THE EFFECTS OF THERAPEUTIC ANTIOXIDANT COMPOUNDS ON THE PRODUCTION OF PROINFLAMMATORY CYTOKINES IN BV2 MICROGLIAL CELLS

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ABSTRACT

Alzheimer's Disease (AD) is a neurodegenerative disease that afflicts the elderly. Three hallmark pathologies of AD include amyloid beta (Aβ) accumulation, inflammation, and oxidative stress. The aggregation of Aβ protein fragments develops plaques in the brain, increasing levels of proinflammatory cytokines. Chronic activation of proinflammatory cytokines increases inflammation, further exacerbating AD pathologies. Oxidative stress is linked to many neurodegenerative diseases, due to an imbalance of the antioxidant system and increased reactive oxygen species (ROS) production, which further damages DNA and chronically activates microglial cells. Past research has shown that antioxidant compounds alleviate proinflammatory cytokine production. The aim of this study was to further explore this by studying the therapeutic capabilities of two potent antioxidant molecules (L2 and L4) in attenuating oxidative stress and inflammation in microglial cells. We hypothesized that L2 and L4 will reduce proinflammatory cytokines induced by LPS treatment.

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Lastly, I would like to thank the undergraduate students. I am honored to have worked with such a great team. I will always cherish the laughs we shared and your friendship. I will look back at my time in Dr. Chumley's lab with reverence. Thank you, everyone.

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INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, memory loss, disorientation, difficulty organizing thoughts, personality changes, and motor dysfunction. AD is the most common cause of dementia, consisting of 80% of the dementia diagnoses in the US. AD is a growing problem as the life expectancy of the world's population continues to increase (Weller and Budson, 2018). The most common biological markers of AD consist of accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) resulting from hyperphosphorylation of the microtubule associated protein, tau (White, et al., 2016).

Amyloid beta (Aβ) plaques are formed when amyloid precursor protein (APP) is cleaved in the incorrect location. One of the fragments after cleavage of APP is amyloid beta. Amyloid beta has a tendency to cluster with other amyloid beta fragments, causing the buildup of plaques between neurons. These plaques contribute to AD pathology by blocking cell signaling at synapses and triggering inflammation, which further contributes to AD pathology (Lakhan 2019). Neurofibrillary tangles are formed by abnormal accumulations of tau, which occur when tau is phosphorylated. In healthy neurons, tau aids in microtubule function which allows for vesicular transport of neurotransmitters and other materials which are necessary for cell survival. When tau is phosphorylated, it detaches from microtubules and sticks to other tau molecules, which join to create tangles in neurons. These tangles interfere with microtubule function and block the neuron's ability to communicate with other neurons and effector cells through synapses (Corey Bloom 2007).

Inflammation and oxidative stress are two of the hallmark pathologies of AD. While inflammation is a natural response to activation of the immune system, neuroinflammation is a

strong contributor to AD pathology. Microglial cells are macrophages in the central nervous system which are responsible for clearing cellular debris and dead neurons through their inflammatory response. Chronic inflammation and subsequent stimulation of microglial cells can lead to more tissue damage and cell death through toxic compounds such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF- α), nitric oxide (NO), prostaglandin E2 ($PGE2_,$) and superoxide (O_2^-) (Block and Hong, 2005). This contributes to a positive feedback loop of activation of microglial cells and neuroinflammation. Oxidative stress, another hallmark pathology of AD, is linked to inflammation. Inflammation caused by Aβ is able to activate NADPH oxidase which can cause the generation of excess reactive oxygen species (ROS) and oxidative stress (Fischer and Maier, 2016). Both dysfunction of the antioxidant system (Birben, et al., 2011) and microglial activation due to neuronal damage create ROS and contribute to oxidative stress in the brain. Oxidative stress can activate microglial cells that lead to the production of proinflammatory cytokines, such as interleukin 1 beta (IL-1β), interleukin-6 (IL-6), and TNF- α (Agostinho, et al., 2010).

Metal ion dysregulation and oxidative stress have been linked to cognitive decline in AD and other neurodegenerative diseases. Metal ions are critical for life and contribute to many essential functions for survival such as regulating gene expression, cell signaling, and catalyzing enzymatic reactions. However, accumulation of these ions is believed to contribute to AD by exacerbating amyloid beta plaques (Fischer and Maier, 2016). Post-mortem analyses of amyloid beta plaques in AD patients revealed an accumulation of copper, iron, and zinc that was significantly higher than levels observed in normal brains. Copper amyloid complexes are able catalyze the formation reactive oxygen species (ROS), leading to neuron death (Cheignon, et al.,

2018). Various trials for AD therapeutics have focused on oxidative stress and metal ion dysregulation. However, these therapeutics have not been successful. (Liu, et al., 2019).

Dr. Kayla Green's lab in the TCU Chemistry Department created two powerful antioxidant molecules, L2 (3,6,9,15-tetraazabicyclo[9.3.1]penta-deca-1(15),11,13-trien-13-ol) and L4 (1,4,11,13-Tetraazabis(2,6-pyridinophane)- 8,17-diol). Both L2 and L4 are N-heterocyclic amines. Dr. Green's lab previously found that L2 and L4 could be promising therapeutic treatments for neurodegenitive diseases through preventing oxidative stress and metal ion dysregulation. However, this experiment was not focused on chelation therapy for metal ion dysregulation due to many failed trials with drugs that were supposed to work in this way. Due to this reason, this experiment focused on the ability of L2 and L4 in reducing oxidative stress and inflammation. They also found that L4 is a stronger antioxidant than L2 and is capable of protecting BV2 microglial cells from cell death after treatment with hydrogen peroxide. Western blot studies found that along with increasing cell viability, L4 also enhanced the cellular antioxidant defense capacity by activating the Nrf2 signaling pathway (Johnston, et al., 2019). The Nrf2 signaling pathway protects against oxidative stress brought on by injury and inflammation through suppressing pro-inflammatory cytokines, inhibiting pro-inflammatory pathways, or expressing antioxidant proteins.

Previous studies have shown that L2 and L4 are able to protect BV2 microglial cells from cell death through preventing oxidative stress and metal ion dysregulation (Lincoln, et al., 2013). This project aims to study the ability of L2 and L4 to block the release of proinflammatory cytokines in BV2 cells after LPS treatment and to demonstrate the connection between oxidative stress and inflammation. In order to do this, the cytokine producing capabilities of BV2 cells treated with LPS were determined. Then, the BV2 cells were treated with L2 and L4 in order to

attempt to reduce cytokine production. LPS is an endotoxin from the cell wall of gram-negative bacteria which is capable of activating the immune system and creating an inflammatory state. For this project, BV2 cells were plated in a 96-well plate and treated with LPS. Using an ELISA, the amount of TNF- α was quantified. Then another assay was carried out by treating the cells various concentrations of L2 and L4 before LPS treatment. The amount of TNF- α was again quantified using an ELISA, and western blots were used to detect the presence of signaling proteins in the TLR-4 signaling pathway, which is known to signal the production of pro-inflammatory cytokines, including TNF- α, IL-1β, and IL-6. Since oxidative stress and inflammation are linked, the goal of this study is to test the therapeutic potential of two potent antioxidant compounds in inhibiting the signaling cascade of pro-inflammatory cytokines that contribute to neuroinflammation and neurodegeneration in microglial cells. This has the potential to not only be used for AD, but also for other diseases that present with increased neuroinflammation and oxidation.

MATERIALS AND METHODS

BV2 Cell Maintenance

BV2 microglial cells were maintained in a cell incubator at 37 degrees Celsius at 5% CO_2. Cells were grown in 10 cm tissue culture dishes in complete cell medium containing 10 mL of DMEM, 5% Pen/Strep, 5% L-Glutamine, and 15% Fetal Bovine Serum. When the cells became around 80-90% confluent, they were passaged. Cells were passaged by first aspirating off the medium and washing with 5 ml of PBS. After washing the cells, 5 ml of complete medium were added and a cell scraper was used to remove the cells from the bottom of the dish. The cells were then spun down in a centrifuge at 1.2 rcf for 10 minutes in a 15 ml conical tube. Once a pellet was formed, the supernatant was aspirated, leaving a 2mL solution in the tube. The cells were counted

using a hemocytometer, adding 10 microliters of the resuspended cells to each side and manually counting using a microscope.

Experimental Treatment of Cells

First, cells were treated with LPS. LPS was added to a 6 well plate of BV2 cells so that the total concentration of LPS was 5 ug/mL, 0.5 ug/mL, 0.05 ug/mL, 0.005 ug/mL, 0.0005 ug/mL, and a control of 0 μg/mL. All treatments lasted 16 hours prior to measurement of baseline levels of TNF- α using an enzyme linked immunosorbent assay (ELISA). Once the cytokine concentrations were determined, it was determined that the rest of the experiment would be carried out using an LPS concentration of 0.0005 ug/mL.

L2 and L4 Compounds

Cells were administered L2 and L4 one hour prior to treatment with LPS. L2 or L4 was added to a 6-well plate at concentrations of 12.5 μM, 1.25 μM, 0.125 μM, along with two control wells (one with LPS only and another with 12.5 uM of L2 or L4 only). All cells were then administered LPS at a concentration of 0.0005 ug/mL. L2 and L4 compounds were provided by Dr. Kayla Green. After 12 hours, the supernatant was collected.

Enzyme Linked Immunosorbent Assays (ELISA)

ELISAs were performed in order to determine the levels of TNF- α in LPS treated BV2 cells that were pre-treated with L2 and L4 as compared to BV2 cells without L2 and L4 pre-treatment. The BioLegend Mouse TNF- α ELISA MAX Deluxe Set was used in this experiment. 96-well plates were used. Each well was treated with 100uL of diluted capture antibody, then incubated overnight. Then, the plate was washed 4 times using a wash buffer of PBS and 0.05% Tween-20. The wells were then blocked using diluted Assay Diluent A and the plate was incubated for 1 hour at room temperature on a plate shaker (500 rpm with a 0.3 cm circular orbit). The plate

was washed 4 times and then treated with the 100 uL of diluted standards and supernatant per well. The plate was sealed and incubated for two hours with shaking. The plate was washed four times and then treated with 100 uL of detection antibody per well. The plate was sealed and incubated for one hour at room temperature with shaking. The plate was then treated with 100uL of Avidin HRP (secondary antibody) per well, sealed, and incubated at room temperature for 30 minutes with shaking. The plate was washed 5 times, soaking 45 seconds per wash. 100uL of TMB substrate solution was added to each well and the plate was incubated for 15 minutes in the dark. Then, 100uL of stop solution (2N H_2SO_4) was added to each well. The plates were then read on a plate reader (BMG LabTech FLUOstar Omega, Cary, NC) at absorbances of 450 nm and 570 nm.

RESULTS

LPS causes an increase in proinflammatory cytokine production:

In order to induce inflammation in the BV2 cells, various concentrations of LPS were added. An ELISA was then completed to measure the amount of TNF- α in each well. A one-way analysis of variance (ANOVA) was used to determine if there was a statistically significant difference between the TNF-α concentration values for each concentration of LPS added. A p-value below 0.05 indicates that the likelihood of the null hypothesis (that the concentration of LPS added does not affect TNF-α production) being true is very small. The light bars indicated that the LPS was not diluted, and the dark bars indicate a 1:10 dilution of LPS. Each set of bars corresponds to a different concentration of LPS. The light bars (neat concentration of LPS) showed a high level of TNF-α production. The dark bars (1:10) dilution showed a relationship between the concentration of LPS added and the production of TNF-α. The results indicated a statistically significant difference between each condition, as well as demonstrating that increasing

concentrations of LPS led to more TNF-α production (Figure 1), supporting the idea that LPS causes inflammation.

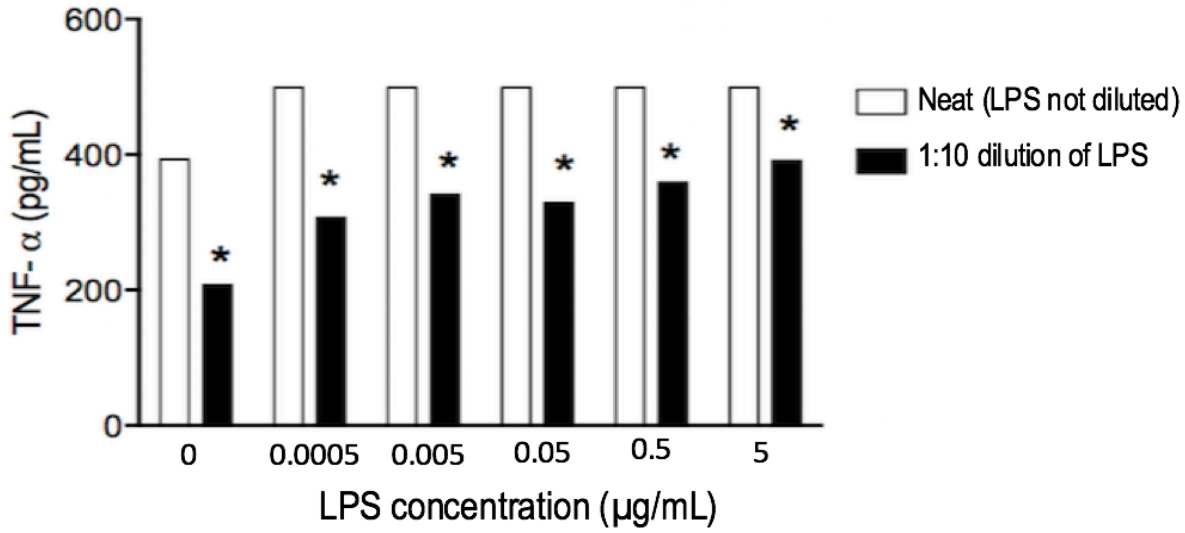

Figure 1: **LPS increased the production of TNF- α**
A one-way ANOVA revealed a significant difference between the various concentration of LPS and the corresponding levels of TNF- α ($p<0.05$) *. For the neat samples, every concentration of LPS above zero led to levels of TNF- α that were at or above the maximum amount that the ELISA could detect. For the diluted samples (1:10), as the LPS concentration was increased, TNF-α levels increased as well.

L2 and L4 administration decreased TNF- α production:

BV2 cells treated with 0.0005 ug/mL of LPS and then treated with various concentrations of L2 and L4. The general trend in the preliminary data showed that L2 and L4 both decreased the production of TNF- α after LPS treatment (data not shown). However, due to the circumstances with COVID-19, the data was unable to be properly analyzed.

DISCUSSION

Oxidative stress and inflammation are two hallmark pathologies of AD. Additionally, oxidative stress and inflammation are linked. Inflammation leads to oxidative stress by activating

NADPH oxidase, which generates excess reactive oxygen species, leading to oxidative stress (Fischer and Maier, 2016). Oxidative stress leads to inflammation by activating microglial cells to produce proinflammatory cytokines (Agostinho, et al., 2010). Dr. Green's lab previously demonstrated that L2 and L4 are effective in protecting BV2 cells from death through their antioxidant capabilities and involvement with the Nrf2 signaling pathway (Johnson, et al., 2019). This experiment sought out to show that L2 and L4 not only attenuated oxidative stress, but that by doing so, it also decreases the production of proinflammatory cytokines. In this study, we tested whether L2 and L4 were able to protect BV2 cells from inflammation due to LPS treatment. To determine this, we first showed that LPS induces inflammation in BV2 cells by treating the cells with various concentrations of LPS and used ELISAs to measure the production of the proinflammatory cytokine, TNF- α. The results showed that increasing LPS concentrations led to increasing concentrations of TNF- α, supporting the idea that LPS induces inflammation in BV2 cells.

Next, TNF- α ELISAs were completed in cells pre-treated with L2 and L4 in order to see if L2 and L4 could protect against LPS induced inflammation. While the data was unable to be analyzed, a general trend in the preliminary data suggested a possible protective effect of L2 and L4 against LPS induced TNF- α production. TNF-alpha levels were generally lower in wells that received higher concentrations of L2 and L4, suggesting less TNF- α in the treated cells, but unfortunately this data was unable to be analyzed due to Covid-19. The preliminary data suggests that before the LPS was added, L2 and L4 may have effectively reduced inflammation before it took place, possibly through the interaction between the TLR4 pathway and the Nrf2 pathway (Figure2). The cells produced less TNF- α before initiating the TLR4 pathway, which could be

due to the Nrf2 pathway inhibiting the TLR4 pathway so that it does not produce proinflammatory cytokines such as TNF- α as efficiently.

One of the goals of the study was to further show the connection between inflammation and oxidative stress. The protective ability of antioxidant compounds such as L2 and L4 against TNF- α production demonstrate that inflammation and oxidative stress are connected. The figure below demonstrates how two pathways related to oxidative stress and inflammation (Nrf2 and TLR4) interact. In futures studies, more ELISAs would be completed to see the effect of L2 and L4 on other pro-inflammatory cytokines, such as IL-1β and IL-6. In addition, western blots would be completed to test for the presence of proteins involved in these signaling pathways at different time intervals. This would hopefully provide more information regarding which proteins involved in these pathways are affected by L2 and L4 administration.

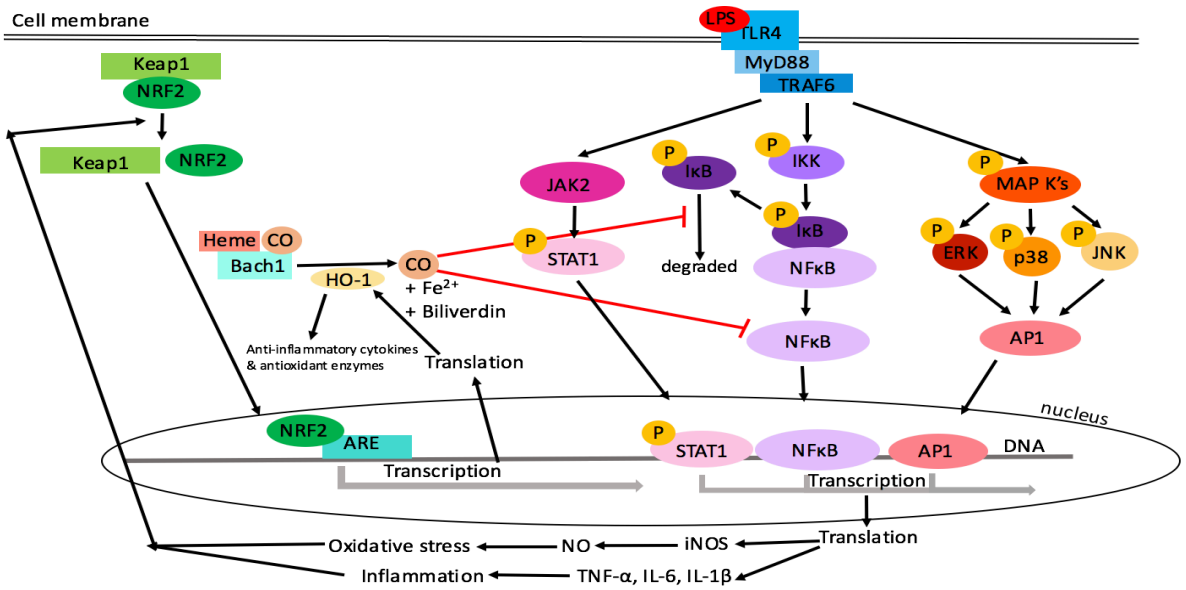

Figure 2: **Signaling pathways involved in inflammation and oxidative stress**
This figure demonstrates the interactions between the Nrf2 pathway and TLR4 pathway. The Nrf2 pathway is activated by oxidative stress and inflammation. The Nrf2 pathway leads to the production of anti-inflammatory cytokines and antioxidant enzymes, as well as inhibiting the degradation of IκB and subsequently preventing the production of proinflammatory cytokines and NO through the TLR4 pathway. (Figure based on information from the following papers: Ahmed et al., 2017; Cai et al., 2018; Dai et al., 2014; Gong et al., 2015; Ko et al., 2016; Wang, et al. 2019).

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