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Antinociceptive effects induced through the stimulation of spinal cannabinoid type 2 receptors in chronically inflamed mice

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ABSTRACT

The stimulation of spinal cannabinoid type 2 (CB_2) receptors is a suitable strategy for the alleviation of experimental pain symptoms. Several reports have described the up-regulation of spinal cannabinoid CB_2 receptors in neuropathic settings together with the analgesic effects derived from their activation. Besides, we have recently reported in two murine bone cancer models that the intrathecal administration of cannabinoid CB_2 receptor agonists completely abolishes hyperalgesia and allodynia, whereas spinal cannabinoid CB_2 receptor expression remains unaltered. The present experiments were designed to measure the expression of spinal cannabinoid CB_2 receptors as well as the analgesic efficacy derived from their stimulation in mice chronically inflamed by the intraplantar injection of complete Freund's adjuvant 1 week before. Both spinal cannabinoid CB_2 receptors mRNA measured by real-time PCR and cannabinoid CB_2 receptor protein levels measured by western blot remained unaltered in inflamed mice. Besides, the intrathecal (i.t.) administration of the cannabinoid CB_2 receptor agonists AM1241, (R,S)-3-(2-Iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole, (0.03-1 μg) and JWH 133, (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran, (3–30 μg) dose-dependently blocked inflammatory thermal hyperalgesia and mechanical allodynia. The analgesic effects induced by both agonists were counteracted by the coadministration of the selective cannabinoid CB_2 receptor antagonist SR144528, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl) methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide, (5 μg) but not by the cannabinoid CB_1 receptor antagonist AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, (10 μg). The effects induced by AM1241 were also inhibited by the coadministration of the opioid receptor antagonist, naloxone (1 μg). These results demonstrate that effective analgesia can be achieved in chronic inflammatory settings through the stimulation of spinal cannabinoid CB_2 receptors even if this receptor population is not up-regulated.

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1. Introduction

Cannabinoids can exert analgesic effects through the stimulation of cannabinoid CB_1 or CB_2 receptors. In particular, cannabinoid CB_2 receptor agonists could be promising analgesics since their administration does not seem to induce psychotomimetic effects (Malan et al., 2001; Valenzano et al., 2005) or tolerance (Leichsenring et al., 2009; Lozano-Ondoua et al., 2010; Romero-Sandoval et al., 2008; Yao et al., 2009).

Cannabinoid CB_2 receptors are mostly expressed at periphery in immune cells (Munro et al., 1993) and nociceptor endings (Anand et al., 2008) and their stimulation alleviates hypernociceptive symptoms in different experimental pain models (Gutierrez et al., 2007; Potenzieri et al., 2008; Zhu et al., 2009). Moreover, the expression of cannabinoid CB_2 receptors at CNS structures related to nociceptive processing, as the spinal cord, has been further described (Alkaitis et al., 2010; Gong et al., 2006; Jhaveri et al., 2008; Morgan et al., 2009; Romero-Sandoval et al., 2008; Van Sickle et al., 2005; Zhang et al., 2003).

In neuropathic settings, both the up-regulation of spinal cannabinoid CB_2 receptor (Romero-Sandoval et al., 2008; Walczak et al., 2005; Wotherspoon et al., 2005; Zhang et al., 2003) and the efficacy of spinally-administered cannabinoid CB_2 receptor agonists are well established (Hsieh et al., 2011; Romero-Sandoval et al., 2008; Yamamoto et al., 2008).

In contrast, the analgesic effect induced by the spinal administration of cannabinoid CB_2 receptor agonists in inflammatory models has been more scarcely explored and seems more limited. Only a weak analgesic effect has been found in rats 2 days after the intraplantar administration of complete Freund's adjuvant (CFA) (Hsieh et al.,

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2011) whereas no analgesic effect was found in the formalin test, a setting involving an inflammatory component (Yoon and Choi, 2003). Besides, it has been shown that spinal cannabinoid CB_2 receptors are not up-regulated in several inflammatory processes such as CFA-induced paw inflammation (Hsieh et al., 2011; Zhang et al., 2003), CFA-induced arthritis (Cox et al., 2007) or acrolein-induced bladder inflammation (Merriam et al., 2008) in rats.

Thus, it might be conceived that the diminished analgesic responses to cannabinoid CB_2 receptor agonists in inflammatory settings could be related to the absence of spinal cannabinoid CB_2 receptor up-regulation. Contrasting with this view, effective analgesia can be achieved through spinal cannabinoid CB_2 receptor stimulation in experimental neoplastic processes (Cui et al., 2011; Curto-Reyes et al., 2010) regardless of changes in cannabinoid CB_2 receptor mRNA or protein expression at the spinal cord (Curto-Reyes et al., 2010; Hald et al., 2008). This fact prompted us to design experiments to study whether it could also be the case in chronic inflammation where, as shown above, studies dealing the analgesic effects driven through spinal cannabinoid CB_2 receptor stimulation are rather scarce. Therefore, in mice receiving intraplantar CFA 1 week before, we have measured the spinal cannabinoid CB_2 receptor mRNA and protein expression and assessed the antihyperalgesic and antiallodynic effects evoked by the spinal administration of two structurally unrelated cannabinoid CB_2 receptor agonists, AM1241 and JWH 133.

2. Materials and methods

2.1. Animals

Experiments were performed in 26–33 g weight Swiss male mice bred in the Animalario of the Universidad de Oviedo (Reg. 33044 13A), maintained on a 12 h dark–light cycle with free access to food and water. All the experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain).

2.2. Drugs

(R,S)-AM1241 (Sigma) was dissolved in 2% Cremophor (Sigma), 10% ethanol and distilled water. JWH 133 (Tocris) was diluted in TocrisolveTM100 (Tocris) and distilled water. SR144528 (kindly donated by Sanofi-Aventis, France) was diluted in 2% dimethyl sufoxide (DMSO), 6% ethanol and distilled water. AM251 (Tocris) was solved in 10% DMSO (Sigma) and distilled water. Naloxone hydrochloride (Sigma) was dissolved in saline. In all cases, control animals received the corresponding solvent.

Intrathecal injections were performed as previously shown (Curto-Reyes et al., 2010) by introducing the tip of a 26 gauge needle inserted in a Hamilton syringe at the level of L5–L6 and injecting a volume of 5 μl. In no case mice exhibited signs of neurological or motor alteration after intrathecal injections.

2.3. Induction of inflammation

Inflammation was induced by the intraplantar (i.pl.) administration of 30 μl of complete Freund's adjuvant (CFA; Sigma) into the right hindpaw 1 week before testing. Control mice received the same volume of saline intraplantarly 1 week before testing into the right paw.

2.4. Analysis of cannabinoid CB_2 mRNA expression by real-time PCR

Seven days after CFA intraplantar injection, mice were exposed to a CO_2 atmosphere and decapitated. The vertebral column was sectioned at thoracic and sacral levels and lumbar spinal cord was extracted by flushing about 3–5 ml of ice-cold saline through the spinal cavity with a syringe. L2–L6 lumbar spinal segments were selected, frozen in liquid nitrogen and conserved at −80 °C.

Total mRNA was extracted from frozen lumbar spinal cord samples using TRI Reagent® (Sigma) following the manufacturer's protocol. Samples were diluted in DEPC-treated water up to 1 μg/μl and RNA purity was assessed by 260/280 ratio measurement using a UV spectrophotometer (GeneQuant Pro, Amersham Biosciences).

First strand cDNA was synthesized with random primers from the whole amount of the isolated RNA using a commercial kit for reverse transcription (Qiagen Iberia). Next, real-time PCR was performed in each sample by using specific primers for the mouse gene of the cannabinoid CB_2 receptor and for the mouse housekeeping gene β-actin, used as control to normalize RNA input. Reactions were conducted on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Duplicates of four independent samples were analyzed in each group. Data were analyzed by the 2-ΔΔCt method (Livak and Schmittgen, 2001) using the expression level of the cannabinoid CB_2 receptor gene in non-inflamed mice as control condition. Primer pairs for mouse cannabinoid CB_2 receptor and β-actin were as follows: cannabinoid CB_2 receptor (Ensembl accession number ENSMUST00000097843; NCBI accession number NM_009924): forward 5′ ATGCAGCTCTTGGGACCTAC 3′ and reverse 5′ ACTGGAGCTGTCCCAGAAGA 3′. β-actin (NCBI accession number NM_007393.3): forward 5′ GCAGCTCCTTCGTTGCCGGT 3′ and reverse 5′ TACAGCCCGGGGAGCATCGT 3′.

2.5. Western blot assays

Lumbar cord segments, either complete or longitudinally hemisected, were harvested as described in the previous section and homogenized in ice-cold buffer containing 60 mM Tris–HCl (pH 7.4), 10% glycerol, 80 mM sodium dodecyl sulfate (SDS) and protease inhibitors (1 tablet/50 ml buffer, Roche Diagnostics) in a volume of 6 μl/mg of tissue and then centrifuged (120 g, 10 min, 4 °C). The supernatant obtained was centrifuged again (26,000 g, 20 min, 4 °C), collected and conserved at −80 °C until its use.

Protein concentrations were determined by a BCA protein assay (Pierce) according to the manufacturer's protocol. As previously described (Curto-Reyes et al., 2010), the volume of homogenate corresponding to 50 μg of protein was vigorously mixed with the volume of sample buffer (200 mM Tris–HCl at pH 6.8, 0.02% bromophenol blue, 8% mercaptoethanol, 40% glycerol, 8% SDS) necessary to obtain 30 μl, placed in an eppendorf tube and heated at 100 °C for 5 min. After this, samples were run on a 10% SDS-PAGE gel at 90 V during 90 min. Samples were then transferred onto a nitrocellulose membrane (Bio-Rad) at 4 °C during 90 min using 100 V. The membrane was blocked in Tris buffered saline-Tween (TBST, Tris 10 mM, NaCl 150 mM (pH = 7.6), Tween 20 0.1%) with 5% non-fat milk for 90 min at RT, washed with TBST and incubated overnight at 4 °C with goat polyclonal anti-CB_2 receptor (1:200 Santa Cruz Biotechnology). After incubation, the membrane was washed with TBST and incubated with the secondary antibody (donkey anti-goat IgG-HRP, 1:20000 Santa Cruz Biotechnology) dissolved in TBST containing 0.1% non-fat milk for 90 min. After final washes, labeled cannabinoid CB_2 receptor protein was detected at 45 kDa by enhanced chemiluminescence detection autoradiography using Immobilon™ Western chemiluminiscent HRP substrate kit (Millipore) according to the manufacturer's protocol. Immune reaction intensity was determined by computer-assisted densitometry (ImageJ, NIH) on exposed LS film (Kodak X-Omat).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed protein of 35 kDa, was also measured by western blotting using a polyclonal rabbit anti-mouse GAPDH antibody (1:30000 Sigma).

Six independent samples of complete lumbar spinal cords were analyzed in each group of inflamed or non-inflamed mice. Three independent samples of hemisected spinal cords, corresponding to

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