Investigating the Potential Therapeutic Effects of CBD in Vitro

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INTRODUCTION

Reactive oxygen species (ROS) are primarily generated by the mitochondria as by-products of oxygen metabolism, a process utilized by cells to create energy (Pizzino, 2017). Common ROS include superoxide radicals ($O^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). At low levels, these metabolic by-products play an important physiological role and are necessary for many cellular processes (Pizzino, 2017). However, at high levels, ROS can be harmful and damage cellular structures such as membranes, proteins, and deoxyribonucleic acid (DNA) (Pizzino, 2017). To protect itself from damage, the cell utilizes antioxidants, which can neutralize ROS. When there is an imbalance between ROS and antioxidants, such that there is a high accumulation of ROS in cells and not enough antioxidant elements to detoxify them, the biological system is said to be in a state of "oxidative stress" (Pizzino, 2017). Oxidative stress is harmful to the human body and has been shown to play a role in the progression of many diseases such as cardiovascular disease, diabetes, cancer, and neurodegenerative diseases (Pizzino, 2017).

One of the mechanisms the cell uses to defend against oxidative stress is the Keap1-Nrf2 pathway (Figure 1) (Baird, 2020). Under normal conditions, a protein and component of an E3 ubiquitin ligase, Keap1, regulates the activity of the transcription factor Nrf2 by binding it in the cytoplasm of the cell and targeting Nrf2 for ubiquitination and proteosome-dependent degradation. Under oxidative stress, the activity of the ubiquitin ligase is inactivated such that Nrf2 can no longer be ubiquitinated and degraded. Nrf2 instead occupies the binding sites of Keap1, and this allows for newly translated Nrf2 to translocate into the nucleus of the cell and bypass binding with Keap1. Inside the nucleus, Nrf2 can act as a transcription factor and bind to an enhancer sequence of DNA known as the antioxidant response element (ARE), which promotes the transcription of antioxidant enzymes such as Heme-oxygenase 1 (HO-1). These antioxidant enzymes can help to neutralize ROS and mitigate the damage caused by oxidative stress (Baird, 2020).

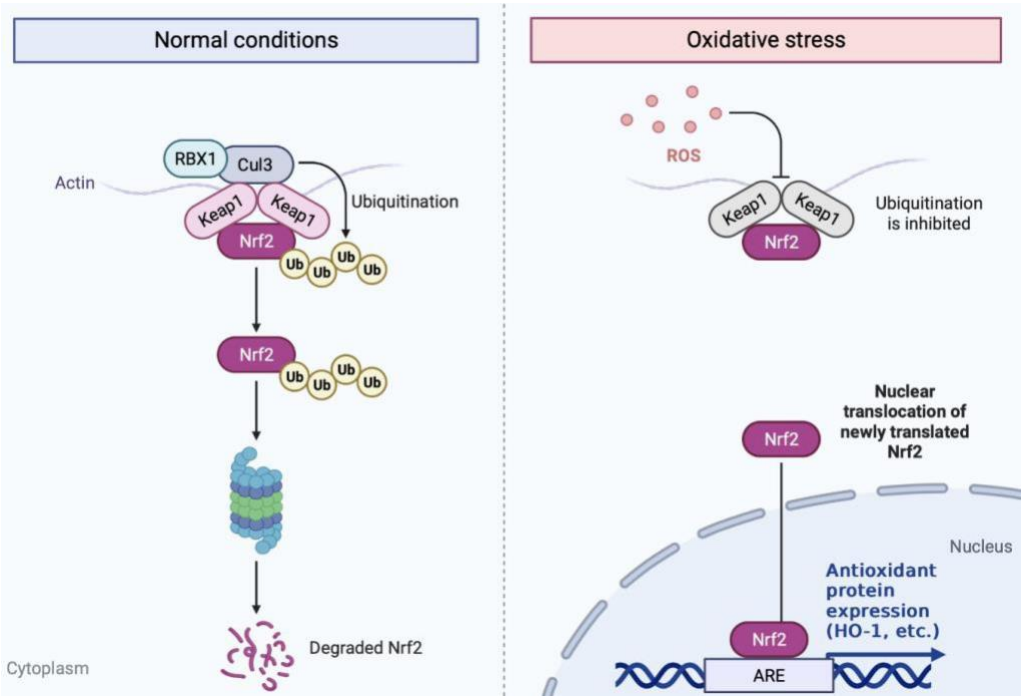

Figure 1. Schematic of the Nrf2 pathway. Created with BioRender.com

Another facet of these oxidative stress-related diseases is chronic inflammation, and many studies show an interdependent relationship between oxidative stress and inflammation (Biswas, 2015). Inflammation is the body's protective response to harmful stimuli like pathogens and toxic compounds such as ROS (Chen, 2018). An acute inflammatory response is important for minimizing injury or infection (Chen, 2018). However, an unregulated inflammatory response can become chronic and induce tissue damage, contributing to many of the same diseases involving oxidative stress. A major part of the inflammatory response is the activation of inflammatory cells that release ROS and chemical mediators called cytokines (Biswas, 2015). Thus, inflammation can induce oxidative stress, but oxidative stress can also induce inflammation through activation of inflammatory pathways, most notably the NF-κB pathway (Figure 2). In this pathway, the transcription factor NF-κB is sequestered in the cytoplasm of the cell by the inhibitory protein IκBα. The NF-κB pathway gets activated when an inflammatory agent such as ROS binds to toll-like receptors (TLR) on the surface of the cell, triggering the activation of a multi-subunit IκB kinase (IKK) complex. Upon activation, IKK phosphorylates IκBα, resulting in IκBα degradation and nuclear translocation of NF-κB. NF–κB in the nucleus of the cell induces the transcription of pro-inflammatory cytokines such as TNF-α.

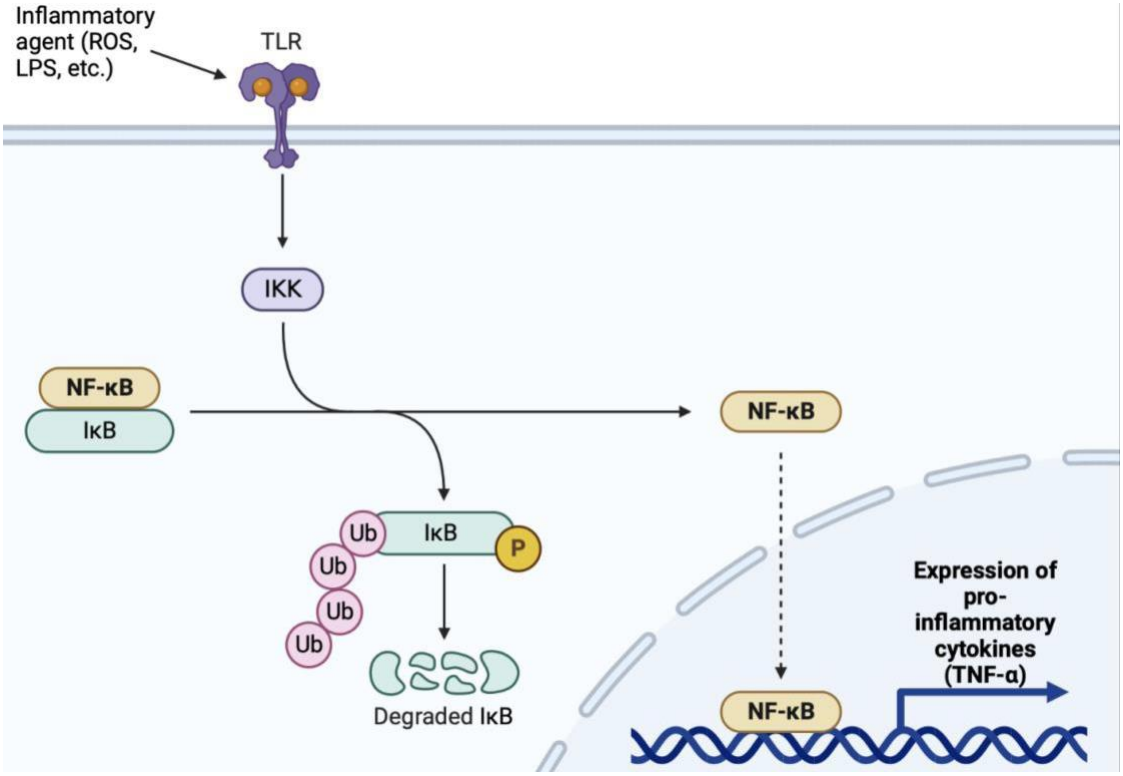

Figure 2. Schematic of the NF-κB pathway. Created with BioRender.com

This tightly linked relationship between oxidative stress and inflammation, which is prevalent in many chronic diseases, is seen in Alzheimer's disease (AD). AD is a tragic, neurodegenerative disease that afflicts more than six million Americans (Alzheimer's Association). It is the most common form of dementia, characterized by cognitive decline and memory loss. There is no curative measure against AD, and by 2050, the number of Americans living with Alzheimer's is projected to increase to 13 million (Alzheimer's Association). This represents a dire need for the scientific community to gain more insight into the pathophysiology of AD and identify potential therapeutic targets (Merighi, 2022). Hallmark pathologies of AD include the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) in the brain. These induce a chronic inflammatory response by microglia, resident immune cells of the central nervous system (CNS), resulting in neurodegeneration (Sun, 2022). Although there is evidence Aβ and NFTs play a crucial role in AD pathology, failure of Aβ-targeted immunotherapies to actually improve cognitive function in AD patients demonstrates a significant role of other pathogenic elements such as neuroinflammation and oxidative stress in the progression of symptoms (Merighi, 2022).

Thus, current approaches to AD therapies are focusing on targets of these elements, specifically the NF-κB and Nrf2 pathways. Cannabidiol (CBD) is one of these potential therapeutics gaining interest because of its known antioxidant and anti-inflammatory effects.

CBD is a non-psychoactive cannabinoid derived from the *Cannabis sativa* L. plant and is being studied for its pharmacotherapeutic potential in treating oxidative stress and inflammation- related diseases (Atalay, 2022). CBD has been shown to have antioxidant effects on several cells. For example, one study showed that CBD protected keratinocytes by preventing the damage to cell membrane composition caused by oxidative stress induced from exposure to UVB irradiation and hydrogen peroxide (Atalay, 2020). The literature suggests that CBD elicits its antioxidant effects both directly and indirectly (Jîtcă, 2023). Directly, CBD's molecular structure with an aromatic nucleus and hydroxyl group can neutralize ROS. Indirectly, CBD influences molecular mechanisms involved in regulating oxidative stress, namely the Nrf2 pathway (Jîtcă, 2023). One study showed that CBD upregulated expression of antioxidant enzymes, such as HO-1 and NQO1 in human oral keratinocytes, increased expression and nuclear translocation of Nrf2, and decreased expression of Keap1 (Li, 2022). Another study also showed that treating endothelial cells with CBD up to 6 μM increased mRNA and protein levels of HO-1 and protein levels of Nrf2 (Böckmann, 2020).

Previously, our lab has been able to show that CBD acts as an anti-inflammatory by decreasing the production of pro-inflammatory cytokines (O'Connor, 2022). In one experiment, BV2 microglia cells were pre-treated with increasing concentrations of CBD. Then, lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria, was used to induce an inflammatory response and levels of the pro-inflammatory cytokine TNF-α were measured. The data (Figure 3) indicates that pre-treatment of 1 μM CBD significantly decreased TNF-α production, reducing inflammation. We hypothesized this to be a result of the downregulation of the NF-κB pathway. One study showed that treatment of mouse microglial cells with 10 μM CBD after LPS treatment reduced the expression of the phosphorylated p65 subunit of NF-κB below control levels, suggesting CBD blocks NF-κB pathway-dependent signaling events (dos-Santos-Pereira, 2019). Another study also showed that pre-treating BV2 microglia cells with a CBD-based compound and then exposing to LPS reduced NF-κB translocation into the nucleus (Borgonetti, 2022).

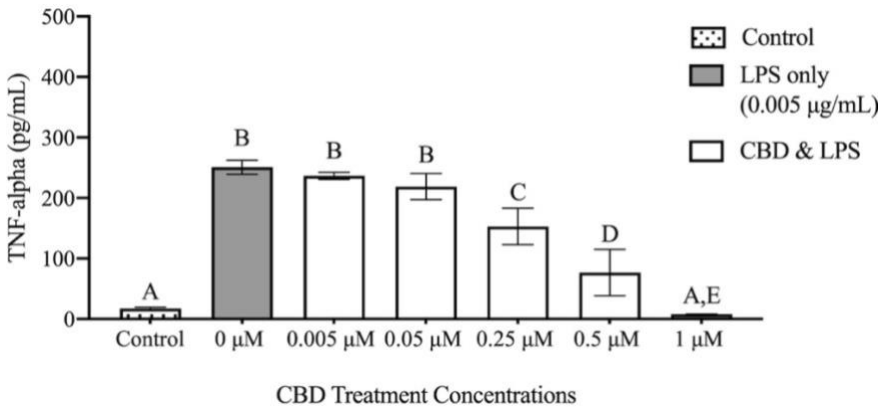

Figure 3. CBD pre-treatment attenuates LPS-induced TNF-alpha production following 8 hours of LPS treatment (0.005 µg/mL of LPS). BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.005 µg/mL). Supernatants were collected 8 hours after LPS treatment. ANOVA revealed a significant main effect of 4 hours of CBD treatment ($p \leq 0.001$) and 8 hours of LPS treatment ($p \leq 0.001$) on TNF- alpha production. Controls did not receive any treatment or drug. Different letters (A, B, C, D) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. Ns = 2 – 6. (Figure and caption from O'Connor, 2022)

Many studies have shown there is a crosstalk between the NF-κB and Nrf2 signaling pathways (Sivandzade, 2018). For example, one study showed that inducing inflammation with LPS in Nrf2 knock out mice increased hippocampal levels of inflammatory markers like TNF-α (Innamorato, 2008). The study also found that treating mice with sulforaphane, a compound known to induce the Nrf2 pathway, increased hippocampal levels of HO-1 while decreasing the abundance of microglial cells and production of TNF–α (Innamorato, 2008). Although the interconnectedness between these two pathways is complex, there are some well-characterized points of molecular crosstalk between them. Increases in HO-1 have been shown to inhibit NF-κB-mediated transcription in endothelial cells (Wardyn, 2015). In another study, p65, a subunit of NF-κB, was shown to physically associate with Keap1 and inhibit Nrf2-dependent transcription by decreasing Nrf2 binding to DNA and enhancing Nrf2 ubiquitination (Yu, 2011). Additionally, p65 is known to compete with Nrf2 for the transcriptional co-activator CBP (CREB-binding protein)-p300 complex, so overexpression of p65 prioritizes transcription of NF-κB driven genes and knockdown of p65 promotes transcription of Nrf2 driven genes (Wardyn, 2015). Also, Keap1 has been found to negatively regulate IKK by triggering its autophagic degradation and preventing its phosphorylation (Wardyn, 2015). Other points of crosstalk include GSK3β, which phosphorylates Nrf2 and p65, β-TrCP, which regulates Ikβ degradation and nuclear Nrf2 degradation, and p62, which

mediates Keap1 degradation and activation of TNF-α receptor-associated factor 6 (TRAF6) (Wardyn, 2015).

Although it is clear in the literature that CBD does affect both the Nrf2 and NF-κB pathways, the mechanism of action of CBD on these pathways is not well defined (Atalay, 2022). Determining how CBD intersects these pathways, which may be that it interferes with an element of the Nrf2 and NF-κB crosstalk, has become a point of interest in our research. Because our lab has previously shown that CBD decreased the production of pro-inflammatory cytokines, most likely by way of the NF-κB pathway, we aim to confirm CBD is also affecting the Nrf2 pathway as a launching point into our investigation of its mechanism. Understanding CBD's role in these pathways is important for assessing the use of CBD as a potential anti-inflammatory and antioxidant therapy. In this study we show preliminary evidence that CBD affects the Nrf2 pathway by increasing Nrf2 protein levels within two cell lines that are commonly used as immune models for studying diseases. BV2 is a microglial cell line derived from C57/BL6 mice and immortalized by infecting with a retrovirus. RAW 264.7 is a macrophage cell line derived from BALB/c mice and immortalized by infecting with Abelson leukemia virus. For this study, both cell lines were grown and maintained and then treated with CBD. Cell lysates were collected, and Western blots were performed to analyze levels of the Nrf2 protein. We hypothesize that treating BV2 and RAW264.7 cells with CBD will increase Nrf2 protein levels.

METHODS

BV2 & RAW 264.7 Cell Culture

BV2 and RAW 264.7 cells were grown in 10 cm tissue culture-treated plates containing 10 mL of a complete cell medium that was comprised of Dulbecco's Modified Eagle Medium with L-Glutamine (DMEM; Caisson Laboratories, Smithfield, UT), 1% Pen/Strep antibiotics, and 10% Fetal Bovine Serum. The plates were placed in a cell culture incubator, which was maintained at 37 degrees Celsius at 5% CO_2, to promote cell growth. When cells reached approximately 70–80% confluency, they were subcloned. BV2 cells were subcloned by aspirating off the old media, scraping the cells into fresh complete media using a cell scraper, and dividing the media and cells (20–30%) between new plates with 10 mL of fresh complete media in each. RAW 264.7 cells were subcloned by aspirating off the old media, washing the plate with 3 mL of phosphate-buffered saline (PBS; Caisson Laboratories), and incubating with 3 mL of trypsin for 5 minutes to lift the cells. Following trypsin incubation, fresh

complete media was added to the plate, and the media and cells were transferred to a conical tube (sometimes gentle scraping with a cell scraper was needed to help detach the cells). The cells were isolated by centrifugation (5 minutes at 1.1 RCF) in conical tubes. After supernatants were aspirated, the cells were resuspended in complete media and divided (20–30%) between new plates with 10 mL of fresh complete media in each. Following subcloning of both cell lines, plates were returned to the incubator.

When there were enough plates to run an experiment, cells were lifted using the subcloning procedures detailed above. To determine cell concentration a sample of the cells and media were mixed with Trypan Blue (Caisson Laboratories) and placed in a cell counter, Countess II FL (Life Technologies). Cells were seeded at 200,000 cells per well in 3.5 cm 6-well plates with 2 mL of complete medium in each well. The 6-well plates were incubated overnight prior to treatment.

CBD Treatment & Lysate Collection

CBD powder isolate (Eureka 93, Eureka, MT) was dissolved in dimethyl sulfoxide (DMSO) to create a 100 mM stock solution. Aliquots of the CBD stock solution were stored at -20 degrees Celsius. The experimental concentration of CBD was prepared just prior to treatment by diluting the stock solution with serum-free media (SFM) so that adding 100 µL of CBD to the wells would result in a concentration of 1 µM of CBD in each well. BV2 and RAW 264.7 were treated with 1 µM of CBD for five different durations of time (30 minutes, 1, 2, 4, and 8 hours). One well on each plate did not receive CBD treatment (no treatment/NT) and was treated with SFM to serve as the negative control.

Cell lysates from each well were collected after their respective treatment times. After aspirating off the supernatant in each well, the wells were washed with PBS. After aspirating off the PBS, the plates were immediately placed on ice, and lysis buffer was added to each well. The lysis buffer consisted of mammalian protein extraction reagent (M-PER; Invitrogen, Waltham, MA) and protease and phosphatase inhibitors. After 5 minutes on ice, lysates were removed by scraping the bottom of the well with a cell scraper. Lysates from each well were aliquoted, placed on ice for 30 minutes, and then stored at -20 degrees Celsius until needed for analysis.

Bradford Assay

To measure the amount of total protein in each cell lysate sample, a Bradford Assay was performed. 5 µL of each lysate sample was added in duplicate to a 96-well plate, along

with 250 µL of Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). After plates incubated in the dark for 40 minutes, the plates were read on a FluoStar Omega plate (BMG Labtech, Cary, NC) reader at an absorbance of 595 nm. Protein concentrations for each sample were reported in mg/mL. These values were used to prepare samples for Western blotting.

Western Blot

Western blots were performed to semi-quantify levels of Nrf2 in the samples. β-actin was used as the loading control. Samples were prepared at 0.25 µg/µL (RAW 264.7 lysates) or 0.5 µg/µL (BV2 lysates) by mixing with MPER lysis buffer and Laemmli sample buffer. The samples were then boiled at 100 degrees Celsius for 5 minutes. 50 µL of sample or 8 µL of ladder (Bio-Rad Laboratories, Hercules, CA) were loaded into BioRad 4–15% Mini-PROTEAN TGX Precast Protein Gels. The gels were run in a gel electrophoresis apparatus with Tris-glycine running buffer for approximately one hour at 120 V, 300 watts, and 3.0 amps. The gels were washed with DI water and then submerged in cold Towbin buffer, rocking for 30 minutes. PVDF membranes (Immobilon-P Transfer Membrane; Sigma-Aldrich, St. Louis, MO) were prepared by briefly hydrating them with methanol, washing with DI water for 2 minutes, and submerging in cold Towbin buffer, rocking for the remainder of the 30 minutes. The filter paper was also saturated in cold Towbin buffer prior to transfer. Semi-dry transfers were then run using the Trans-Blot SD transfer cell at 18 V and 0.3 amps per gel for 30 minutes. After the transfer was complete, the membranes were cut and blocked in TBST+5% BSA for 2–4 hours, rocking at room temperature. Mouse polyclonal primary antibodies for Nrf2 (Santa Cruz Technology, Dallas, TX) were diluted 1:1000 in TBST+5% BSA. Rabbit polyclonal primary antibodies for β-actin (Proteintech Group Inc, Rosemont, IL) were diluted 1:75,000 or 100,000 in TBST+5% BSA. Primary antibodies were added to the membrane strips and shaken overnight at 4°C. The next day, primary antibodies were removed, and the membrane strips were washed with TBST 4-5 times for 15 minutes each wash. Goat-anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for Nrf2 were diluted 1:10,000 in TBST. Goat-anti-rabbit secondary antibodies (Jackson ImmunoResearch) for β-actin were diluted 1:75,000 or 1:100,000 in TBST. Secondary antibodies were added to the membrane strips, rocking at room temperature for 1.5 hours. The membrane strips were washed again, as described above. The strips were treated with Super Signal West Pico chemiluminescent reagent (Thermo Scientific, Rockford, IL) for two minutes and imaged using Syngene GeneSys Image Acquisition Software (Bangalore, India).

Lastly, densitometry was performed using ImageJ software (Rasband et al., 1997) to compute the pixel densities of the Nrf2 and β-actin bands.

RESULTS

Cannabidiol increases Nrf2 protein levels in BV2 and RAW264.7 cell lysates

To gauge the antioxidant potential of CBD, protein levels of Nrf2 were semi-quantified by Western blotting and densitometry. CBD (1μM) was added to BV2 microglia cells and RAW 264.7 macrophage cells for increasing durations of time. These data illustrate that the relative density of Nrf2 in BV2 lysates generally increases as a function of treatment duration (see Figure 4A). The exception to this trend is the drop in Nrf2 levels at the 2-hour treatment duration before increasing again. A similar trend is also observed in the RAW 264.7 lysates with exceptions at the 2- and 8-hour mark (see Figure 4B).

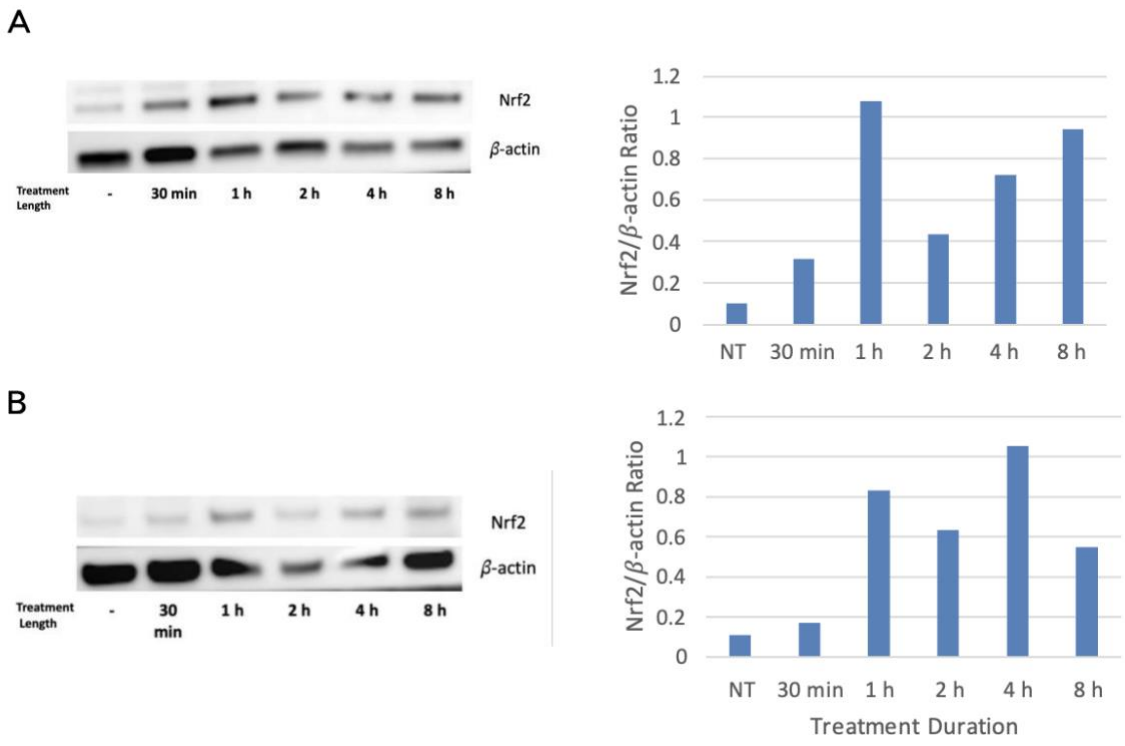

Figure 4. CBD treatment increases Nrf2 protein levels. A) BV2 microglia cells and **B)** RAW 264.7 macrophage cells were incubated with CBD (1 μM) for different durations of time (30 min, 1 h, 2 h, 4 h, 8 h) or with serum-free media (NT). The left panels show the Western blots for Nrf2 and β-actin proteins. β-actin was used as a loading control to normalize these data. The right panels show

the graph obtained from densitometry of the Western blot to semi-quantify relative Nrf2 protein levels within the lysates. This relative density of Nrf2 is reported as the ratio of Nrf2 to β-actin on the y-axis as a function of increasing treatment duration on the x-axis.

DISCUSSION

The results obtained from this experiment indicate that CBD is affecting the Nrf2 pathway. This is seen by the general increase in Nrf2 protein levels in both BV2 and RAW 264.7 cells after treating them with 1 μM of CBD and subsequent Western blot analyses. We chose 1 μM of CBD as our concentration for this experiment because this was the concentration our lab found to be effective for reducing inflammation (O'Connor, 2022). We are unsure as to why Nrf2 levels decrease at 2 h in BV2 cells and 2 and 8 h in RAW264.7 cells, but we hypothesize this might be that the Nrf2 present within the cell at the time of treatment is mainly used up by those time points, and the cell must go through the process of transcription and translation for newly Synthesized Nrf2, which takes time. The data reported are from only one experiment run over the course of the year. This experiment will be replicated to confirm these results and generate enough data to run statistical analyses so that we can say there is a significant effect of CBD treatment on Nrf2 levels. Additional experiments were conducted, and lysate samples were collected, but one challenge we faced was reliably getting Nrf2 bands to appear on the Western blots. Some of these additional experiments included treating BV2 and RAW 264.7 with different concentrations of CBD only (10 μM, 20 μM, and 50 μM), LPS only, CBD pre-treatment before LPS stimulation, and LPS before a CBD post-treatment. Unfortunately, analyses of these lysates will be part of a future project, but these results will begin our investigation into CBD's mechanism of action on the Nrf2 and NF-κB crosstalk. We are curious to see how LPS, a known activator of the NF-κB pathway, affects the Nrf2 pathway and how CBD modulates this as a pre-treatment and post-treatment.

To further investigate CBD's mechanism of action, we also want to analyze levels of other proteins involved in the Nrf2/NF-κB crosstalk as described in the introduction, such as HO- 1, Keap1, GSK3β, p65, and p62. Perhaps CBD's mechanism involves influencing one of these elements. Additionally, Western blots only provide a semi-quantitative measure of Nrf2 protein levels. Further analyses can include more quantitative measures, such as RT-qPCR, which can provide deeper insight into Nrf2 gene expression as a result of CBD treatment. Also, fractionation experiments can be performed to separate cytosolic and nuclear lysates.

This can tell us whether the Nrf2 increased by CBD is going into the nucleus of the cell and acting as a transcription factor like we think it is. Looking at protein or transcript levels of downstream antioxidant enzymes, such as HO-1, can also give us an indication of this.

Although the antioxidant and anti-inflammatory effects of CBD are well-researched, the mechanism of action of CBD on the Nrf2 and NF-κB pathways is not well understood. Understanding exactly how CBD affects these pathways is important in developing CBD as a therapy for people afflicted with chronic inflammation and oxidative stress-related diseases, such as AD. Because inflammation and oxidative stress are tightly linked pathophysiological processes, focusing on just one or the other may not be enough to be an effective treatment for these diseases (Biswas, 2015). This increases the attractiveness of CBD as a potential therapeutic because it seems to be acting as both an antioxidant and anti-inflammatory. Although more research is needed, the present study shows CBD's potential as an antioxidant by influencing Nrf2 protein levels within the cell, which supplements previous research in our lab that shows CBD's potential as an anti-inflammatory by downregulating the NF-κB pathway.

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