EFFECTS OF POTENT ANTIOXIDANT COMPOUNDS ON PROINFLAMMATORY CYTOKINE PRODUCTION IN BV2 MICROGLIAL CELLS

by

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ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that is the most common form of dementia. It is expected that AD cases worldwide will exceed 100 million by 2050. There are two key pathologies to AD which are extracellular β-amyloid (Aβ) plaques and intracellular hyperphosphorylated, neurofibrillary tau tangles. The main brain regions impacted by AD damage are the cerebral cortex and the hippocampus; areas that are in charge of learning and memory. The presence of the Aβ plaques can activate microglial cells in the brain. Microglial cells are macrophages in the central nervous system and are responsible for the innate immune response in the brain. Activation of these microglial cells can induce an inflammatory response and oxidative stress. Inflammation is a natural response to fighting off pathogens, however, chronic inflammation can be detrimental to tissues and this damage plays a pivotal role in neurodegeneration. Inflammation and oxidative stress are two hallmarks of AD pathogenesis and are connected. Dysfunction of the antioxidant system can cause accumulation of reactive oxygen species (ROS) which causes oxidative stress. The presence of ROS activates an inflammatory response leading to the production of proinflammatory mediators and cytokines. ROS and proinflammatory cytokines can lead to more Aβ plaques that cause more inflammation, thus, causing a self-perpetuating process. Previous research has shown that agents that lead to reduction in inflammation and oxidative stress are potential drug therapies for AD and other neurodegenerative disorders. This study was done in collaboration with Dr. Green's lab in the TCU Chemistry Department. Dr. Green has successfully created compounds, one being L4, that act as potent antioxidants. Prior studies have demonstrated that L4 has the capability to rescue BV2 microglial cells and increase cell survival during oxidative stress. This research aimed to examine the potential therapeutic properties of the antioxidant compound L4 against the

inflammatory response *in vitro*, using BV2 microglial cells, following stimulation by lipopolysaccharide (LPS). LPS is a known element to induce inflammation in microglial cells. First, it was determined that LPS treatment can successfully induce the secretion of the proinflammatory cytokine TNF-α. This was done by treating cells with different concentrations of LPS for specific treatment periods. Next, the experiment was repeated, except cells were pretreated with the compound L4 for one hour before adding the LPS treatment. This was done to study the ability of L4 to block the proinflammatory cytokine production. The hypothesis was that L4 pretreatment would reduce proinflammatory cytokine production, however, the concentrations of L4 used in this study were likely not high enough to block the inflammatory response. Therefore, further research needs to be done to determine the appropriate concentration of the L4 that would be the most therapeutic against the inflammatory response.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

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INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that is the most common form of dementia and accounts for nearly 70% of dementia cases around the world. The total number of cases worldwide is expected to exceed 100 million by 2050 (Liu et al., 2019). As the world's life expectancy continues to increase, AD will stay a major healthcare issue. AD symptoms include, but are not limited to, cognitive decline, memory loss, loss of language skills, loss of motor function, and personality changes (Weller & Budson, 2018). The two common biological markers of AD are the deposition of extracellular β-amyloid (Aβ) plaques and intracellular hyperphosphorylation of tau proteins (Liu et al., 2019). The Aβ plaques are formed from proteolytic cleavage of the amyloid precursor protein, in which one of the fragments is Aβ (Finder & Glockshuber, 2007). These fragments of Aβ aggregate forming the senile plaques that are believed to be part of the cause of AD, rather than being a result of the disease. The Aβ plaques are thought to block signaling at cell synapses and initiate inflammation, further contributing to AD pathogenesis (Lahkan 2019).

Two hallmarks of AD are inflammation and oxidative stress. Inflammation is a natural response within the immune system and its goal is to eliminate the cause of cellular damage, in addition to removing dead cells and initiating repair mechanisms (Ahmed et al., 2017). There are two forms of inflammation: acute and chronic. Acute inflammation is self-limiting and can be beneficial to the host. On the other hand, chronic inflammation, especially when uncontrolled, can be harmful to cells and lead to cellular damage (Ahmed et al., 2017). Prolonged chronic inflammation and the presence of damaged tissue leads to the activation of microglia and astrocytes, which generate a neuroinflammatory response (Fischer & Maier, 2015). Astrocytes

and microglia are the two main cellular mediators involved with the inflammatory response (Wyss-Coray & Rogers, 2012).

Microglial cells are macrophages in the central nervous system (CNS) that are responsible for the innate immune response in the brain and are critical for the immunological insult to neurons (Block & Hong, 2005). Microglia account for 10-15% of CNS cells that, along with immune response responsibilities, aids in trophic support and maintaining homeostasis (Zheng et al., 2018). When activated, microglia undergo morphologic alternations changing from resting microglia to activated microglia (Block & Hong, 2005). Activation of microglial cells occurs through inflammation in response to Aβ plaque accumulation (Johnston et al., 2019). Microglia release a variety of proinflammatory soluble factors through the TLR4 pathway,

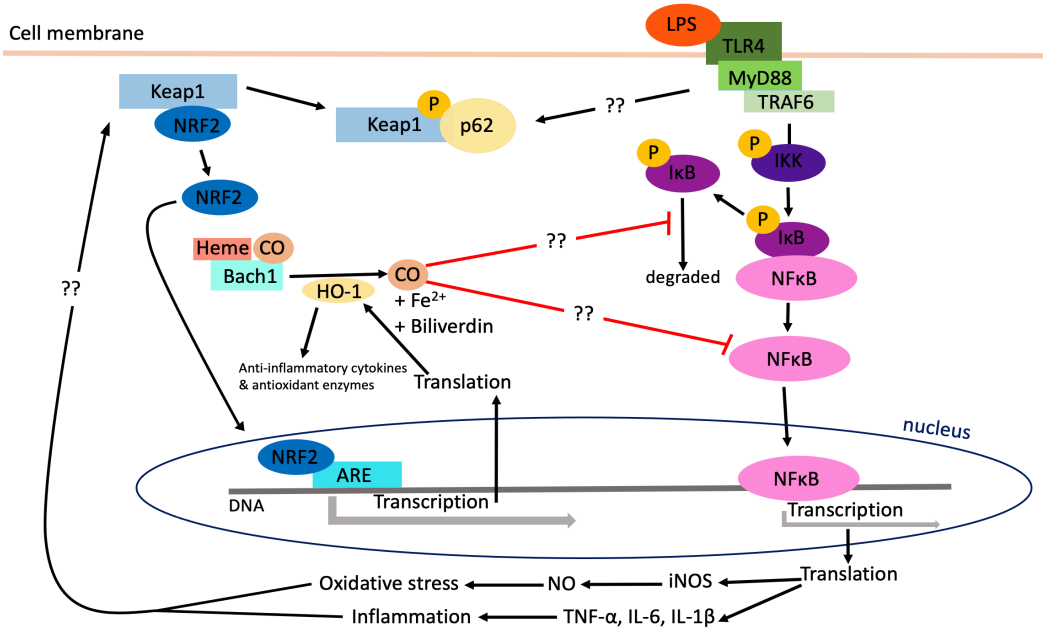

Figure 1. Signaling pathways involved in inflammation and oxidative stress, simplified for the purpose of this study. This figure demonstrates the interaction between theTLR4 pathway and the Nrf2 pathway. The TLR4 pathway is activated by LPS and through the signaling cascade, NFκB is activated and translocates into the nucleus where it acts as a transcription factor for proinflammatory mediators and cytokines. Oxidative stress and inflammation activate the Nrf2 pathway. Nrf2, once activated, translocates into the nucleus and acts as a transcription factor that leads to anti-inflammatory cytokines and antioxidant enzymes. (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. Layout based on pathway from Caitlyn Vilas, TCU Departmental Honors Project 2020.)

Figure 1, such as prostaglandin E2, nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin-(IL-6), IL-1β, and tumor necrosis factor (TNF-α). These mediators generate an inflammatory response and lead to oxidative stress, which contributes to the progression and pathogenesis of AD (Lee & Kang, 2014). In response to inflammation and oxidative stress, there are anti-inflammatory factors released such as nuclear factor (erythroid-derived-2)-like 2 (Nrf2) (Lee & Kang, 2014; Block & Hong, 2005). Nrf2 plays a critical role in the regulation of the antioxidant response. When activated, Nrf2 will translocate into the nucleus, where it binds to the antioxidant response element (ARE) and act as a transcription factor that initiates expression of antioxidant enzymes (Johnston et al., 2019). Nrf2 signaling protects against oxidative stress as a result of inflammation or injury by inhibiting the proinflammatory cytokines, downregulating the proinflammatory pathways, or by expressing antioxidant proteins such as HO-1 (Johnston et al., 2019).

Oxidative stress, which is linked to inflammation, is another important contributor to the pathogenesis of AD. Aβ plaque deposition leads to inflammation. The inflammatory response activates NADPH oxidase that causes an increased production of ROS (Fischer & Maier, 2015). Oxidative stress can occur due to the activation of microglial cells and the dysfunction of the antioxidant system leading to expression of proinflammatory cytokines and ROS

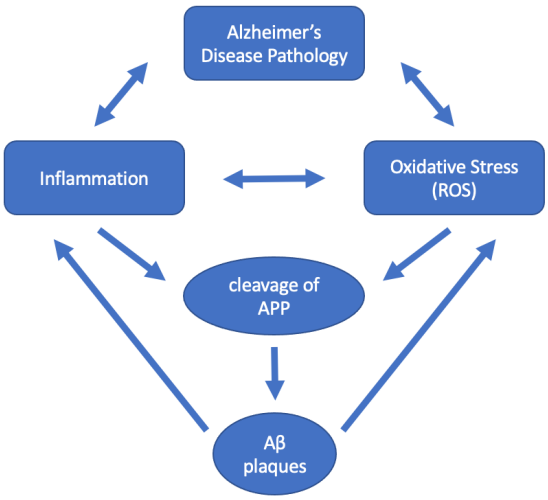

Figure 2. The relationship of AD pathology, it is all connected. Inflammation and ROS lead to the cleavage of APP which generates Aβ plaques. Presence of these plaques furthers inflammation and oxidative stress, it is a positive feedback loop that exacerbates the progression of neurodegenerative diseases, like AD.

(Agostinho et al., 2010). Inflammation and ROS are highly regulated processes, and both can lead to the cleavage of APP, therefore, the production of Aβ. The production and presence of Aβ plaques will lead to further inflammation and oxidative stress, Figure 2. Thus, in AD, this is a self-perpetuating process that plays a role in the progression and pathogenesis of the disease. Therefore, agents that lead to a reduction in NO, iNOS, and proinflammatory cytokines and mediators are a target for drug therapies (Lee & Kang, 2014).

Metal ion dysregulation has also been linked to neurodegenerative diseases. Abnormal concentrations of certain metals may contribute to pathogenesis of diseases like AD (Liu et al., 2019). Metal ions are important for essential life functions because they bind to metalloproteins which allows for maintenance of cell structure, mediation of cell signaling, regulation of gene expression, and catalyzation of enzymatic activity (Kim et al., 2018). The accumulation of these metal ions, such as zinc and copper, is believed to be a contributor to Aβ plaque in AD pathogenesis (Fischer & Maier, 2015). For example, zinc has been shown to promote the resistance of cleavage of Aβ peptides, therefore allowing Aβ to accumulate and adding to further oxidative stress and inflammation (Kim et al., 2018). Unregulated redox-active metals, such as iron and copper, react with molecular oxygen and produce excess ROS (Johnston et al., 2019). The use of metal chelators is proving to be a promising way to reduce redox stress and therefore use as a potential therapeutic for neurodegenerative disorders (Liu et al., 2019).

Dr. Kayla Green's lab in the TCU Chemistry Department has successfully created a family of compounds that act as powerful antioxidants that have the capability of radical scavenging and metal ion capture. L4 (1,4,11,13-Tetraazabis(2,6-pyridinophane)-8,17-diol), shown in Figure 3, is an N-heterocyclic amine that can chelate metal ions and act as an

antioxidant (Johnston et al., 2019). In a previous study, Dr. Green's lab showed that L4 pretreatment could protect HT-22 cells from oxidative stress induced cellular damage in a dose dependent manner (Johnston et al., 2019). L4 can also activate the Nrf2 pathway that promotes antioxidant properties. Nrf2 signaling protects against oxidative stress as a result of inflammation or injury by inhibiting the proinflammatory cytokines, downregulating the proinflammatory pathways, or by expressing antioxidant proteins (Johnston et al., 2019).

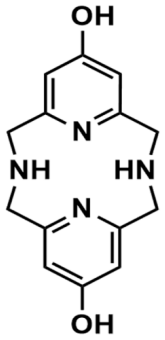

Figure 3. L4 Compound (figure taken from Johnston et al., 2019)

This research focused on the ability of L4 in reducing oxidative stress and specifically, inflammation. BV2 microglial cells, a common model for neurodegenerative diseases, are immortalized murine neonatal cells. These cells are able to induce neuroinflammation via inflammatory responses during endogenous chemical stimulation (Stansley et al., 2012). Microglial cells can be stimulated with lipopolysaccharide (LPS) which results in a neuroinflammatory response (Subedi et al., 2019). LPS is an endotoxin from the cell wall of a gram-negative bacteria which can activate the immune system and induce an inflammatory state (Chen et al., 2012). In this study we used BV2 microglia treated with LPS to induce an inflammatory response to determine the capabilities of L4 to block the release of proinflammatory cytokines. BV2 cells were seeded in 6-well plates and treated with five different concentrations of LPS at five different time intervals. To quantify the amount of TNF-α produced, an Enzyme Linked Immunosorbent Assay (ELISA) was run. Western blotting was done to see the expression of Nrf2 in the LPS treatment. Another experiment was carried out pretreating the cells with L4 at two concentrations for 1 hour prior to LPS treatment. A TNF-α

ELISA was run again to measure the levels of TNF-α in the pretreatment study. This study focused TNF-α, since this study aimed to examine the potential therapeutic properties of the antioxidant compound L4 against the inflammatory response of BV2 microglial cells, following stimulation by LPS treatment.

MATERIALS AND METHODS

BV2 Cell Maintenance

The BV2 microglial cells, Figure 4, were maintained in a cell incubator at 37 degrees Celsius and 5% CO_2. The cells were grown in 10cm tissue culture dishes, Figure 5, in complete medium containing 10mL of Dulbecco's Modified Eagle Medium (DMEM), 5% Penicillin-Streptomycin, 5% L-glutamine, and 15% Fetal Bovine Serum. The cells were monitored and when they reached 80-90% confluency, they were passaged.

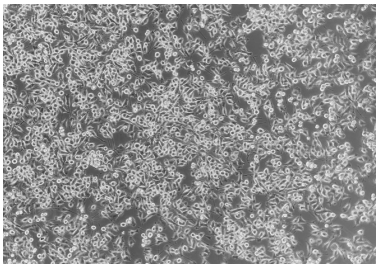

Figure 4. Photo of a plate during LPS treatment experiment, showing BV2 microglial cells.

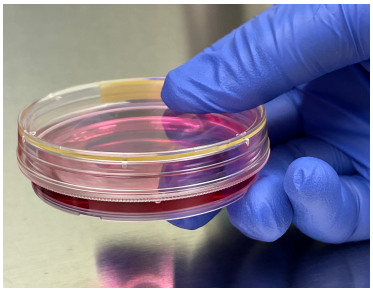

Figure 5. Photo of a tissue culture dish with complete DMEM medium and cells growing at the bottom.

Cells were passaged by first aspirating off the medium and washing with 5-10mL PBS. After washing the cells, 5mL of complete medium was added and a cell scraper was used to loosen the cells from the bottom of the dish. Cells were pipetted up and down to break up clumps before being transferred into a new 10cm plate which contained fresh complete medium and were then returned to the incubator.

Prior to experiments, cells were seeded in 6-well plates. To prepare for cell seeding, the old medium was aspirated off, the cells were washed in 5-10mL of PBS, and 10mL of complete medium was added before using a cell scrapper to remove cells from the bottom of the dish. Cells were pipetted into a 15mL conical tube and centrifuged for 10 minutes at 1.2 rcf where the cells spun down into a pellet. The supernatant was aspirated off, leaving 2mL solution in the conical tube, where the pellet was broken up. The cells were counted using a hemocytometer, by adding 10µL of the resuspended cells to each side and manually counted using a microscope.

LPS Treatment of Cells

Cells were first treated with LPS to determine which concentration and time of treatment would be sufficient to elicit an inflammatory response. For this experiment, cells were seeded in 6-well plates at 200,000 cells per well and treated with six different concentrations of LPS and five different treatment times. The LPS concentrations were 5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, and a control of 0µg/mL and had treatment times of 4 hours, 8 hours, 12 hours, 14 hours, and 24 hours. The timeline of the experiment is shown in Figure 6A. Cells were collected and aliquoted into microcentrifuge tubes. Cell lysates were stored in the lysis buffer MPER which also contained a protease inhibitor and a phosphatase inhibitor and then stored in the -20 degrees Celsius freezer. The supernatant was stored separately in the -20 degrees Celsius freezer.

We measured TNF-α using an ELISA and once these cytokine concentrations were determined, it was decided that LPS concentrations of 0.5µg/mL and 0.05µg/mL with a treatment time of 8-hours would be used for the following experiment with the L4 compound.

L4 Pretreatment of Cells

The same protocol for cell seeding was followed. Two 6-well plates were prepared by seeding 200,000 cells per well. After 24 hours of incubation, these cells were pretreated with five different concentrations of L4 for 1 hour prior to LPS activation. These concentrations were 1µM, 0.1µM, 0.01µM, 0.001µM, and 0.0001µM, and a control of 0µM, no L4. Plate 1 had an LPS concentration of 0.5µg/mL and plate 2 had an LPS concentration of 0.05µg/mL. Both plates were treated with LPS for 8 hours before cells were collected. Figure 6B shows the timeline for this experiment. Collected cells were aliquoted into microcentrifuge tubes and stored in the -20

degrees Celsius freezer. Supernatant was collected separately from the cell lysates, which were stored in the lysis buffer MPER with the added protease inhibitor and phosphatase inhibitor.

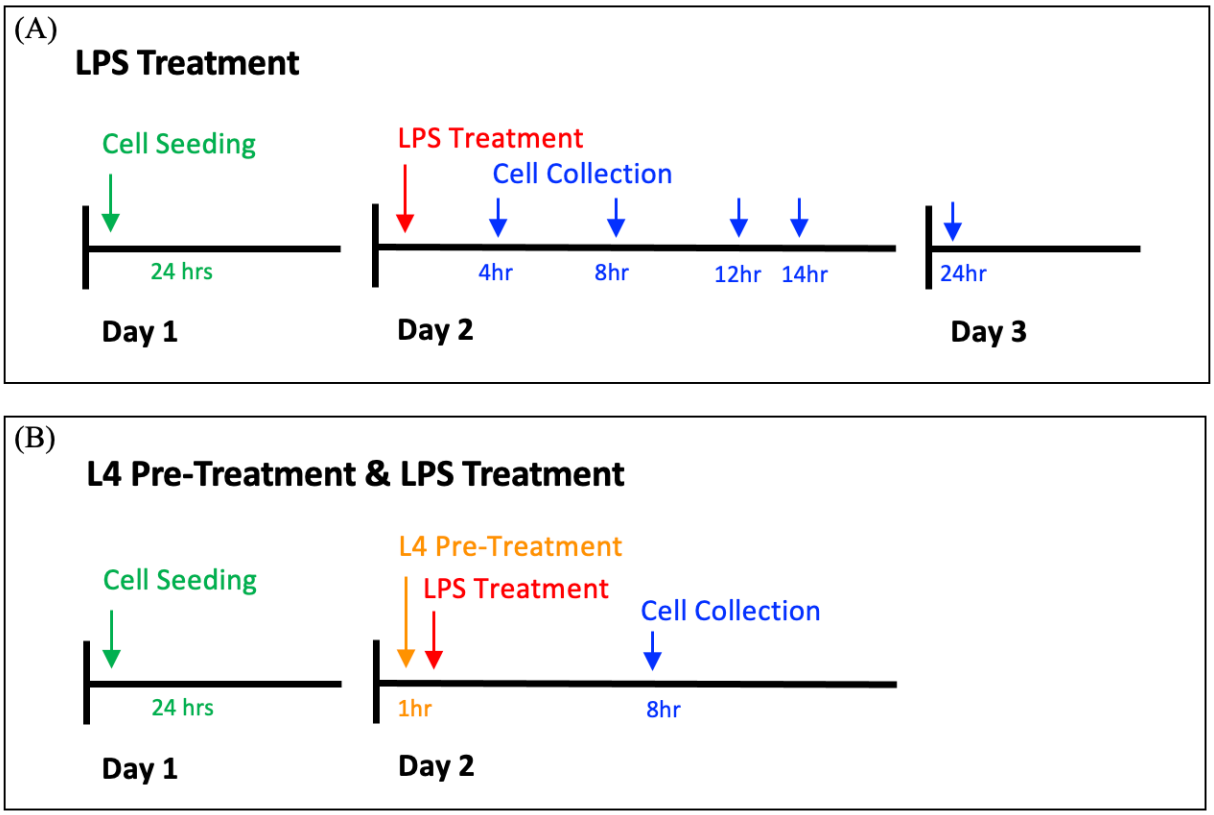

Figure 6. Timeline of experimental procedure. (A) 24 hours after seeding, BV2 microglial cells were treated with six different LPS concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, & 0µg/mL) for five different treatment times (4hr, 8hr, 12hr, 14hr, & 24hr). Following each treatment time, cell lysates and supernatant was collected. (B) BV2 Microglia were seeded and 24 hours later were pretreated with five concentrations of L4 (1uM, 0.1uM, 0.01uM, 0.001uM, & 0.0001uM) for 1 hour before LPS activation for 8 hours, where two LPS concentrations were used 0.5µg/mL and 0.05µg/mL.

Enzyme Linked Immunosorbent Assays (ELISA)

ELISAs were performed in order to determine the levels of TNF-α in both the LPS treated BV2 cells experiment and the experiment when BV2 cells were pretreated with L4. The BioLegend Mouse TNF-α ELISA MAX Deluxe Set was used to perform this assay. For the LPS treatment samples, two plates were needed to run the samples neat and two dilutions, 1:10 and 1:100. To prepare for the assay, two 96-well plates were used and coated overnight with 100µL

of diluted capture antibody. Then each plate was washed four times using a wash buffer of PBS and 0.05% Tween-20. The wells were then blocked using diluted Assay Diluent A and incubated for an hour at room temperature on a plate shaker (500 rpm with a 0.3cm circular orbit). The plates were washed four times again, followed by loading 100µL of sample or standard to the appropriate wells after which the plates were sealed and incubated for two hours with shaking. The plates were washed four times and then 100µL of detection antibody was added to each well before they were sealed and incubated for an hour with shaking. Next, the plates were treated with 100µL of Avidin HRP (secondary antibody) per well, the plates were sealed and incubated at room temperature for 30 minutes with shaking. The plates were washed five times, letting the washing buffer soak for one minute every wash. 100µL of TMB substrate solution was added to each well and the plates were incubated for 15 minutes in the dark. Finally, 100µL of stop solution (2N H_2SO_4) was added to each well. The plates were then read on the plate reader, using the protocol for BioLegend TNF-α and BMG plate at an absorbance of 450.

The same protocol was followed for the TNF-α ELISA run for the L4 pretreatment experiment. The cells were also run neat and 1:10 and 1:100 dilutions.

Bradford Assay & Western Blotting

A Bradford Assay was preformed to measure the protein levels in each sample of cell lysates. Cell lysates were run with dilutions 1:2 and 1:4 for the Bradford assay. Samples were diluted in MPER lysis buffer. 96-well plates were used. 5µL of sample or standard were added to the appropriate well followed by 250µL of Bradford reagent. The plates were incubated in the dark for five minutes before being read on the plate reader. The Bradford Assay protocol was used which is 595nm absorbance with shaking for 15 seconds. Upon analysis, it was determined

that 1:2 dilutions exceeded the standard so 1:4 dilution protein concentrations were used to calculate the appropriate sample to buffer ratio for running Western Blots.

Western blotting was used to analyze Nrf2 protein levels in the samples. β-actin was used a loading control. Samples were mixed with sample buffer and boiled for five minutes in the 100-degree Celsius dry bath before adding 20µL of sample or 20µL of all-blue ladder to the any kD gel and run in the SDS Page apparatus at 200 volts (held constant), 3 amps, 300 watts, for 40 minutes. Once the run was complete, the gel was prepared for transfer by soaking in Towbin for 16 minutes. During this time, the PVDF membrane was cut and washed with methanal, distilled water, and then washed in TBST. After setting up the appropriate filter paper, gel and membrane orientation, the transfer machine was run at 25 volts, 0.3 amps, and 300 watts for 1:05 minutes. After transfer was complete, the membrane was washed in TBST before ponceau staining and then cutting the membrane. The membrane was then put in a 5% BSA in TBST blocking buffer for two hours on the shaker. The primary antibody for Nrf2 was a rabbit polyclonal, 1:250 concentration and the primary for β-actin was a mouse monoclonal, 1:500 concentration. Both primaries were placed on the membrane overnight and placed in the fridge on a rocker. On day two, the primary antibody was removed and an hour worth of TBST washes was completed before adding the secondary antibody. A goat anti-rabbit Nrf2 (1:25,000) and goat anti-mouse β-actin (1:15,000) were added and the membrane was put on the shaker for two hours. Another hour worth of TBST washes was done before the membrane was prepped for imaging.

RESULTS

LPS Induces Production of the Proinflammatory Cytokine TNF-α

In order to induce an inflammatory response in the BV2 cells, LPS was added to the cells at increasing concentrations and for increasing treatment times. TNF-α production was measured using an ELISA and illustrated a dose-dependent relationship between LPS concentration and TNF-α production. Increasing the concentrations of LPS increased the production of TNF-α. Figure 7 shows the results from the ELISA. The 8-hour LPS treatment period generated the largest amount of TNF-α. The 12-hour and 14-hour LPS treatment periods showed that TNF-α production dropped off. This drop in TNF-α could be due to cell death, consumption of LPS which then slows production of TNF-α, or more likely, that TNF-α is a small protein and degrades rapidly.

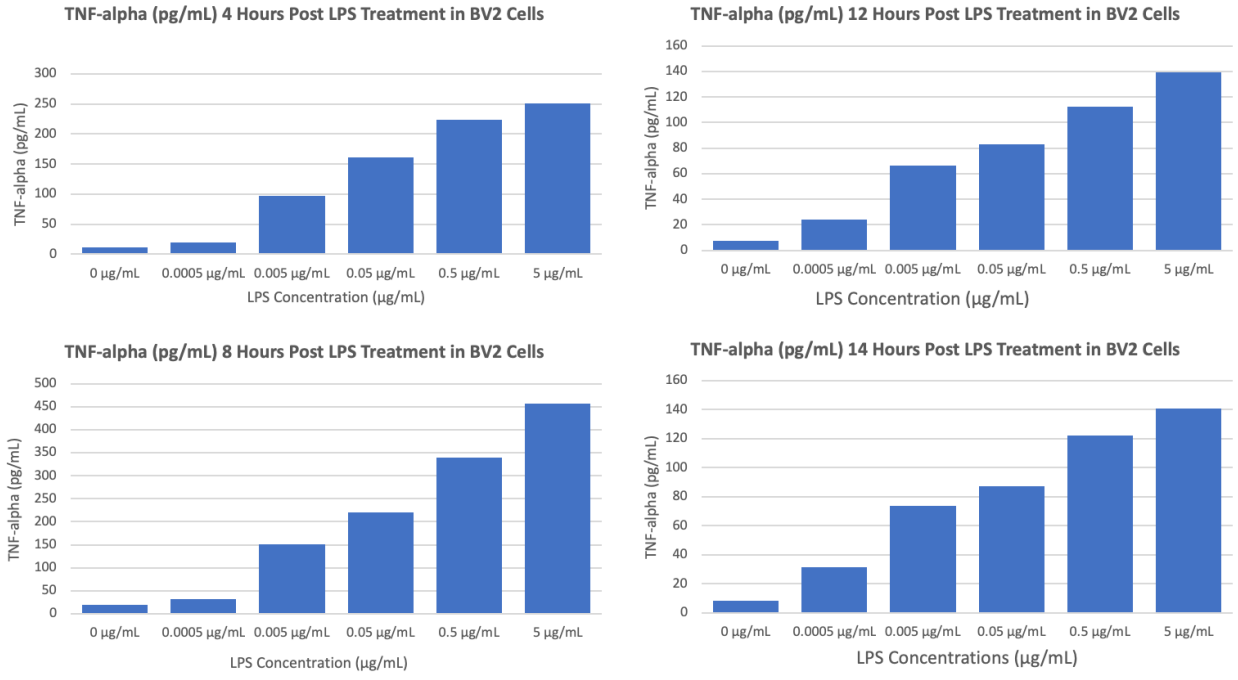

Figure 7. TNF-α ELISA results following LPS treatment. BV2 microglial cells were treated with six different LPS concentrations (5µg/mL, 0.5µg/mL, 0.05µg/mL, 0.005µg/mL, 0.0005µg/mL, & 0µg/mL) for five different treatment times (4hr, 8hr, 12hr, 14hr, & 24hr). Results are shown in pg/mL. LPS treatment of 8-hours induced the largest production of TNF-α.

Nrf2 Western Blots from LPS Treatment

Microglia can also release anti-inflammatory mediators like Nrf2. Nrf2 signaling protects against oxidative stress as a result of inflammation or injury by inhibiting the proinflammatory cytokines. Nrf2 Western Blotting was done to see Nrf2 production in the cells following LPS inducing an inflammatory response. The initial piloting stage results from these western blots, show that there is the presence of Nrf2 in our samples, Figure 8. B-actin was the loading control, and the bands are present around 40kDa. It appears that Nrf2 bands, 60-68kDa, get stronger with increased concentrations of LPS. The highest concentration of LPS is on the left and decreases as you move to the right. Due to COVID-19 constraints, a brief period out of the lab due to a snowstorm, and difficultly getting consistent and usable results, densitometry was not able to be completed for these samples. In addition, due to these circumstances, western blotting for the samples in the L4 pretreatment experiment were not performed.

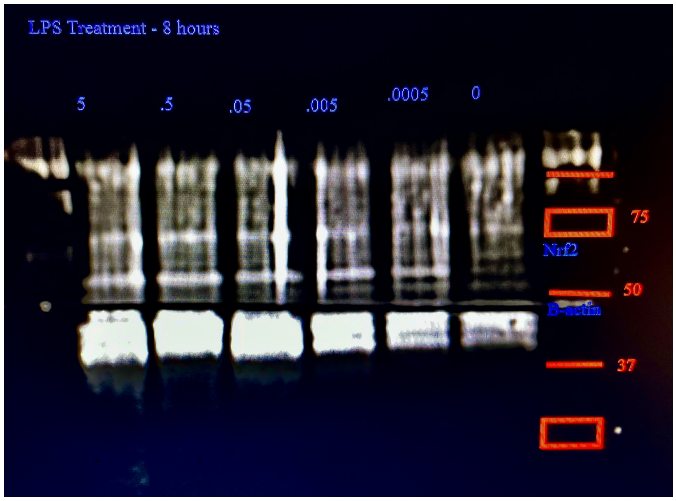

Figure 8. Nrf2 Western Blot. Nrf2 at 60-68kDa. B-actin used as a loading control at 40kDa. It appears that higher concentrations of LPS show a stronger Nrf2 band.

L4's Capability at Blocking the Inflammatory Response

The LPS only experiment was repeated only this time, a 1-hour pretreatment with L4 at two concentrations was added to the protocol. It was decided to go ahead with the L4 pretreatment experiment while we continued with assays for just LPS treatment to get some

preliminary data for this project. The two concentrations of LPS used, 0.5µg/mL and 0.05µg/mL, were used because they produced a strong response, but not the greatest response. It was of concern that using our highest concentration of LPS, 5µg/mL, that it would produce a ceiling effect, where we would not be able to see an effect of L4 pretreatment. TNF-α was again measured using an ELISA, results in Figure 9. The preliminary data shows that the concentrations of L4 were likely not high enough to block proinflammatory production.

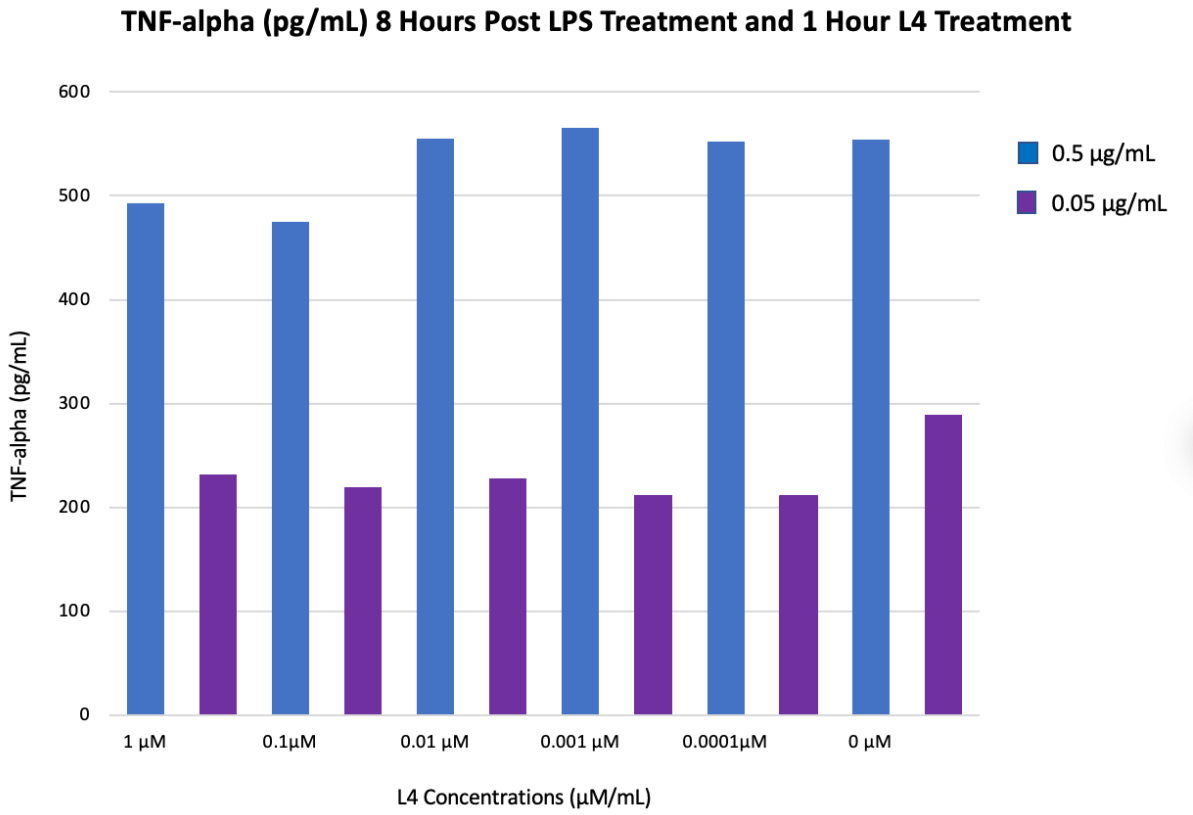

Figure 9. TNF-α ELISA results following L4 pretreatment. BV2 Microglia were pretreated with five concentrations of L4 (1µM, 0.1µM, 0.01µM, 0.001µM, 0.0001µM, and 0µM) for 1 hour before LPS stimulation for 8 hours. Two LPS concentrations were used 0.5µg/mL and 0.05µg/mL. The results are shown in pg/mL. These preliminary results show these concentrations of L4 most likely were not high enough to block the inflammatory response.

DISCUSSION

Two hallmark pathologies of AD are oxidative stress and inflammation. Oxidative stress leads to inflammation by inducing microglial cells to produce proinflammatory cytokines, proinflammatory mediators, NO, and iNOS expression (Lee & Kang, 2014; Agostinho et al., 2010). Inflammation can generate oxidative stress by activating NADPH oxidase, which generates ROS (Fischer & Maier, 2015). ROS and inflammation lead to the cleavage of APP which generates Aβ plaques, which in turn increases inflammation and oxidative stress. In previous studies, Dr. Green's lab has shown that L4 is effective in protecting BV2 cells through its antioxidant properties involved with the Nrf2 pathway (Johnston et al., 2019).

This study sought out to show that L4 attenuated oxidative stress in addition to decreasing the expression of proinflammatory cytokines. In this experiment, we first demonstrated that BV2 cells stimulated with various concentrations of LPS, induces an inflammatory response. ELISAs were used to measure the production of the proinflammatory cytokine TNF-α, and the results showed that increasing concentrations of LPS, increased expression of TNF-α. This supports other studies that demonstrated that LPS induces an inflammatory response in BV2 microglial cells. Next, we pretreated BV2 microglial cells with two concentrations of L4 and found that these concentrations did not seem to have an effect on decreasing the production of TNF-α. These results could be due to a number of factors besides L4 concentrations not being high enough to block the inflammatory response. The pretreatment time of only 1-hour may not have been long enough, we may need to increase this time. Additionally, the LPS concentration and treatment time may have resulted in too high of an inflammatory response that it would have overpowered the L4 pretreatment effect. Therefore, we need to reevaluate the LPS concentrations and treatment time, potentially decreasing to 2- or 4-

hour treatment times versus the 8-hour period used in this study. Lastly, another possible reason we saw a reduction in TNF-α levels is that the Nrf2 pathway, which is activated by L4, may not interact with the TLR4 pathway that leads to the production of proinflammatory cytokines. These two signaling pathways could be separate within the cell, thus, the effects of LPS and L4 may be occurring independently. It could be one, or a combination, of these factors that resulted in us not seeing a decrease in TNF-α levels after LPS stimulation following L4 pretreatment.

Future directions for this project can go a number of directions. One being that the LPS only treatment experiment must be repeated, and more assays run to be able to complete statistical analysis on the results. The results from this research show only one assay's results, which is not enough to run statistics on. In addition, more work needs to be done with L4. This would involve increasing the concentrations of L4 to see if higher dosages would be more efficient at reducing the inflammatory response. In addition to the L4 concentrations, the treatment period can also be adjusted, 1-hour might not have been sufficient. In these studies, looking at proinflammatory markers like TNF-α, IL-1β, or IL-6, as well as anti-inflammatory markers, like IL-10, Nrf2, or HO-1 through ELISAs and Western Blotting will be beneficial in understanding the bigger picture of inflammation and oxidative stress. These experiments and corresponding results will help support the idea that L4 has antioxidant and anti-inflammatory properties. If proven to be successful, L4 could reveal to be a possible therapeutic agent for AD and other neurodegenerative diseases that present with oxidative stress and inflammation.

REFERENCES

Agostinho, P., Cunha, R. A., & Oliveira, C. (2010). Neuroinflammation, Oxidative Stress and the Pathogenesis of Alzheimers Disease. *Current Pharmaceutical Design*, *16*(25), 2766–2778. https://doi.org/10.2174/138161210793176572

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1863*(2), 585–597. https://doi.org/10.1016/j.bbadis.2016.11.005

Block, M. L., & Hong, J.-S. (2005). Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. *Progress in Neurobiology*, *76*(2), 77–98. https://doi.org/10.1016/j.pneurobio.2005.06.004

Cai, B., Seong, K.-J., Bae, S.-W., Chun, C., Kim, W.-J., & Jung, J.-Y. (2018). A synthetic diosgenin primary amine derivative attenuates LPS-stimulated inflammation via inhibition of NF-κB and JNK MAPK signaling in microglial BV2 cells. *International Immunopharmacology*, *61*, 204– 214. doi: 10.1016/j.intimp.2018.05.021

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., … Trapp, B. D. (2012). Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury Is Independent of Hematogenous TLR4. *Journal of Neuroscience*, *32*(34), 11706–11715. https://doi.org/10.1523/jneurosci.0730-12.2012

Dai, X.-J., Li, N., Yu, L., Chen, Z.-Y., Hua, R., Qin, X., & Zhang, Y.-M. (2014). Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. *Cell Stress and Chaperones*, *20*(2), 321–331. doi: 10.1007/s12192-014-0552-1

Finder, V. H., & Glockshuber, R. (2007). Amyloid-β Aggregation. *Neurodegenerative Diseases*, *4*(1), 13–27. https://doi.org/10.1159/000100355

Fischer, R., & Maier, O. (2015). Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease: Role of TNF. *Oxidative Medicine and Cellular Longevity*, *2015*. https://doi.org/10.1155/2015/610813

Gong, X., Ivanov, V. N., & Hei, T. K. (2015). 2,3,5,6-Tetramethylpyrazine (TMP) down-regulated arsenic-induced heme oxygenase-1 and ARS2 expression by inhibiting Nrf2, NF-κB, AP-1 and MAPK pathways in human proximal tubular cells. *Archives of Toxicology*, *90*(9), 2187–2200. doi: 10.1007/s00204-015-1600-z

Johnston, H. M., Pota, K., Barnett, M. M., Kinsinger, O., Braden, P., Schwartz, T. M., … Green, K. N. (2019). Enhancement of the Antioxidant Activity and Neurotherapeutic Features through Pyridol Addition to Tetraazamacrocyclic Molecules. *Inorganic Chemistry*, *58*(24), 16771–16784. https://doi.org/10.1021/acs.inorgchem.9b02932

Kim, A., Lim, S., & Kim, Y. (2018). Metal Ion Effects on Aβ and Tau Aggregation. *International Journal of Molecular Sciences*, *19*(1). https://doi.org/10.3390/ijms19010128

Ko, S. H., Rho, D. J., Jeon, J. I., Kim, Y.-J., Woo, H. A., Kim, N., & Kim, J. M. (2016). Crude Preparations of Helicobacter pylori Outer Membrane Vesicles Induce Upregulation of Heme Oxygenase-1 via Activating Akt-Nrf2 and mTOR–IκB Kinase–NF-κB Pathways in Dendritic Cells. *Infection and Immunity*, *84*(8), 2162–2174. doi: 10.1128/iai.00190-16

Lakhan, S. E. (2019, May 9). *Alzheimer Disease*. Practice Essentials, Background, Anatomy. https://emedicine.medscape.com/article/1134817-overview.

Lee, S.-G., & Kang, H. (2014). Inhibition of Lipopolysaccharide-Stimulated Neuro-Inflammatory Kuntze in BV-2 Microglial Cell Mediators by Tetragonia tetragonoides (Pall). *Tropical Journal of Pharmaceutical Research*, *13*(12). https://doi.org/10.4314/tjpr.v13i12.8

Liu, Y., Nguyen, M., Robert, A., & Meunier, B. (2019). Metal Ions in Alzheimer's Disease: A Key Role or Not? *Accounts of Chemical Research*, *52*(7), 2026–2035. https://doi.org/10.1021/acs.accounts.9b00248

Stansley, B., Post, J., & Hensley, K. (2012). A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, *9*(1). https://doi.org/10.1186/1742-2094-9-115

Subedi, L., Lee, J., Yumnam, S., Ji, E., & Kim, S. (2019). Anti-Inflammatory Effect of Sulforaphane on LPS-Activated Microglia Potentially through JNK/AP-1/NF-κB Inhibition and Nrf2/HO-1 Activation. *Cells*, *8*(2). https://doi.org/10.3390/cells8020194

Wang, L.-Y., Huang, C.-S., Chen, Y.-H., Chen, C.-C., Chen, C.-C., & Chuang, C.-H. (2019). Anti- Inflammatory Effect of Erinacine C on NO Production Through Down-Regulation of NF-κB and Activation of Nrf2-Mediated HO-1 in BV2 Microglial Cells Treated with LPS. *Molecules*, *24*(18), 3317. doi: 10.3390/molecules24183317

Weller, J., & Budson, A. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Research*, *7*, 1161. https://doi.org/10.12688/f1000research.14506.1

Wyss-Coray, T., & Rogers, J. (2012). Inflammation in Alzheimer Disease--A Brief Review of the Basic Science and Clinical Literature. *Cold Spring Harbor Perspectives in Medicine*, *2*(1). https://doi.org/10.1101/cshperspect.a006346

Zheng, Q., Sun, W., & Qu, M. (2018). Anti-neuro-inflammatory effects of the bioactive compound capsaicin through the NF-κB signaling pathway in LPS-stimulated BV2 microglial cells. *Pharmacognosy Magazine*, *14*(58), 489. https://doi.org/10.4103/pm.pm_73_18