluated 20, 60, 180, 240, 300, 360, 420, 480, 540, and 1440 min after carrageenan injection (Supplementary Figure 1).

The doses of AM630, MAFP and minocycline used in this study were according to previous study conducted by Dos Santos *et al*.[25].

2.8. Statistical analysis

The data were presented as mean ± SEM of the evaluated parameter and were analysed for statistical significance as follows: two-way ANOVA for repeated measurements, followed by Bonferroni's *post hoc* test for multiple comparisons of nociceptive threshold measurements; and by one-way ANOVA followed by Bonferroni's *post hoc* test for analysis of immunofluorescence results. Significance was considered when $P < 0.05$. Statistical analyses and preparation of figures were done using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA).

2.9. Ethical statement

This study was previously approved by the Animal Care and Use Committee at the Federal University of Alfenas (protocol number: 683/2015). Experiments were carried out following the International Association for the Study of Pain guidelines for animal studies[26].

3. Results

3.1. Effect of Tg on inflammatory pain induced by carrageenan

Figure 1 shows that i.pl. administration of carrageenan induced significant mechanical hyperalgesia ($P < 0.001$, $F_{3,24} = 17.4$), which lasted from 1 to 7 h. This effect was reversed ($P < 0.001$, $F_{3,24} = 17.4$) by Tg at 5.3, 15.9 and 53 mg/kg doses, characterising an antinociceptive effect. Tg vehicle did not alter the nociceptive threshold.

3.2. Spinal participation of CB_2 receptor in Tg-induced antinociception

After we found the antinociceptive effect induced by Tg, the next step was to investigate the involvement of the CB_2 receptor in this effect at spinal level. The result showed that Tg-induced antinociception was significantly reversed ($P < 0.001$, $F_{3,24} = 32.89$) by the CB_2 receptor antagonist AM630 at the 4 μg dose (Figure 2), suggesting the participation of this receptor in this effect. The vehicle did not alter the nociceptive threshold.

3.3. Involvement of the endocannabinoids in the Tg-induced antinociception

In addition to participation of the CB_2 receptor, we investigated the involvement of the endocannabinoid in the antinociceptive effect induced by Tg. The pretreatment with MAFP (2 μg, i.t.), an inhibitor of the FAAH enzyme, potentiated and prolonged the antinociception induced by Tg ($P < 0.001$, $F_{3,24} = 48.42$; Figure 3). The vehicle did not alter the nociceptive threshold.

3.4. Spinal involvement of microglia in the antinociception induced by Tg

As the CB_2 receptor is present in microglia, we assume that it may interfere in the pain process by inhibiting these cells. Thus, to demonstrate the involvement of spinal microglia in the genesis of inflammatory pain, minocycline was i.t. administered-a selective microglial inhibitor drug.

We thus verified that minocycline prolonged and potentiated the antinociceptive effect of Tg ($P < 0.001$, $F_{3,4} = 60.62$) after 420 min in the inflammatory pain group (Figure 4).

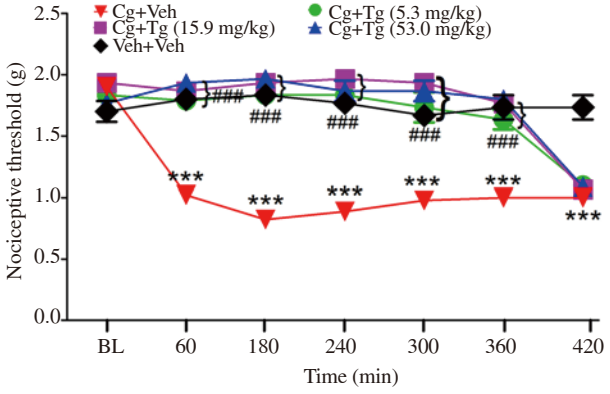

Figure 1. Effect of tingenone on hyperalgesia induced by carrageenan. Data are expressed as mean ± SEM of the nociceptive threshold of animals per group. $^{***}P < 0.001$ versus the control group (Veh+Veh), which received only vehicles; $^{###}P < 0.001$ between the group that received carrageenan and groups that received carrageenan plus tingenone (Cg+Tg). Two-way ANOVA followed by Bonferroni's multiple comparison test. BL: Baseline latency, Tg: tingenone; Cg: carrageenan; Veh: vehicle.

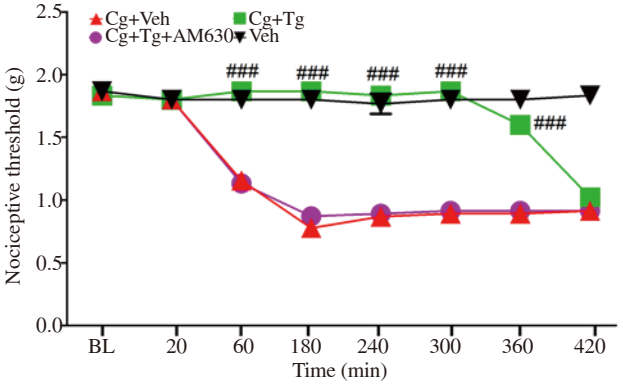

Figure 2. Participation of the cannabinoid CB_2 receptor in the tingenone-induced antinociception. Data are expressed as mean ± SEM of the nociceptive threshold of six animals per group. $^{###}P < 0.001$, versus the carrageenan plus tingenone group (Cg+Tg). Two-way ANOVA followed by Bonferroni's multiple comparison test.

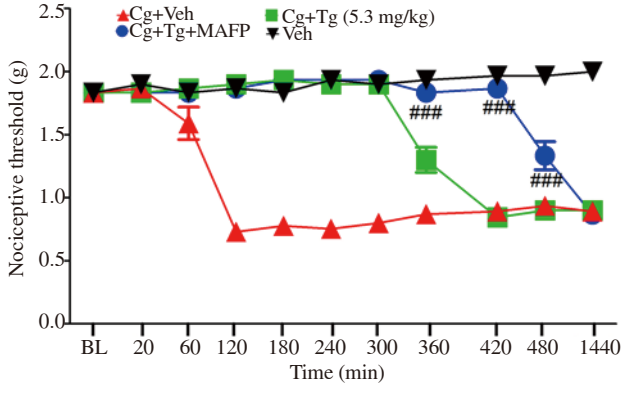

Figure 3. Involvement of endocannabinoids in the tingenone-induced antinociception. Data are expressed as mean ± SEM of the nociceptive threshold of six animals per group. $^{###}P < 0.001$, versus the carrageenan plus tingenone group (Cg+Tg). Two-way ANOVA followed by Bonferroni's multiple comparison test.

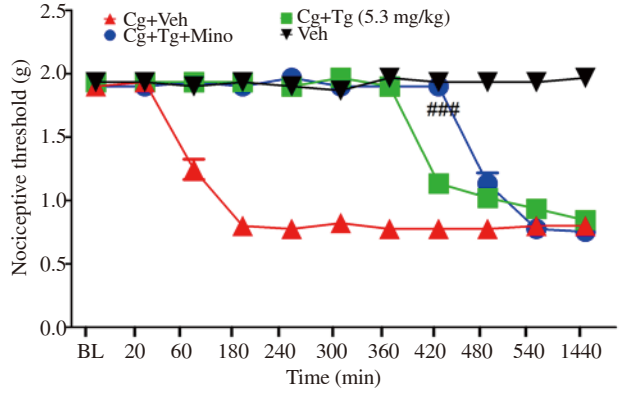

Figure 4. Participation of the spinal microglia in the antinociceptive effect induced by tingenone. Data are expressed as mean ± SEM of the nociceptive threshold of six animals per group. $^{###}P < 0.001$, versus the carrageenan plus tingenone group (Cg+Tg). Two-way ANOVA followed by Bonferroni's multiple comparison test. Mino: minocycline.

3.5. Effect of Tg on the CB_2 receptor and microglia immunoreactivity in spinal cord

After we demonstrated that the spinal CB_2 receptor participates in the Tg-induced antinociception, and that spinal microglia are involved in inflammatory pain, we evaluated the effect of Tg on the expression of this receptor and microglial cells in the spinal cord. Figure 5 shows that the group received carrageenan and Tg induced a significant increase ($P < 0.001$) of the microglia and CB_2 receptor expression in the spinal cord. Furthermore, the increased spinal microglia expression was reduced ($P < 0.001$) in mice that received carrageenan and pretreatment with minocycline, MAFP, and AM630. Moreover, the CB_2 expression was inhibited in mice pre-treated with AM630 (Figure 5).

4. Discussion

This study demonstrated that Tg inhibits inflammatory pain and that this effect involves the participation of the spinal CB_2 receptor. This study also verified that spinal microglia may participate in the hyperalgesia induced by carrageenan, suggesting that the inhibition of these cells potentiated the Tg-induced antinociception.

Several studies have demonstrated that CB_2 receptor activation inhibits pain at spinal level[25,27,28]; however, none have investigated this effect induced by herbal medicines such as Tg. A previous study showed that Tg promoted antinociception *via* CB_2 receptor activation, but this effect was evaluated peripherally[7]. Type 2 cannabinoid receptors are widely found in immune cells[29], although studies have described these receptors in the CNS[30]. Once activated by endocannabinoids, the firing rate of excitable cells is reduced, leading to the inhibition of neurotransmitter release and reduction in the nociceptive response[31]. Other authors reported an association between increased CB_2 receptor expression and reduced spinal microglia activation in rodents with inflammatory and neuropathic pain[32,33]. According to these findings, we suggest that Tg may activate the CB_2 receptors on the spinal microglia, facilitating the inhibition mechanism of hyperalgesia.

This activation is likely to occur directly at the receptors, or indirectly by increasing levels of endocannabinoids. We found that MAFF-a FAAH inhibitor-is an enzyme that hydrolyses

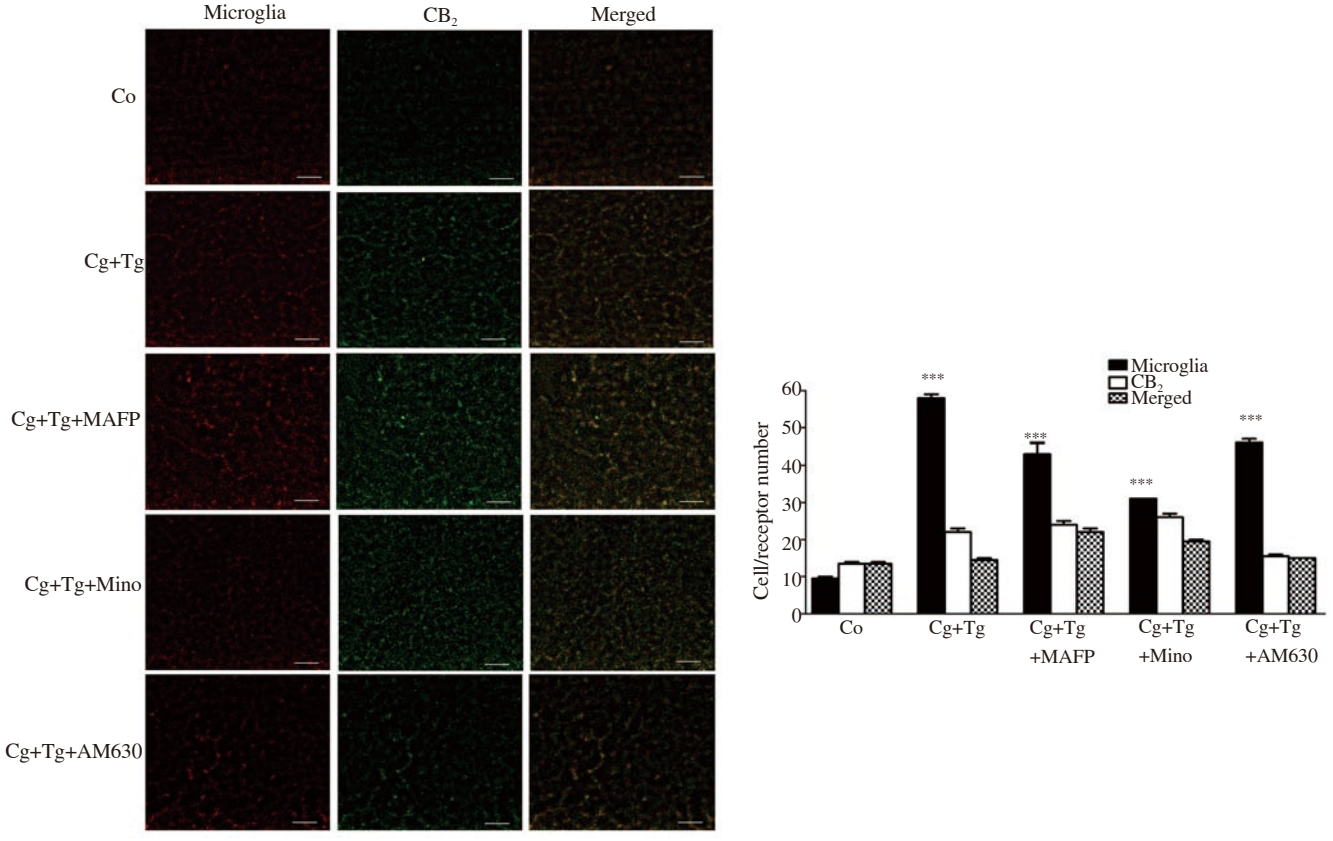

Figure 5. Microglia and cannabinoid CB_2 receptor immunoreactivity in mice with inflammatory pain pretreated with tingenone. Representative immunofluorescence staining of microglia (red) and cannabinoid CB_2 receptor (green) in the spinal cord dorsal horn. Scale bars, 20 μm. Data are expressed as mean ± SEM of 4-5 animals per group. $^{***}P < 0.001$, versus the control group (Co). One-way ANOVA followed by the Bonferroni test.

endocannabinoids anandamide and that 2-AG, potentiated and prolonged the Tg-induced antinociception after 360 to 480 min of its administration. In a previous study conducted by Veloso *et al*.[7], it was verified that MAFP injected peripherally did not alter the antinociceptive effect of Tg. Thus, the effect produced by MAFP and Tg in the present study may be restricted to the spinal level and involve the release and increase of endocannabinoids. Furthermore, i.t. injection of MAFP also potentiated the antinociception induced by exercise in mice with muscle inflammatory pain[25], reinforcing the hypothesis that this inhibitor increased the supply of endocannabinoids during Tg-induced antinociception.

Moreover, the present study suggests that all these events occur together with spinal microglia involvement. Microglia are macrophage-like cells in the CNS that regulate homeostasis in the brain and spinal cord[34]. After tissue or nerve injury, synaptic plasticity in the brain and spinal cord increases, an event known as central sensitisation; microglia are the main cells involved in this response[34]. Once activated, a signaling cascade occurs in the microglia, which results in the release of pronociceptive molecules responsible for sensitising neurons in the dorsal horn of the spinal cord and facilitating the transmission of the nociceptive impulse[34]. Thus, we suggest that Tg may activate CB_2 receptors on the spinal microglia, reducing the nociceptive impulse.

Studies also found an association between the increase in CB_2 receptor expression and the reduction of spinal microglia activation in animals with paw incision and peripheral nerve injury[28,32,33,35,36]. This hypothesis was supported by different studies that used different CB_2 receptor agonists (HU-210, WIN55, 212–2, and JWH-133) and found that nociceptive molecules, such as tumour necrosis factor and nitric oxide were reduced, as well as increases in anti-inflammatory cytokines caused by microglia[32,33,37–39]. In this study, MAFP, AM630, and minocycline reduced the spinal microglia immunoreactivity in mice that received i.pl. injection of carrageenan, supporting the hypothesis that these cells are involved in the inflammatory pain model used and that the endocannabinoid system may inhibit these cells.

The main limitations of the present study were that it is not yet possible to carry out the antinociceptive effect in humans, mainly due to ethical issues for conducting controlled trials, and in addition, Tg is not commercially available.

Thus, the present study suggests that spinal microglia participate in the inflammatory pain induced by carrageenan and that Tg may inhibit this process by activation of the endocannabinoid system. These findings may be important for future studies that investigate the effect of herbal medicines in the control of pain.

Conflict of interest statement

We declare that there is no conflict of interest.

Funding

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (Finance Code 001).

Authors' contributions

CCVM developed theoretical formalism, contributed to the original, revision, and final version of the manuscript, as well as supervised the project. GG performed the analytic calculation and formal analysis, and contributed to the original, revision, and final version of the manuscript. RSS contributed to the original version of the manuscript, as well as performed the analytic calculation and formal analysis. LPD ceded tingenone for pharmacological studies.

References

[1] Cohen M, Quintner J, van Rysewyk S. Reconsidering the international association for the study of pain definition of pain. *Pain Rep* 2018; **3**(2): e634.

[2] Arruda APN, Ayala AP, Lopes LC, Bergamaschi CC, Guimarães C, Del Grossi M, et al. Herbal medications for surgical patients: A systematic review protocol. *BMJ Open* 2017; **7**(7): e014290.

[3] Fukumitsu S, Villareal MO, Fujitsuka T, Aida K, Isoda H. Anti-inflammatory and anti-arthritic effects of pentacyclic triterpenoids maslinic acid through NF-κB inactivation. *Mol Nutr Food Res* 2016; **60**(2): 399-409.

[4] Veloso CC, Rodrigues VG, Azevedo AO, Oliveira CO, Gomides LF, Duarte LP, et al. Duarte, Antinociceptive effects of *Maytenus imbricata* Mart. ex. Reissek (Celastraceae) root extract and its tingenone constituent. *J Med Plants Res* 2014; **8**: 68-76.

[5] Veloso CC, Rodrigues VG, Ferreira RCM, Duarte LP, Klein A, Duarte ID, et al. Tingenone, a pentacyclic triterpene, induces peripheral antinociception due to opioidergic activation. *Plant Med* 2014; **80**: 1615-1621.

[6] Veloso CC, Rodrigues VG, Ferreira RCM, Duarte LP, Klein A, Duarte ID, et al. Tingenone, a pentacyclic triterpene, induces peripheral antinociception due to NO/cGMP and ATP-sensitive K(+) channels pathway activation in mice. *Euro J Pharmacol* 2015; **755**: 1-5.

[7] Veloso CC, Ferreira RCM, Rodrigues VG, Duarte LP, Klein A, Duarte ID, et al. Tingenone, a pentacyclic triterpene, induces peripheral antinociception due to cannabinoid receptors activation in mice. *Inflammopharmacology* 2018; **26**(1): 227-233.

[8] Hossain MZ, Ando H, Unno S, Kitagawa J. Targeting peripherally restricted cannabinoid receptor 1, cannabinoid receptor 2, and endocannabinoid-degrading enzymes for the treatment of neuropathic pain including neuropathic orofacial pain. *Int J Mol Sci* 2020; **21**(4): 1423.

[9] Yuill MB, Hale DE, Guindon J, Morgan DJ. Anti-nociceptive interactions between opioids and a cannabinoid receptor 2 agonist in inflammatory

pain. *Mol Pain* 2017; **13**: 1744806917728227.

[10]Starowicz K, Finn DP. Cannabinoids and pain: Sites and mechanisms of action. *Adv Pharmacol* 2017; **80**: 437-475.

[11]Romero TRL, Galdino GS, Silva GC, Resende LC, Perez AC, Cortes SF, et al. Involvement of the *L*-arginine/nitric oxide/cyclic guanosine monophosphate pathway in peripheral antinociception induced by *N*-palmitoyl-ethanolamine in rats. *J Neurosci Res* 2012; **90**: 1474-1479.

[12]Romero TRL, Duarte ID. *N*-Palmitoyl-ethanolamine (PEA) induces peripheral antinociceptive effect by ATP-sensitive K^+-channel activation. *J Pharmacol Sci* 2012; **118**: 156-160.

[13]Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. *Science* 2016; **354**(6312): 572-577.

[14]Inoue K, Tsuda M. Microglia in neuropathic pain: Cellular and molecular mechanisms and therapeutic potential. *Nat Rev Neurosci* 2018; **19**(3): 138-152.

[15]Chung WS, Allen NJ, Eroglu C. Astrocytes control synapse formation, function, and elimination. *Cold Spring Harb Perspect Biol* 2015; **7**(9): a020370.

[16]Fumagalli M, Lombardi M, Gressens P, Verderio C. How to reprogram microglia toward beneficial functions. *Glia* 2018; **66**(12): 2531-2549.

[17]Loggia ML, Chonde DB, Akeju O, Arabasz G, Catana C, Edwards RR, et al. Evidence for brain glial activation in chronic pain patients. *Brain* 2015; **138**(3): 604-615.

[18]Vanderwall AG, Milligan ED. Cytokines in pain: Harnessing endogenous anti-inflammatory signaling for improved pain management. *Front Immunol* 2019; **10**: 3009.

[19]Zhang P, Bi RY, Gan YH. Glial interleukin-1β upregulates neuronal sodium channel 1.7 in trigeminal ganglion contributing to temporomandibular joint inflammatory hypernociception in rats. *J Neuroinflammation* 2018; **15**(1): 117.

[20]Zou S, Kumar U. Cannabinoid receptors and the endocannabinoid system: Signaling and function in the central nervous system. *Int J Mol Sci* 2018; **19**(3): 833.

[21]Rodrigues VG, Duarte LP, Silva GDF, Silva FC, Góes JF, Takahashi JA, et al. Evaluation of antimicrobial activity and toxic potential of extracts and triterpenes isolated from *Maytenus imbricata*. *Quím Nova* 2012; **35**(7): 1375-1380.

[22]Wagner H, Bladt S. *Plant drug analysis. A thin layer chromatography atlas.* 2nd ed. Springer; 2001.

[23]Romero TLR, Castor MGM, Parrella C, Piscitelli F, Di Marzo V, Duarte IDG. α2-Adrenoceptor agonist induces peripheral antinociception *via* the endocannabinoid system. *Pharmacol Rep* 2020; **72**: 96-103.

[24]Wang HC, Cheng KI, Chen PR, Tseng KY, Kwan AL, Chang LL. Glycine receptors expression in rat spinal cord and dorsal root ganglion in prostaglandin E_2 intrathecal injection models. *BMC Neurosci* 2018; **19**(1): 72.

[25]Dos Santos RS, Sorgi CA, Peti APF, Veras FP, Faccioli LH, Galdino G. Involvement of spinal cannabinoid CB_2 receptors in exercise-induced antinociception. *Neuroscience* 2019; **418**: 177-188.

[26]Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; **16**: 109-110.

[27]Ikeda H, Ikegami M, Kai M, Ohsawa M, Kamei J. Activation of spinal cannabinoid CB_2 receptors inhibits neuropathic pain in streptozotocin-induced diabetic mice. *Neuroscience* 2013; **1250**: 446-454.

[28]Niu J, Huang D, Zhou R, Yue M, Xu T, Yang J, et al. Activation of dorsal horn cannabinoid CB_2 receptor suppresses the expression of P2Y12 and P2Y13 receptors in neuropathic pain rats. *J Neuroinflammation* 2017; **14**(1): 185.

[29]Liu YJ, Fan HB, Jin Y, Ren CG, Jia XE, Wang L, et al. Cannabinoid receptor 2 suppresses leukocyte inflammatory migration by modulating the JNK/c-Jun/Alox5 pathway. *J Biol Chem* 2013; **288**(19): 13551-13562.

[30]Onaivi ES, Ishiguro H, Gu S, Liu QR. CNS effects of CB_2 cannabinoid receptors: Beyond neuro-immuno-cannabinoid activity. *J Psychopharmacol* 2012; **26**: 92-103.

[31]Barrie N, Manolios N. The endocannabinoid system in pain and inflammation: Its relevance to rheumatic disease. *Eur J Rheumatol* 2017; **4**(3): 210-218.

[32]Romero-Sandoval A, Eisenach JC. Spinal cannabinoid receptor type 2 activation reduces hypersensitivity and spinal cord glial activation after paw incision. *Anesthesiology* 2007; **106**: 787-794.

[33]Romero-Sandoval MD, Chai N, Nutile-McMenemy N, Deleo JA. A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res* 2008; **1219**: 116-126.

[34]Chen G, Zhang YQ, Qadri YJ, Serhan CN, Ji RR. Microglia in pain: Detrimental and protective roles in pathogenesis and resolution of pain. *Neuron* 2018; **100**: 1292-1311.

[35]Mao Y, Huang Y, Zhang Y, Wang C, Wu H, Tian H, et al. Cannabinoid receptor 2-selective agonist JWH015 attenuates bone cancer pain through the amelioration of impaired autophagy flux induced by inflammatory mediators in the spinal cord. *Mol Med Rep* 2019; **20**(6): 5100-5110.

[36]Gu X, Mei F, Liu Y, Zhang R, Zhang J, Ma Z. Intrathecal administration of the cannabinoid 2 receptor agonist JWH015 can attenuate cancer pain and decrease mRNA expression of the 2B subunit of *N*-methyl-*D*-aspartic acid. *Anesth Analg* 2011; **113**(2): 405-411.

[37]Ehrhart J, Obregon D, Mori T, Hou H, Sun N, Bai Y, et al. Stimulation of cannabinoid receptor 2 (CB_2) suppresses microglial activation. *J Neuroinflammation* 2005; **2**: 29.

[38]Tanaka M, Sackett S, Zhang Y. Endocannabinoid modulation of microglial phenotypes in neuropathology. *Front Neurol* 2020; **11**: 87.

[39]Jean-Gilles L, Braitch M, Latif ML, Aram J, Fahey AJ, Edwards LJ, et al. Effects of pro-inflammatory cytokines on cannabinoid CB_1 and CB_2 receptors in immune cells. *Acta Physiol* 2015; **214**(1): 63-74.

Cannabinoid CB_2 receptors and spinal microglia are implicated in tingenonemediated antinociception in mice

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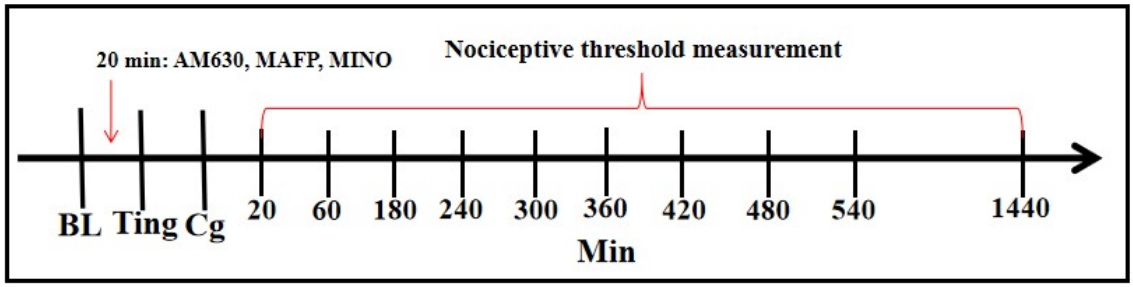

Suppl Figure 1: Experimental protocol to nociceptive threshold measurements. BL: baseline latency; Tg: tingenone; Cg: carrageenan.