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Research paper

Mechanisms of action of cannabidiol in adoptively transferred experimental autoimmune encephalomyelitis

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ABSTRACT

Cannabidiol (CBD) is one of the most important compounds in *Cannabis sativa*, lacks psychotropic effects, and possesses a high number of therapeutic properties including the amelioration of experimental autoimmune encephalomyelitis (EAE). The aim of this study was to analyse the relative efficacy of CBD in adoptively transferred EAE (at-EAE), a model that allows better delineation of the effector phase of EAE. Splenocytes and lymph nodes from mice with actively induced EAE were cultured in the presence of $MOG_{35–55}$ and IL-12 and inoculated intraperitoneally in recipient female C57BL/6J mice. The effects of CBD were evaluated using clinical scores and magnetic resonance imaging (MRI). In the central nervous system, the extent of cell infiltration, axonal damage, demyelination, microglial activation and cannabinoid receptors expression was assessed by immunohistochemistry. Lymph cell viability, apoptosis, oxidative stress and IL-6 production were measured *in vitro*. Preventive intraperitoneal treatment with CBD ameliorated the clinical signs of at-EAE, and this improvement was accompanied by a reduction of the apparent diffusion coefficient in the subiculum area of the brain. Inflammatory infiltration, axonal damage, and demyelination were reduced, and cannabinoid receptor expression was modulated. Incubation with CBD decreased encephalitogenic cell viability, increasing early apoptosis and reactive oxygen species (ROS) and decreasing IL-6 production. The reduction in viability was not mediated by CB_1, CB_2 or GPR55 receptors. CBD markedly improved the clinical signs of at-EAE and reduced infiltration, demyelination and axonal damage. The CBD-mediated decrease in the viability of encephalitogenic cells involves ROS generation, apoptosis and a decrease in IL-6 production and may contribute to the therapeutic effect of this compound.

1. Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) and is characterized by the infiltration of inflammatory leukocytes into the CNS, followed by demyelination and axonal loss (Seehusen and Baumgärtner, 2010; Sospedra and Martin, 2005). This results in a variety of neurological deficits that frequently lead to progressive disability (Prat and Antel, 2005). The pathogenesis of MS is not fully understood, and despite the existence of approved medications, there remain a significant number of unmet therapeutic needs.

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model of MS and constitutes a versatile system in translational neuroimmunopharmacology. Active EAE is induced with self-antigens or CNS homogenates emulsified in adjuvant (Wekerle et al., 1994; Mendel et al., 1995; Amor et al., 1996). In addition, EAE can be induced by adoptive transfer (at-EAE) of lymphoid encephalitogenic cells specific for CNS antigens (Zamvil et al., 1986;

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Kuchroo et al., 1992).

Compared with the classic active EAE, at-EAE is a more useful model to analyse the effector phase of the disease. Moreover, the encephalitogenic cells can also be manipulated *in vitro* to study the roles of specific biological agents before the cells are transferred to recipients.

The endocannabinoid system consists of the CB_1 and CB_2 receptors, a number of endocannabinoid ligands and their degradation molecules. However, endocannabinoids and phytocannabinoids can stimulate other receptors such as the transient receptor potential vanilloid 1 (TRPV1) ion channel and the orphan G protein-coupled receptor 55 (GPR55) (Console-Bram et al., 2012). In several neurologic disorders such as MS (Baker et al., 2001; Moreno Torres et al., 2014), Alzheimer's, Parkinson's diseases (Fernández-Ruiz et al., 2015a) and amyotrophic lateral sclerosis (Pryce and Baker, 2015), the anti-inflammatory and neuroprotective effects of cannabinoids (CBs) have been examined. Furthermore, studies performed in EAE have shown that CBs ameliorate symptoms (spasticity, tremor, ataxia and pain), reduce inflammation and favour remyelination *via* CB_1- and CB_2-mediated mechanisms (Arevalo-Martin et al., 2003; Pryce et al., 2003; Fernández-Ruiz et al., 2008). However, the therapeutic potential of many of the CBs is limited due to their psychotropic effects, which are mediated by CB_1 receptors (Klein and Newton, 2007). The major non-psychoactive plant-derived cannabinoid, cannabidiol (CBD), which does not act through CB_1 or CB_2 receptors, shares many beneficial effects with classic cannabinoids, including anti-inflammatory and immunomodulatory properties (Hammell et al., 2015; Ribeiro et al., 2015). Due to its lack of psychotropic effects, the therapeutic potential of CBD is currently under intensive investigation (Ibeas Bih et al., 2015; Lee et al., 2016).

Although CBD treatment was reported to decrease the severity of active EAE (Kozela et al., 2011), information about the effect of this compound on the effector phase of the disease is limited. Consequently, we designed this study to evaluate the potential therapeutic effect of CBD against adoptively transferred EAE and to elucidate its mechanisms of action in encephalitogenic spleen cells. We report here that CBD ameliorates clinical signs of at-EAE and triggers apoptosis in primary encephalitogenic spleen cells *in vitro* by inducing oxidative stress and reducing IL-6 levels in these cells.

2. Methods

2.1. Animals

Pathogen-free male and female C57BL/6 mice, 8 weeks of age, were purchased from Charles River Breeding Laboratories. On arrival, the mice were randomized, transferred to plastic cages containing 5 animals/cage, and quarantined for 1 week. The animals were given food and water *ad libitum*. The mouse holding rooms were kept at 21–24 °C and 40–60% relative humidity with a 12-hour/12-hour light/dark cycle. The animals were handled in accordance with the European Union animal care guidelines (2010/63/EU) and Spanish Government Regulations (Real Decreto 53/2013). All studies were carried out in accordance with the ARRIVE guidelines for animal research (Kilkenny et al., 2010; McGrath et al., 2010).

2.2. Induction of active EAE

EAE was induced in male mice using $MOG_{35–55}$ (Bionova) emulsified in a 1:1 ratio in complete Freund's adjuvant (CFA) containing 10 mg ml^{-1} of *Mycobacterium tuberculosis* (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 250 μg of $MOG_{35–55}$ was dissolved in 100 μl of phosphate-buffered saline (PBS) (Lonza) and mixed with an equal volume of CFA. On days 0 and 7, the MOG-CFA emulsion was subcutaneously (s.c.) injected (200 μl/mouse). The immune adjuvant pertussis toxin (PTX) (Sigma-Aldrich) was injected intraperitoneally (i.p.) (500 ng per mouse) on day 0 and 48 h later.

2.3. Encephalitogenic cells and at-EAE induction

After active induction of EAE, mice were sacrificed and lymph nodes and spleens were aseptically removed. Erythrocytes from the spleens were lysed with ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Single-cell suspensions were prepared, and cells were cultured in a 1:5 ratio (lymph node cells:spleen cells) in DMEM (Lonza) supplemented with 100 units ml^{-1} penicillin/streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM nonessential amino acids, 4 mM L-glutamine (Lonza) and 10% foetal bovine serum (Biowest).

Encephalitogenic cells (5×10^6 cells ml^{-1}) were primed for 72 h with $MOG_{35–55}$ (25 μg ml^{-1}) and IL-12 (25 ng ml^{-1}) and then transferred i.p. to naïve mice.

2.4. At-EAE and treatment groups

All drugs and their respective vehicles were injected i.p. immediately after EAE induction. The following groups of at-EAE mice were used in this study:

1) Control group (n = 5): at-EAE mice treated with cannabinoid vehicle (Solutol:ethanol:PBS at a ratio of 1:1:8).
2) CBD low-dose group (n = 7): at-EAE mice treated with 5 to 10 mg kg^{-1} CBD.
3) CBD high-dose group (n = 7): at-EAE mice treated with 50 mg kg^{-1} CBD.

The doses and duration of preventive treatment were based on previous research and published data.

CBD (GW pharma) solutions were prepared before the experiment according to the manufacturer's instructions and stored for up to 1.5 months.

2.5. At-EAE monitoring

Mice were examined daily to record behavioural and neurological signs for 24 days after the immunization. Disease scores were assigned as follows: 0, asymptomatic; 1, partial loss of tail tonicity or soft weakness in hind limbs; 2, tail paralysis or mild weakness in hind limbs; 3, hind limb weakness or ataxia; 4, partial hind limb paralysis and ataxia; 5, total hind limb paralysis; 6, moribund.

On day 24, spinal cords were collected for further pathological and expression studies.

2.6. Magnetic resonance imaging (MRI)

Brain MRI was performed using a 7-Tesla scanner (7-T Bruker Pharmascan) on day 17. During imaging, the mice were anaesthetized with isoflurane and placed in a birdcage coil. An MR-compatible small animal monitoring system was used during imaging to monitor the respiratory and heart rates of the anaesthetized mice.

The following MRI parameters were calculated: quantitative T2 maps and apparent diffusion coefficient (ADC). Using guidance from mice brain atlases (Badea et al., 2007; Ullmann et al., 2013) we segmented the brain into the corpus callosum, hippocampus, subiculum, and basal ganglia, which were our defined regions of interest (ROI) in each slice. Histogram analysis was performed in the ROIs with ImageJ software (NIH).

2.7. Tissue preparation for H & E and immunohistochemical staining

After the mice were euthanised, lumbar spinal cords were collected and fixed in paraformaldehyde, embedded in OCT (Tissue-Tek Sakura) and cryosectioned for immunostaining.

For the haematoxylin and eosin (H & E) staining protocol, 3-μm-