Inhibitory Effect of Cannabidiol on the Activation of NLRP3 Inflammasome Is Associated with Its Modulation of the P2X7 Receptor in Human Monocytes

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**ABSTRACT:** Cannabidiol (CBD), a phytocannabinoid, has been reported to have anti-inflammatory effects associated with NLRP3 inflammasome activation, but its mechanism of anti-inflammasome action remains unclear. Herein, we report CBD’s effect on NLRP3 inflammasome activation and its modulation of P2X7, an inflammasome activation-related receptor, in human THP-1 monocytes. CBD (0.1, 1, and 10 μM) exerted anti-inflammasome activity in LPS-nigericin-stimulated THP-1 monocytes by reducing media IL-1β concentration (by 63.9%, 64.1%, and 83.1%, respectively), which was similar to the known NLRP3 inflammasome inhibitors oridonin and MCC950 (16.9% vs 20.8% and 17.4%, respectively; at 10 μM). CBD (10 μM) decreased nigericin-alone- and nigericin-lipopolysaccharide-induced potassium efflux by 13.7% and 13.0%, respectively, in THP-1 monocytes, strongly suggesting P2X7 receptor modulation. Computational docking data supported the potential for CBD binding to the P2X7 receptor via interaction with GLU 172 and VAL 173 residues. Overall, the observed CBD suppressive effect on NLRP3 inflammasome activation in THP-1 monocytes was associated with decreased potassium efflux, as well as in silico prediction of P2X7 receptor binding. CBD inhibitory effects on the NLRP3 inflammasome may contribute to the overall anti-inflammatory effects reported for this phytocannabinoid.

Inflammation is an immune response to harmful stimuli and can be mediated by several biological pathways including the activation of inflammasomes, intracellular protein complexes that serve as signaling platforms to regulate the production of pro-inflammatory cytokine interleukin-1β (IL-1β).1 One of the most characterized and clinically implicated inflammasome protein complexes is known as the NLRP3 inflammasome, which contains members of the nucleotide-binding oligomerization domain-like receptor (NLR) and pyrin domain containing receptor 3.2 Activation of the NLRP3 inflammasome can be triggered in macrophages by inflammatory stimuli in two steps: (1) stimulation by multiprotein complex assemblies, known as pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide (LPS), to synthesize and mature pro-inflammatory cytokine IL-1β;3 (2) a secondary stimulation by the stimulus nigericin to induce the assembly and activation of the inflammasome complex.4 As a response to detrimental stimuli, activation of inflammasomes leads to various forms of inflammatory cell death including pyroptosis and necroptosis and further leads to numerous autoinflammatory or autoimmune diseases.5 Activation of the NLRP3 inflammasome has been linked to a group of purinergic type 2 receptors including the P2X7 receptor, a ligand-gated ion channel modulating intracellular K⁺ efflux.6 It has been reported that nigericin, an activator of the NLRP3 inflammasome, can bind to P2X7 and increase the permeability of the plasma membrane for K⁺ flux, which further facilitates the secretion of IL-1β.6 Therefore, the P2X7 receptor has attracted interest as a potential therapeutic target, and growing research efforts have been directed toward investigating small molecules as anti-inflammatory agents through the blockade of P2X7.7

Cannabidiol (CBD) is a phytocannabinoid with several reported beneficial pharmacological effects including antioxidant, antidiabetic, anticancer, and anti-inflammatory effects.8 Published studies have reported that CBD ameliorated several inflammatory-associated diseases including diabetes and diabetic complications, hypertension, ischemia-reperfusion injury, and neurodegenerative diseases through modulation of key pro-inflammatory transcription factors including NF-κB, iNOS, and TNFα.9,10 Recently, published data suggest that the anti-inflammatory effects of CBD are associated with the regulation

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of inflammasome activation.\textsuperscript{11,12} For instance, CBD was reported to show hepatoprotective effects against nonalcoholic steatohepatitis in a mouse model.\textsuperscript{12} Further in vitro studies in murine RAW264.7 macrophages demonstrated that CBD’s antihepatitis effect was involved with the NLRP3 inflammasome pathway.\textsuperscript{12} However, the effects of CBD on the activation of the NLRP3 inflammasome in human THP-1 monocytes and its mechanisms of action remain unclear. Herein, the inhibitory effects of CBD on inflammasome activation were evaluated and compared to the known NLRP3 inflammasome inhibitors, namely, oridonin (a diterpenoid from Rabdosia rubescens)\textsuperscript{13} and MCC950 (a synthetic inhibitor; see Chart 1 for the chemical structures of CBD, oridonin, and MCC950).\textsuperscript{14} In addition, CBD’s effects on the modulation of the P2X7 receptor, as a function of intracellular K$^+$ flux, in human THP-1 monocytes were investigated.

**CBD Inhibited LPS-Induced IL-1β Secretion in THP-1 Monocytes.** Prior to the evaluation of the anti-inflammasome activity of CBD, its effects on the viability of THP-1 monocytes with and without the presence of LPS were assessed. Treatment with CBD (0.1, 1, 10, and 100 µM) did not induce significant cytotoxicity in THP-1 monocytes, as they maintained cell viability at 96.5%, 97.6%, 103.1%, and 99.9%, respectively, as compared to the control group (Supporting Information). Similarly, in the presence of LPS (100 ng/mL), no signs of cytotoxicity were observed with the treatment of CBD (0.1–100 µM; cell viability >97%; Supporting Information). Next, the anti-inflammatory effects of CBD in THP-1 monocytes were evaluated. Stimulation by LPS significantly increased the concentration of IL-1β and TNF-α in THP-1 monocytes by 38.7-fold (from 1.3 to 51.2 pg/mL) and 93.1-fold (from 6.9 to 647.2 pg/mL), respectively, compared to the control group. Treatment with CBD (0.1, 1, and 10 µM) decreased the LPS-induced secretion of IL-1β to 41.2, 43.5, and 32.9 pg/mL, respectively, while not affecting the level of TNF-α (Figure 1).

It has been reported that CBD can modulate inflammatory responses by both up- and down-regulation of inflammatory cytokines in murine RAW264.7 macrophages. Treatment of hemp oil containing CBD (10.6 µM) significantly increased several inflammatory cytokines including IL-1α, IL-6, IL-27, and TNF-α in murine RAW264.7 macrophages.\textsuperscript{15} Conversely, CBD (25 µM) showed anti-inflammatory activity by effectively reducing LPS-induced production of the pro-inflammatory cytokine, TNF-α, in RAW264.7 macrophages.\textsuperscript{16} In the current study, CBD reduced the level of the pro-inflammatory cytokine IL-1β, while not affecting TNF-α in LPS-stimulated THP-1 monocytes, supporting its anti-inflammatory effect. The selective inhibitory effect on IL-1β by CBD (0.1–10 µM) suggested that this effect could be associated with the activation of the NLRP3 inflammasome, a multiprotein complex that is responsible for the cleavage and production of IL-1β. Therefore, CBD’s inflammasome-specific inhibitory effects were further evaluated in human monocytes.

**CBD Inhibited LPS-Nigericin-Induced Activation of the NLRP3 Inflammasome.** Specific inhibition of NLRP3 inflammasome activation by CBD was evaluated by measuring LPS-nigericin-induced IL-1β in THP-1 monocytes. Stimulation by LPS and nigericin significantly induced the production of IL-1β as compared to the LPS-alone treated group (492.1 vs 51.2 pg/mL). Stimulation by LPS-nigericin also increased the concentration of TNF-α as compared to the LPS-alone treated group (957.6 vs 647.2 pg/mL), indicating that the stimulation by LPS-nigericin activated the NLRP3 inflammasome (Figure 2). CBD (0.1, 1, and 10 µM) reduced the concentration of IL-1β to 177.5, 176.9, and 83.0 pg/mL, respectively, as compared to the LPS-nigericin treated group (957.6 vs 647.2 pg/mL), suggesting that the stimulation by LPS-nigericin activated the NLRP3 inflammasome (Figure 2A). At equivalent concentrations of 10 µM, CBD showed similar inhibitory activity to the known NLRP3 inflammasome inhibitors oridonin and MCC950 (16.9% vs 20.8% and 17.4%, respectively; Figure 3A). As expected, CBD, oridonin, and MCC950 (all at 10 µM) did not inhibit the LPS-nigericin-induced elevation of TNF-α (661.9, 777.2, and 957.6 vs 957.6 pg/mL, respectively; Figure 2B), suggesting that CBD, similar to oridonin and MCC950, was a specific inhibitor of NLRP3 inflammasome activation.
Figure 2. CBD’s specific inhibition of the NLRP3 inflammasome. LPS (100 ng/mL) was added to THP-1 monocytes for 4 h followed by treatment with CBD (0.1, 1, and 10 μM), oridonin (10 μM), or MCC950 (10 μM) for 1 h. Nigericin (10 μM) was then added and incubated for 1 h. Supernatant was collected and measured for the level of IL-1β (A) and TNF-α (B) using ELISA kits.

Figure 3. Effects of CBD and nigericin on intracellular potassium in THP-1 monocytes. Nigericin (1, 5, and 10 μM) was added to THP-1 monocytes for 30 min followed by treatment with CBD (10 μM) for 30 min. The cells were stained with ion potassium Green-2 AM dye, and the fluorescent intensity was measured by flow cytometry.

Previously reported studies have shown that the anti-inflammatory effects of CBD may be attributed to its ability of deactivating the NLRP3 inflammasome.11,12 CBD was shown to suppress the expression of several inflammasome-related genes in human gingival mesenchymal stem cells.11 This effect was supported by Western blot analysis showing that CBD was able to suppress the expression of proteins including NALP3, IL-18, and CASP1 in the same cell line. However, the level of inflammasome-specific pro-inflammatory cytokines, such as IL-1β, was not examined. It was also reported that CBD showed hepatoprotective and anti-inflammatory effects by regulation of NFkB and NLRP3 inflammasome pathways in murine RAW264.7 macrophages.12 However, solely using murine RAW264.7 macrophages to evaluate CBD’s anti-inflammasome activity is insufficient to delineate its inhibitory effects since these cells do not release mature IL-1β nor do they express apoptotic proteins with a caspase-activating recruiting domain, which are critical factors for the activation of the NLRP3 inflammasome. Therefore, the utilization of human THP-1 monocytes has been adopted by other researchers to investigate anti-inflammasome activity.13-15 This is the first report to show that CBD, similar to the known NLRP3 inflammasome inhibitors oridonin and MCC950, selectively reduced the secretion of IL-1β in human THP-1 monocytes.

CBD Alleviated LPS and Nigericin-Stimulated Increase of Intracellular Potassium but Did Not Affect Intracellular Calcium in THP-1 Monocytes. The anti-inflammasome activity of CBD was further evaluated by assessment of its effects on the P2X7 receptor, a ligand-gated cation channel, which modulates the level of intracellular potassium (K+) in THP-1 monocytes. Stimulation by nigericin, a K+/H+ ionophore, led to the formation of cell membrane pores and increased K+ efflux in THP-1 monocytes as the intensity of fluorescent dye Green-2 increased, as shown by cell flow cytometry. Incubation of THP-1 monocytes with nigericin (1, 5, and 10 μM) led to increased release of intracellular K+ by 18.9%, 14.0%, and 17.9%, respectively, as compared to the control group (Figure 3). This effect was alleviated by treatment with CBD (10 μM) by reducing nigericin (1, 5, and 10 μM)-induced K+ efflux by 11.0%, 9.1%, and 13.7%, respectively.

Inflammatory stimuli, such as LPS, can potentiate nigericin-induced cell membrane pore formation, which increases the cell membrane permeabilization for K+ efflux.19 To verify the modulatory effects of CBD on the P2X7 receptor, LPS (100 ng/mL) was co-incubated with nigericin in THP-1 monocytes, followed by CBD treatment. The level of intracellular K+ in THP-1 monocytes was determined by measuring the fluorescent intensity of Green-2 dye with flow cytometry. Stimulation by LPS-nigericin increased intracellular K+ by 27.5% as compared to the control group, and CBD (1 and 10 μM) alleviated LPS-nigericin induced intracellular K+ efflux by 9.2% and 13.3%, respectively (Figure 4).

In addition, the effect of CBD on intracellular calcium (Ca2+) was evaluated with flow cytometry. Stimulation by nigericin (10 μM) effectively increased the permeability of the cell membrane as it increased the cell uptake of fluorescent dye Fluo-3 and intracellular Ca2+ in THP-1 monocytes by 84.3% compared to the control group. However, a modulatory effect of CBD (1, 10, and 100 μM) on intracellular Ca2+ in THP-1 monocytes was not observed (Supporting Information). CBD’s anti-inflammatory effects have been reported to be linked to its up-regulation of intracellular Ca2+ in RBL-2H3 mast cells, leading to cell activation via yet-to-be identified receptors.20 Therefore, further studies on whether CBD’s modulation of cellular ion channels in various cell lines can contribute to its anti-inflammasome effects are warranted.

CBD Interacted with the P2X7 Receptor. Molecular modeling was used to study the interactions between CBD and the P2X7 receptor to predict possible binding sites. The most favorable binding pocket was identified by comparing the calculated free binding energy in different binding modes. As depicted in Figure 5A and B, the most suitable binding site of CBD was at the upper body, between the head and left flipper of the P2X7 receptor, with the lowest free binding energy of −6.62 kcal/mol. CBD was able to bind to the P2X7 receptor by forming a hydrogen bond between its hydroxy group and residue GLU 172, and the stabilization of the CBD-P2X7 complex may be facilitated by forming a pi-sigma covalent bond between the
Cannabidiol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The CellTiter-Glo (CTG) 2.0 assay kit was purchased from Promega (Fitchburg, WI, USA). Dimethyl sulfoxide (DMSO), nigericin, phorbol 12-myristate 13-acetate (PMA), and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co.

**Cell Culture and Differentiation.** Human THP-1 monocytes were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Gaithersburg, MD, USA) and 1% P/S solution (100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Life Technologies, Grand Island, NY, USA)). THP-1 monocytes were differentiated by incubation with PMA (25 nM) for 48 h. After that, PMA was removed and cells were cultured with PMA-free medium for another 24 h.

**Cell Viability Assay.** Cytotoxicity of CBD in THP-1 monocytes was measured by using a CTG 2.0 assay.23 Briefly, differentiated THP-1 monocytes were seeded into a 96-well plate at 1 × 10⁴ cells per well and incubated with PMA-free complete medium for 24 h. Cells were then treated with CBD at concentrations of 0.1, 1, 10, and 100 μM in the presence or absence of LPS (100 ng/mL) for 24 h. CTG 2.0 reagent (100 μL) was then added in each well and incubated for 10 min at room temperature. Luminescence intensity was measured using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Measurement of IL-1β and TNF-α.** THP-1 monocytes were seeded at a density of 5 × 10⁴ cells per well in a 48-well plate and differentiated with PMA (25 ng/mL). For the evaluation of LPS-induced cytokine secretion, CBD at concentrations of 0.1, 1, 10, and 100 μM was added to cells for 1 h followed by adding LPS (100 ng/mL). After incubation for 24 h, the cell culture supernatant was collected for the measurement of IL-1β and TNF-α. The in vitro anti-inflammamome activity of CBD was evaluated by measuring the concentrations of LPS- and nigericin-induced cytokines according to a previously reported method.23 THP-1 monocytes were treated with LPS (100 ng/mL) for 4 h, followed by treatment with CBD (0.1, 1, 10, and 100 μM) for 1 h. Then, nigericin (10 μM) was added with the cells for 1 h, and the cell culture supernatant was collected. The levels of IL-1β and TNF-α were determined using specific ELISA kits (BioLegend, San Diego, CA, USA).

**Intercellular Potassium and Calcium Measurement.** THP-1 monocytes were seeded at a density of 5 × 10⁴ cells per well in six-well plates. The differentiated THP-1 monocytes were incubated with CBD (10 μM) for 30 min prior to incubating with nigericin (10 μM) for 30 min. To measure the intracellular potassium or calcium, fluorescent dye Green-2 or Fluo-3 (Abcam; Cambridge, MA, USA) was added into each well and incubated for 30 min, followed by washing with PBS. The level of intracellular potassium in THP-1 monocytes was measured by a flow cytometry assay.23

**Molecular Docking.** The 3D structural coordinates of CBD were obtained from the human metabolome database (www.HMDB.ca). Biovia Discovery Studio Visualizer 4.5 was used to convert to the PDB
file format. The structural coordinates of the P2X7 receptor were retrieved in PDB format from the RCSB protein data bank (www.rcsb.org; PDB ID: SU1L). AutodockTools 1.5.6 was used to perform molecular docking with the Autodock 4.2 algorithm.

**Statistical Analysis.** Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Data are shown as mean ± standard deviation. One-way analysis of variance with multiple comparisons and Student–Newman–Keuls test were performed. A p-value less than 0.05 was defined as statistical significance between the two groups.

# ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00138.

Data for the effects of CBD on the viability of THP-1 monocytes and the intracellular calcium in THP-1 (PDF)

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**Notes**

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