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Anti-inflammatory effects of the cannabidiol derivative dimethylheptyl-cannabidiol – studies in BV-2 microglia and encephalitogenic T cells

DOI 10.1515/jbcpp-2015-0071
Received June 18, 2015; accepted September 13, 2015; previously published online November 5, 2015

Abstract

Background: Dimethylheptyl-cannabidiol (DMH-CBD), a non-psychoactive, synthetic derivative of the phytocannabinoid cannabidiol (CBD), has been reported to be anti-inflammatory in RAW macrophages. Here, we evaluated the effects of DMH-CBD at the transcriptional level in BV-2 microglial cells as well as on the proliferation of encephalitogenic T cells.

Methods: BV-2 cells were pretreated with DMH-CBD, followed by stimulation with the endotoxin lipopolysaccharide (LPS). The expression levels of selected genes involved in stress regulation and inflammation were determined by quantitative real-time PCR. In addition, MOG35–55-reactive T cells (TMOG) were cultured with antigen-presenting cells in the presence of DMH-CBD and MOG 35–55 peptide, and cell proliferation was determined by measuring [3H]thymidine incorporation.

Results: DMH-CBD treatment downregulated in a dose-dependent manner the mRNA expression of LPS-upregulated pro-inflammatory genes (Il1b, Il6, and Tnf) in BV-2 microglial cells. The expression of these genes was also downregulated by DMH-CBD in unstimulated cells. In parallel, DMH-CBD upregulated the expression of genes related to oxidative stress and glutathione homeostasis such as Trb3, Slt7a1l/xCT, Hmox1, Atf4, Chop, and p8 in both stimulated and unstimulated microglial cells. In addition, DMH-CBD dose-dependently inhibited MOG35–55-induced TMOG proliferation.

Conclusions: The results show that DMH-CBD has similar anti-inflammatory properties to those of CBD. DMH-CBD downregulates the expression of inflammatory cytokines and protects the microglial cells by inducing an adaptive cellular response against inflammatory stimuli and oxidative injury. In addition, DMH-CBD decreases the proliferation of pathogenic activated TMOG cells.

Keywords: anti-inflammation; cannabidiol; cannabinoids; cystine/glutamate transporter; dimethylheptyl-cannabidiol; gene expression; glutathione; microglia; oxidative stress; proliferation; T cells.

To the memory of Prof. Itai Bab.

Introduction

Preparations derived from Cannabis sativa (marijuana and hashish) have become widespread since ancient times, both as therapeutic agents and in recreational smoking [1–3]. Among the more than 60 phytocannabinoids identified in Cannabis extracts, the two most abundant are Δ9-tetrahydrocannabinol (THC), the major psychotropic constituent, and cannabidiol (CBD), the major non-psychoactive component. Cannabinoids were shown to exert a wide range of therapeutic effects, and many of the cannabinoids, especially CBD, were shown to possess potent anti-inflammatory and immunomodulatory activities [4–15]. In addition, it was shown that several cannabinoids have pro-apoptotic, neuroprotective, and antitumor properties [16, 17].

The biological activities of cannabinoids are mostly mediated through cannabinoid receptors, the CB1 (primarily, but not exclusively, expressed in the central nervous system) and the CB2 (mainly expressed in immune cells) [18, 19, and references therein]. However, cannabinoids were also shown to act via mechanisms independent of CB1 and CB2. For example, CBD has very low affinity for both CB1 and CB2 receptors and is therefore devoid of the psychotropic effects produced by Cannabis or THC that are...
mediated via CB1 [4, 20]. Therefore, the anti-inflammatory activities of CBD do not seem to be mediated via either CB1 or CB2 [21 and references therein].

Several groups including ours have shown that various microglial cell functions are affected by cannabinoids, including CBD. For example, our group reported that THC and CBD differentially inhibit the lipopolysaccharide (LPS)-activated nuclear factor κB (NF-κB) and interferon β/signal transducers and activators of transcription (IFN-β/STAT) pro-inflammatory pathways in a CB1/CB2-independent manner [7] and that CBD affects the expression of genes related to oxidative stress and glutathione (GSH) deprivation via the general control non-derepressible 2 (GCN2)/eukaryotic initiation factor 2α (eIF2α)/p8/ATF4/CHOP-tribbles homologue 3 (Trb3) pathway [11, 12]. In addition, CBD-stimulated genes were shown to be controlled by nuclear factors known to be involved in the regulation of stress response and inflammation, mainly through the [electrophiile response element (EpRE)/antioxidant response element (ARE)-nuclear factor-erythroid 2-related factor 2 (Nrf2)/ATF4 system and the Nrf2/Hmox1 axis [11, 12].

CBD was also shown to alleviate the pathological symptoms in a demyelinating disease, the experimental autoimmune encephalomyelitis (EAE), commonly used as a model of multiple sclerosis (MS). Our group reported that CBD ameliorated the severity of EAE in myelin oligodendrocyte glycoprotein 35–55 (MOG35–55)-immunized mice and attenuated microglial activation and T-cell recruitment in these CBD-treated, MOG35–55-induced EAE mice [8]. Moreover, we were able to show that CBD inhibited the in vitro MOG-induced proliferation of MOG35–55-reactive T cells (TMOG) cells and markedly reduced their Th1 inflammatory phenotype [8, 9].

Several CBD derivatives were also shown to have anti-inflammatory and anti-proliferative properties [22, 23]. In view of the therapeutic potential of CBD, we were interested in looking into the activity of its synthetic non-psychoactive derivative, the dimethylethyl-cannabidiol (DMH-CBD). DMH-CBD has been reported to induce apoptosis of human acute myeloid leukemia HL-60 cells [24] and was reported to inhibit the production of reactive oxygen intermediates, nitric oxide, and tumor necrosis factor alpha (TNFα) in LPS-activated RAW macrophages [25]. In the present work, we studied the transcriptional effects mediated by DMH-CBD within genes involved in the regulation of stress response and inflammation in LPS-activated BV-2 microglial cells. In addition, we studied the effects of DMH-CBD on the proliferation of MOG35–55-activated TMOG cells. The results show that DMH-CBD induces similar anti-inflammatory, anti-proliferative, and stress response effects to those previously observed for CBD.

**Materials and methods**

**Reagents**

LPS (Escherichia coli serotype 055:B5) was obtained from Sigma (St. Louis, MO, USA). LPS was dissolved in water, stock solutions (1 mg/mL) were stored in aliquots at −20 °C and diluted in culture medium before experiments. Lyophilized MOG35–55 peptide (MEVGWYRSPRESVRVHLYRNGK) purchased from GenScript (Piscataway, NJ, USA) was reconstituted in sterile phosphate buffered solution (PBS) and the stock solution stored in aliquots at −20 °C. (-)-5′-DMH-CBD was synthesized as previously described [26]. Stock solution of DMH-CBD was prepared in ethanol and diluted into culture medium before experiments. The final concentrations of ethanol in the experiments did not exceed 0.1%–0.2% and had no effect on the results. Fetal calf serum and other tissue culture medium and reagents were obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

**Microglial cell culture**

The BV-2 mouse microglial cell line was kindly provided by Prof. E. J. Choi from Korea University (Seoul, South Korea). Cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/L glucose and 1 mM sodium pyruvate and supplemented with 5% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) under a humidified 5% CO2 atmosphere at 37 °C. Cells (1×104 cells/well) were pretreated for 2 h with DMH-CBD, at the doses indicated in the text, followed by addition of LPS (100 ng/mL) for another 4 h.

**RNA extraction and quantitative real-time PCR (qPCR)**

Extraction, quantification, and quality of extracted RNA were performed and analyzed as described previously [11]. copy DNA (cDNA) was synthesized using the Quantitect Reverse Transcription kit according to the manufacturer’s instructions (Qiagen AG, Basel, Switzerland). qPCR was carried out as detailed previously [11]. Expression levels of genes were normalized to the reference gene β2-microglobulin (B2m), whose expression has been found not to be affected by the treatments and are expressed as fold change as reported earlier [11]. Primer sets were designed using the PrimerQuest, an online tool provided by Integrated DNA Technologies (http://eu.idtdna.com/Primerquest/Home/Index) and synthesized by Metabion International (Planegg-Steinkirchen, Germany). GenBank™ accession numbers for the different genes and the primer sequences used for amplification were reported earlier [9, 11]. The qPCR runnings were repeated three times using mRNA preparations from three independent experiments.

**Encephalitogenic T-cell proliferation**

Tmog, raised from MOG35–55 immunized mice was obtained and maintained as previously described [8, 9, 27]. To assay the effect of DMH-CBD on Tmog proliferation, Tmog cells were cultured in 96-well plates (1.25×104 cells/well) together with splenic antigen-presenting...
cells (APCs; 5×10^5 cells/well). APCs were irradiated (25 Gy) before co-culturing to prevent them from proliferation. The co-cultured cells (TMOG/APCs) were maintained in 0.2 mL of RPMI medium containing 2.5% fetal calf serum, supplemented with streptomycin (100 µg/mL), penicillin (100 U/mL), 2 mM L-glutamine, and 50 µM β-mercaptoethanol. DMH-CBD at final doses of 0.1, 1, 5, and 10 µM was added to the cells together with the MOG35–55 peptide at either 1 or 2.5 µg/mL. The cells were then incubated for 72 h at 37 °C in 5% CO2-humidified air. Cell proliferation was measured by pulsing the cells with [3H]thymidine (0.5 µCi/well; Perkin Elmer, Boston, MA, USA) for the last 16 h of the incubation period, and the cells were then harvested and counted using a Matrix 96 Direct β-counter (Packard Instruments, Meriden, CT, USA). The proliferative responses of the TMOG cells in each well were converted to percentage values with MOG35–55 effect expressed as 100%. Percentage values are calculated from the stimulation index data, which are the fold changes in mean counts per minutes (cpm) of MOG35–55 cultures over mean cpm of cultures without MOG (spontaneous proliferation). Statistical analysis was performed on percentage values [8, 27].

Statistical analysis

Data are expressed as the mean±SEM of three independent experiments and analyzed for statistical significance using Student’s t-test or one-way analysis of variance (ANOVA), followed by Dunnett’s or Newman-Keul’s post hoc test. p<0.05 was considered significant. GraphPad Prism 5 software (La Jolla, CA, USA) was used for statistical analysis of the data.

Results

DMH-CBD treatment downregulates mRNA expression of LPS-upregulated pro-inflammatory genes in BV-2 cells

BV-2 cells were pretreated for 2 h with DMH-CBD (1, 5, and 10 µM) and then stimulated with LPS (100 ng/mL) for an additional 4 h. The control treatment with DMH-CBD alone lasted for 6 h and with LPS alone for 4 h. These treatment time points were chosen following our previous reports [7, 11]. The cells were then collected, mRNA extracted, and the expression of several genes determined by qPCR. qPCR analysis showed strong upregulation in the mRNA levels of Il1b, Il6, and Tnf following LPS stimulation of microglial cells (Figure 1A–C). Treatment with DMH-CBD significantly decreased, in a dose-dependent manner, the LPS-upregulated expression of Il1b mRNA [by 88% at 10 µM DMH-CBD; ANOVA F(4,10)=187.9, p<0.001] and of Il6 [by 82% at 10 µM DMH-CBD; ANOVA F(4,10)=9.97, p<0.001] (Figure 1A,B). The level of LPS-upregulated Tnf mRNA was also decreased by DMH-CBD, reaching a 48% reduction at 10 µM DMH-CBD, a result that unexpectedly was found to be not statistically significant [ANOVA F(4,10)=4.6, p<0.05] (Figure 1C). As shown in Figure 2, DMH-CBD by itself (without LPS) also reduced the basal transcript levels of Il1b, Il6, and Tnf in a dose-dependent manner. At a dose of 10 µM, DMH-CBD decreased the expression of Il1b mRNA by 82% [ANOVA F(3,18)=27.2, p<0.001], Il6 by 67% [ANOVA F(3,18)=14.4, p<0.01], and Tnf by 66% [ANOVA F(3,18)=43.4, p<0.001].
DMH-CBD treatment upregulates mRNA expression of genes related to oxidative stress and GSH homeostasis in BV-2 cells

qPCR analysis demonstrated that incubation of BV-2 cells with DMH-CBD (at 10 μM) leads to upregulation of several genes related to stress and GSH homeostasis (Figure 3A–F). These included tribbles homologue 3 [Trb3; by 10-fold; ANOVA F(3,8)=94.1, p<0.001], solute carrier family 7, member 11 [cystine/glutamate transporter subunit Slc7a11/xCT; by 5.4-fold; ANOVA F(3,12)=132.4, p<0.001], and heme oxygenase 1 [Hmox1; by 4-fold; ANOVA F(3,8)=49.1, p<0.001]. DMH-CBD also increased the expression of several other genes, but to a significantly lower extent, such as the activating transcription factor 4 [Atf4; by 60%; ANOVA F(3,8)=60.3, p<0.001], C/EBP homologous protein [Chop/Ddit3/Gadd153; by 50%; ANOVA F(3,8)=49.1, p<0.001], and nuclear protein I [Nupr1/p8; by 36%; ANOVA F(3,8)=240.1, p<0.001]. The expressions of Trb3, Hmox1, and Atf4 mRNAs were not affected by LPS. Meanwhile, Slc7a11/xCT mRNA (Figure 3B) was highly upregulated by LPS (by 8.2-fold, p<0.05) and synergistically upregulated by the combined DMH-CBD+LPS treatment (by 34-fold vs. control, p<0.001, and by 3.7-fold vs. LPS, p<0.001). p8 expression was upregulated by LPS by 3.4-fold (p<0.01) and by the combination of DMH-CBD+LPS by 4.8-fold vs. control (p<0.01) and by 40% vs. LPS (p<0.01) (Figure 3F). In contrast, Chop mRNA was slightly downregulated by LPS (by 30%, p<0.01) (Figure 3E).

DMH-CBD inhibits MOG<sub>35-55</sub>-induced T<sub>M</sub>O<sub>G</sub> cell proliferation

We previously showed that CBD ameliorates clinical symptoms of MOG<sub>35-55</sub>-induced EAE [8]. Moreover, we showed that CBD inhibits the MOG<sub>35-55</sub>-induced proliferation of T<sub>M</sub>O<sub>G</sub> cells co-cultured with irradiated APCs in the presence of MOG<sub>35-55</sub> [8]. Therefore, we have analyzed the effect of DMH-CBD in this in vitro model of MOG<sub>35-55</sub>-stimulated encephalitogenic T cells. T<sub>M</sub>O<sub>G</sub>/APCs were co-cultured in the presence of MOG<sub>35-55</sub> at a dose of 1 or 2.5 μg/mL. These concentrations were chosen following our previous reports [8, 9]. T-cell proliferation was analyzed using [3H]thymidine incorporation as previously reported [8]. Incubation with 1 μg/mL MOG<sub>35-55</sub> resulted in a 24±11-fold increase (p<0.05, Student’s t-test) in T<sub>M</sub>O<sub>G</sub> proliferation and incubation with 2.5 μg/mL MOG<sub>35-55</sub> produced a 42±10-fold increase (p<0.01, Student’s t-test) in T<sub>M</sub>O<sub>G</sub> proliferation vs. non-stimulated cells (Figure 4A). Results presented in Figure 4B,C shows the inhibitory effect of DMH-CBD at various doses (0.1-10 μM) on the MOG<sub>35-55</sub>-induced proliferation of T<sub>M</sub>O<sub>G</sub> cells stimulated by either 1 or 2.5 μg/mL of MOG<sub>35-55</sub>. As shown in Figure 4B, addition of 5 μM DMH-CBD to 1 μg/mL MOG<sub>35-55</sub>-stimulated T<sub>M</sub>O<sub>G</sub>/APCs resulted in inhibition of T<sub>M</sub>O<sub>G</sub> proliferation by 51%, whereas 10 μM DMH-CBD inhibited T<sub>M</sub>O<sub>G</sub> proliferation by 97% (p<0.01). Similar results were observed when T<sub>M</sub>O<sub>G</sub> cells were stimulated with 2.5 μg/mL of MOG<sub>35-55</sub> (Figure 4C). In this case, 5 μM DMH-CBD inhibited the proliferation by 62% (p<0.01) and 10 μM DMH-CBD by 97% (p<0.001). Lower doses of DMH-CBD (e.g. 0.1 and 1 μM) had no significant effect on T<sub>M</sub>O<sub>G</sub> proliferation induced by MOG<sub>35-55</sub> at either 1 or 2.5 μg/mL.
Discussion

The present study describes the effects of DMH-CBD on inflammatory and stress-related genes in BV-2 microglial cells and on the proliferation of MOG35–55-activated T MOG cells.

We found that DMH-CBD downregulates in a dose-dependent manner the mRNA expression of LPS-upregulated Il1b and Il6 transcripts. Moreover, DMH-CBD dose dependently downregulates the expression of these transcripts in unstimulated BV-2 cells. These findings show that DMH-CBD possesses anti-inflammatory and protective properties. DMH-CBD effects are similar to those found for CBD in our previous work [7, 11, 13] where we reported that CBD decreases the production and release of the pro-inflammatory cytokines IL-1b and IL-6, but not of TNFα in LPS-stimulated BV-2 microglial cells [7, 13].

In addition, we show here that DMH-CBD strongly upregulates the expression of the genes Trb3, Slc7a11/xCT, and Hmox1 and, to a lesser extent, of the genes Atf4, Chop/Ddit3/Gadd153, and p8/Nupr1. Previous work from our laboratory showed that these genes were also significantly upregulated by CBD [11]. Most of these genes (Trb3, Slc7a11/xCT, Atf4, Chop/Ddit3/Gadd153, and p8/Nupr1) are known to contain amino acid response elements and to be regulated in response to amino acid deprivation [28]. The
The effect of MOG35–55 stimulation on TMOG proliferation expressed as a fold change of mean values compared to non-stimulated cells. Each experiment (n=2) was carried out in triplicate. (A) Effect of MOG35–55 stimulation on TMOG proliferation expressed as fold change of mean values±SEM, vs. non-stimulated cells (*p<0.05 and **p<0.01, Student’s t-test). (B, C) Effect of various concentrations of DMH-CBD on TMOG proliferation expressed as mean percent±SEM, vs. non-stimulated cells (*p<0.05, **p<0.01, and ***p<0.001 vs. MOG35–55-treated cells. (A) and (B) show the effect of DMH-CBD inhibiting MOG35–55-induced TMOG cell proliferation. This result is in agreement with our previous work, which shows that CBD inhibits the MOG35–55-induced proliferation of TMOG cells stimulated with MOG35–55 [8].

Cannabinoids, including CBD, have been shown to possess anti-proliferative properties in highly proliferative cell lines, including transformed T-cell lines [44–46]. For example, CBD inhibits proliferation by inducing cell death in cancer cell lines via the transient receptor potential vanilloid channel 2 [47, 48] and THC reduces proliferation and leads to cell death of C6 glioma cells via CB1 receptor [49]. As for DMH-CBD, it was shown that DMH-CBD (as well as CBD) in combination with γ-irradiation induces apoptotic cell death of HL-60 myeloblastic leukemia cells [24]. Here, we show that DMH-CBD in similarity to p8 represses the expression of ATF4 [33], other cannabinoids like CBD, THC, and the synthetic WIN 55,212-2 were also shown to induce p8 expression [11, 34, 35]. In addition, our results show upregulation of p8 mRNA expression by LPS, an effect also shown by others [36], and by the combined treatment of DMH-CBD+LPS.

As described above, DMH-CBD highly upregulated the expression of the mRNA of the transporter Slc7a11/ xCT and of the antioxidant inducible enzyme Hmox1, both known to be modulated in response to oxidative stress via the ARE/EpRE-Nrf2 system [37]. The Slc7a11/xCT constitutes the stress inducible subunit of the cystine/glutamate transport system x− c [38, 39] whose activation will produce an increase in the levels of GSH in the cells [40]. In addition, we show that LPS strongly upregulates Slc7a11/xCT mRNA expression and that the effect of LPS and DMH-CBD applied together on Slc7a11/xCT expression is synergistic. This result is in agreement with Sato et al. [41], who reported that LPS induces the activity of the cystine/glutamate transporter in macrophages, increasing GSH levels in the cells. Moreover, they showed partial synergism when macrophages were incubated with LPS together with TNFα.

Redox homeostasis was reported to modulate the microglia and macrophages M1/M2 polarization [42]. In this regard, it was shown that Hmox1 induction drives a shift to M2 macrophage phenotype, characterized by its intracellular redox status, consisting of high expression of scavenging molecules and GSH [42, 43]. Thus, upregulation of Slc7a11/xCT and Hmox1 by DMH-CBD may serve to protect the cells by inducing an adaptive cellular response against inflammatory stimuli and oxidative injury, utilizing GSH as antioxidant or conjugating/detoxifying agent.

In the present study, we also report that DMH-CBD significantly decreases MOG35–55-induced TMOG cell proliferation. This result is in agreement with our previous work, which shows that CBD inhibits the MOG35–55-induced proliferation of TMOG cells stimulated with MOG35–55 [8].

Another gene upregulated by DMH-CBD is Nupr1/ p8 (36%). Nupr1/p8 was found to be induced by ATF4 in response to various cellular stressors, and its expression was associated with enhanced transcriptional activation of genes downstream of ATF4 (such as Chop and Trb3). This suggests that p8 promotes the transcription of stress-regulated genes via a positive feedback on ATF4 pathway [33]. Other cannabinoids like CBD, THC, and the synthetic WIN 55,212-2 were also shown to induce p8 expression [11, 34, 35]. In addition, our results show upregulation of p8 mRNA expression by LPS, an effect also shown by others [36], and by the combined treatment of DMH-CBD+LPS.

Upregulation of these amino acid-response genes suggests an effect of DMH-CBD on GNC2/elF2α/ATF4-mediated stress pathway [29–31]. These results demonstrate that DMH-CBD is inducing a metabolic adaptation program typical for nutrient limiting conditions, characterized by repression of protein synthesis and upregulation of amino acid biosynthesis and transporter genes [32].

Figure 4: DMH-CBD inhibits MOG35–55-induced TMOG cell proliferation. TMOG/APC co-cultures were exposed to MOG35–55 (1 or 2.5 μg/mL), and TMOG proliferation was determined by [3H]thymidine incorporation assay in the presence or absence of DMH-CBD at the indicated concentrations. Each experiment (n=2) was carried out in triplicate. (A) Effect of MOG35–55 stimulation on TMOG proliferation expressed as fold change of mean values±SEM, vs. non-stimulated cells (*p<0.05 and **p<0.01, Student’s t-test). (B, C) Effect of various concentrations of DMH-CBD on TMOG proliferation expressed as mean percentage values±SEM. The MOG35–55 effect (in the absence of DMH-CBD) is expressed as 100%. (B) One-way ANOVA F(4,5)=18.2, p<0.01; (C) one-way ANOVA F(4,5)=52.2, p<0.001; Dunnett’s post hoc test: **p<0.01 and ***p<0.001 vs. MOG35–55-treated cells.
previous results with CBD [8, 9, 50] can prevent the proliferation of primary T cells.

Altogether, our results show that DMH-CBD, a non-psychoactive, synthetic CBD derivative, has anti-inflammatory and anti-proliferative properties. DMH-CBD induces the expression of genes related to oxidative stress and amino acid deprivation, genes that are controlled by Nrf2 and ATF4 transcription factors. In addition, DMH-CBD inhibits the proliferation of MOG35–55-activated TMOG cells. In similarity to CBD, DMH-CBD could be of high therapeutic value in neuro-inflammatory diseases and related syndromes.

Acknowledgments: A.J. is supported by the Israeli Ministry for Absorption in Science.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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