THE EFFECTS OF ANTIOXIDANT THERAPY ON NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 (NRF2) EXPRESSION IN PHAGOCYTIC CELLS

by

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ABSTRACT

Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidant defenses resulting in cell damage and chronic inflammation. ROS are unstable oxygen molecules produced during normal cellular metabolic processes or accumulated from exogenous sources, including radiation, infection, or a high-fat diet. Chronic oxidative stress contributes to many disease state pathologies, including neurodegenerative disorders, cardiovascular disease, diabetes, and cancer. As an important facet of one's antioxidant defense system, all cells express nuclear factor-erythroid 2-related factor 2 (Nrf2) to help counteract excess ROS levels. Nrf2 is a transcription factor that promotes the expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1). Our study targets the expression and activation of Nrf2 in cells treated with L2, a compound developed by Dr. Kayla Green (TCU Department of Chemistry) and her colleagues. Our lab previously demonstrated the antioxidant capability of L2 and its ability to protect microglial and neuronal cells from oxidative stress. Our current research aims to examine the therapeutic potential of this compound by monitoring Nrf2 and antioxidant levels in phagocytic cells. This research could provide preliminary evidence for the efficacy of this compound as a treatment option for oxidative stress diseases.

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INTRODUCTION

Oxidative phosphorylation is a cellular metabolic pathway that produces ATP and reactive oxygen species (ROS). ROS are generated from the reduction of O_2 to the superoxide anion ($O_2^{\bullet-}$) via enzymatic NADPH oxidases or the mitochondrial electron transport chain (1). $O_2^{\bullet-}$ further generates other oxidative species, including hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), and nitric oxide (NO). ROS are highly reactive oxygen molecules vital to cellular functions, including hippocampal synaptic plasticity, regulation of vascular tone, and signal transduction cascades (2,3,4). Despite the benefits, ROS have the potential to be harmful. Homeostatic levels are maintained by protective antioxidant mechanisms that neutralize radicals or inhibit oxidative molecules. For example, heme is a prevalent porphyrin found in hemoglobin that carries oxygen within red blood cells. When heme is free in the cytosol, it is cytotoxic, and catalyzes the production of ROS (5). To counteract these effects, cells produce heme oxygenase-1 (HO-1), an antioxidant enzyme that catalyzes the rate-limiting step of heme catabolism to diminish cytosolic availability (6).

Imbalance between ROS production and the protective antioxidant defenses results in a state of oxidative stress. Oxidative stress damages biomolecules, targeting proteins, lipids, polysaccharides, and DNA, and is associated with many disease states, including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders. The cellular injury induced by ROS activates the body's innate immune response, leading to inflammation. There are two distinct forms of inflammation: acute and chronic. Acute inflammation is beneficial to the host and active for a limited duration to mediate the damage caused by a pathogen or cellular trauma. However, chronic inflammation is the result of prolonged immune system activation that results in compounding cellular damage and oxidative stress. The activation of phagocytic immune cells

is a key response to cellular damage caused by both inflammation and oxidative stress. In addition to phagocytosis of pathogens, macrophage cells, or microglia when localized in the brain, are responsible for respiratory bursts. In the inflammatory response, internal NADPH oxidases are activated and produce $O_2^{\bullet -}$ and H_2O_2 (7). These ROS are released by phagocytes in a respiratory burst that degrades engulfed pathogens. Additionally, the ROS serve as second messengers to activate further inflammation pathways (8). The interaction between chronic inflammation and oxidative stress becomes a vicious cyclical process that, if left unchecked, will lead to eventual cell death.

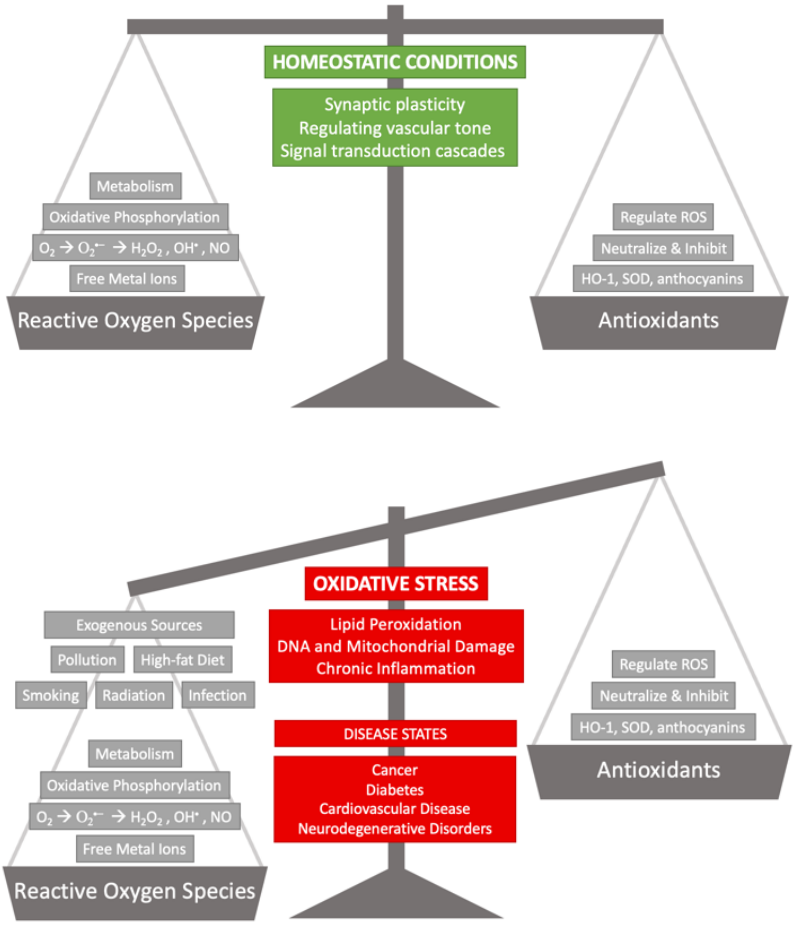

Figure 1. A representation of the balance between ROS production and antioxidant defenses. When ROS overwhelm the system, this results in oxidative stress and the pathology of numerous disease states.

Metal ion dysregulation is another facet of oxidative stress. Metal ions play an important role in the normal function of many metalloproteins. For example, copper contributes to the catalytic property of superoxide dismutase 1 (SOD1), an antioxidant enzyme that degrades $O_2^{\bullet-}$. However, mis-regulated free metal ions promote the production of ROS, leading to biomolecule damage and cell death. For instance, ferroptosis is a form of cell death resulting from iron-dependent production of ROS, and consists of three factors: excessive intracellular iron, lipid peroxidation, and reduced levels of glutathione. Iron ions within cells easily undergo redox reactions to convert between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. One conversion pathway is the Fenton reaction, in which Fe^{2+} catalytically decomposes H_2O_2 to produce ferric iron (Fe^{3+}) and $OH^{\bullet}$. $OH^{\bullet}$ is significantly more reactive than H_2O_2. It promotes ferroptosis via lipid peroxidation (9), in which lipids containing carbon-carbon double bonds are degraded (10), disrupting cellular membrane stability and triggering apoptosis (11). Some of these lipid peroxides can be neutralized by reduced glutathione (GSH), an antioxidant with high affinity for Fe^{2+} and the only enzyme that can reduce phospholipid hydroperoxides (9).

One major focus of this research is the connection between oxidative stress and neurodegenerative disorders. The brain is highly susceptible to oxidative stress due to its high energy consumption, and consequentially, high metabolic ROS production. Additionally, neurons have extensive plasma membranes rich in phospholipids, especially polyunsaturated fatty acids (PUFAs). The brain's high PUFA content, specifically docosahexaenoic acid (DHA), supports biogenesis and neuronal membrane maintenance (12). Due to their bond structure, PUFAs are more susceptible to autoxidation and Fenton reaction oxidation. This leads to lipid peroxidation and membrane instability, resulting in ferroptosis (9,14). Upon peroxidation, DHA-

containing phospholipids are converted to F4-isoprostanes. These products have been found in higher concentrations in the brains of patients with Alzheimer's Disease, indicating a correlation between oxidative stress damage and disease pathology (15).

Alzheimer's Disease (AD) is the seventh leading cause of death in the United States (16). More than six million people in the US are currently living with the disease, and that number is expected to double by 2050 (17). There is no cure for AD; current treatments are not preventative and instead only address the symptoms. AD is characterized by memory loss, cognitive decline, and loss of motor function. Two key biological markers are tau tangles and amyloid plaques. Tau protein stabilizes axon microtubules. Upon hyperphosphorylation, as seen in AD pathology, tau detaches from the microtubules and forms deposits within the neurons, disrupting intracellular transport and communication (18). Tau tangles have been found to induce oxidative stress and reciprocally oxidative stress induces pathways that result in tau hyperphosphorylation (19). Amyloid plaques are extracellular aggregates of amyloid beta (Aβ) protein often found in the hippocampus, cortex, and other brain regions. Aβ is produced by the irregular cleavage of amyloid precursor protein. In the hippocampus and cortex, elevated levels of Aβ were correlated with increased presence of oxidative stress damage to proteins, lipids, and DNA (20,21). Another study found elevated concentrations of ROS free metal ions, specifically zinc, iron, and copper, in Aβ plaques of AD patients (22).

In response to oxidative stress, a key cellular antioxidant pathway is activated to reestablish balance between ROS production and antioxidant defenses. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that interacts with the nuclear antioxidant response element (ARE) in the promoter region of target genes. The ARE is a cis-acting

enhancer in the promoter region that promotes transcription of antioxidants defenses, such as heme oxygenase-1 (HO-1) and GSH biosynthesis enzymes (9).

Low levels of Nrf2 are regularly expressed in cells. Although some translocate into the nucleus, most Nrf2 is sequestered by the Kelch-like ECH-associated protein 1 (Keap1) homodimer in the cytoplasm. Keap1 is a subunit of an E3 ubiquitin ligase complex composed of cullin-3 (Cul3) and RING Box Protein 1 (Rbx1) (23). It binds the Nrf2 ECH homology 2 (Neh2) domain of free, unstable Nrf2 via a "latch mechanism" (24). In the "open conformation," newly synthesized Nrf2 binds the ETGE motif of one Keap1 molecule with high affinity, preventing degradation. Nrf2 then interacts with the second Keap1 molecule's DLG motif with low affinity, adopting the "closed conformation." In this formation, Nrf2 is ubiquitinated by Cul3 and labeled for degradation by 26S proteasomes. Once Nrf2 is degraded, the Keap1 complex is free to bind a new Nrf2 molecule (25).

There are numerous methods of Nrf2 induction in response to oxidative stress. Certain ROS chemically modify Keap1 cysteine residues, locking the Nrf2 in the "closed formation" but blocking ubiquitination. Without the ubiquitin tag, Nrf2 remains bound to the Keap1 binding sites and is not degraded by the proteasome. Consequentially, Keap1 is less available for cytoplasmic regulation and an increased proportion of free Nrf2 translocates into the nucleus (26). A second method of Keap1 regulation is through competitive inhibition via phosphorylated p62/SQSTM1 (p-p62). p62 is a protein involved in protein degradation via selective autophagy and the ubiquitin-proteasome system (UPS) (27). Under oxidative stress conditions, p62 is phosphorylated and interacts with the Keap1 binding site with a higher affinity than Nrf2. Ichimura et al. demonstrated that cells overexpressing p-p62 showed negligible Nrf2-Keap1

interaction, significant accumulation of Nrf2 in the nucleus, and increased expression of Nrf2 target genes (25).

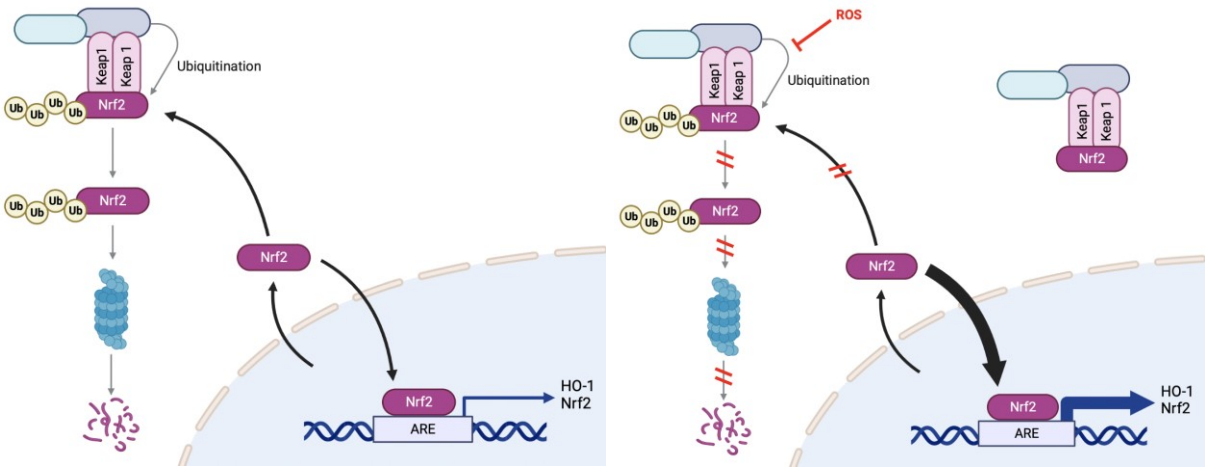

Figure 2. The Nrf2 Pathway. (**a**) Nrf2 is regularly expressed in all cell types at low levels in homeostatic conditions. While some Nrf2 translocates to the nucleus as a transcription factor, most newly synthesized Nrf2 is regulated in the cytoplasm by Keap1-mediated proteasomal degradation. (**b**) In the presence of ROS, the Keap1 complex cannot ubiquitinate Nrf2 to target it for degradation. Instead, Nrf2 occupies the Keap1 binding sites and more Nrf2 translocates to the ARE in the nucleus, promoting the expression of antioxidant target genes.

In addition to metabolic processes, there are numerous exogenous sources of ROS. These include pollutants, high fat diets, radiation, smoking, and infection. Although the Nrf2 pathway can naturally combat acute oxidative stress, these additional stressors may tip the scale to a chronic level of ROS and contribute to disease pathology. Therefore, the Nrf2 pathway has been investigated as a therapeutic target. Studies with rat models have shown that natural bioflavonoids, such as kaempferol, attenuate renal damage, oxidative stress, and inflammation with the downregulation of Keap1 and increased activation and expression of Nrf2 (29). Nrf2 pathway activation has also demonstrated cytoprotective effects in neuronal cell lines, as evident

by decreased ROS accumulation, cellular apoptosis, and mitochondrial dysfunction (30), and increased expression of target genes (31).

Building on this evidence, our research examined the therapeutic potential of a novel drug targeting the Nrf2-Keap1 complex. Dr. Kayla Green, of the TCU Chemistry Department, and her colleagues have developed a family of pyridinophane molecules that target ROS and metal-ion dysregulation. One of her compounds, L2 ($^{OH}PyN_3$), has demonstrated a multimodal approach to oxidative stress, making it a promising therapeutic target (Figure 3). The heterocyclic nitrogen-containing core binds unregulated metal ions, with the highest affinity for Cu^{2+} (32). *In situ*, the hydroxyl group of the pyridol reduced radicals in a dose-dependent manner, and counteracted metal ion redox cycling (33). In HT-22 hippocampal neuronal cells, L2 protected against hydrogen peroxide-induced cytotoxicity in a dose dependent manner. The study also found an increase in Nrf2 concentration after 24 hours of L2 stimulation (34). Based on this finding, our research aims to determine the mechanism by which L2 increases Nrf2 levels. To this, we have a few hypotheses. The first was that L2 induces oxidative stress. However, previous studies demonstrated that L2 was not toxic to HT-22 cells at the concentration used in the experiments (32), rendering this hypothesis is unlikely. Therefore, the two proposed mechanisms that we are exploring in our studies are that L2 increases the expression of Nrf2 or disrupts the Keap1 regulation of Nrf2.

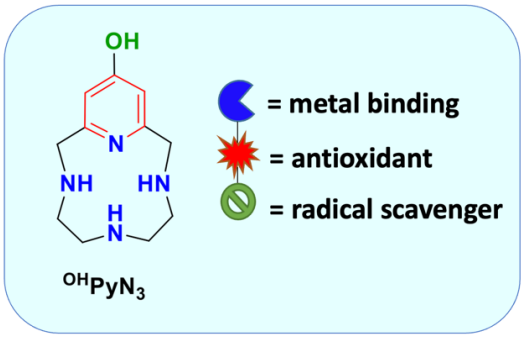

Figure 3. The chemical composition of L2. The novel compound presents a multimodal, therapeutic approach to oxidative stress.

To further evaluate the antioxidant potential of Dr. Green's molecule, we tested the compound in two phagocytic cell lines. RAW 264.7 cells are Abelson leukemia virus-transformed macrophages derived from BALB/c mice, used as an immune model for peripheral diseases (35). BV2 hippocampal microglial cells derive from C57/BL6 mice and are immortalized by a v-raf/v-myc carrying J2 retrovirus (36). BV2 cells are commonly used for *in vitro* studies of neurodegenerative disease models. In this study, cells were treated with varying doses of L2 to analyze the antioxidant effects. Western blots were conducted to analyze the expression of antioxidant markers, including Nrf2 and HO-1.

MATERIALS AND METHODS

Cell Culture

BV2 microglial cells were cultured in 10 cm tissue culture dishes in 10 mL of complete cell medium containing Dulbecco's Modified Eagle's Medium (DMEM; Caisson, Smithfield, UT), 5% Penicillin-Streptomycin, 5% L-Glutamine, and 10% Fetal Bovine Serum (FBS). Cells were maintained at 37ºC in a humidified cell incubator with 5% CO_2 and sub-cloned upon reaching 70–80% confluency. To sub-clone the cells, old media was aspirated off and 3 mL of fresh complete medium was added to the plate. Cells were detached from the bottom of the plate using a cell scraper. 20-30% of the cells were transferred into 10 mL of complete medium in a new 10 cm dish and placed in the incubator.

RAW 264.7 murine macrophages were cultured in the same conditions previously mentioned. To sub-clone, old media was removed, and the plate was washed with 5 mL phosphate-buffered saline (Dulbecco's PBS; Caisson Laboratories, Smithfield, UT). PBS was aspirated off and the cells were lifted by incubating with 3 mL of trypsin for 5 minutes. Depending on the adherence of the cells, a cell scraper was sometimes used following trypsin incubation. 20-30% of the lifted cells were transferred into 10 mL of complete medium in a new 10 cm dish and returned to the incubator.

Prior to treatment experiments, cells were lifted following the procedures listed above and counted in Trypan Blue (Caisson Laboratories, Smithfield, UT) using Countess II FL (Life Technologies, Carlsbad, CA). The cells were seeded in 6-well plates at 200,000 cells/well with 2 mL of complete cell medium.

L2 Preparation

A 2 mM stock of L2 in ultrapure water was acquired from Dr. Kayla Green (TCU Department of Chemistry) and stored at 4 ºC. Serial dilutions of the stock were used to prepare 1, 10 and 50 μM concentrations in ultrapure water.

L2 Treatment of Cells

Cells were plated in 6-well plates at a density of 200,000 cells per well with complete cell medium. After 12 hours of incubation, cells were treated. L2 was added at different concentrations (1, 10 and 50 μM) for varying timepoints (1, 2, 3, 4, 5, 6, 12, and 24 hours). One well in each experiment received no treatment and served as a negative control. Following treatment time periods, cells were lysed in mammalian protein extraction reagent (MPER; Thermo Scientific, Rockford, IL) lysis buffer with protease and phosphatase inhibitors (Abcam, Boston, MA) and stored at -20 ºC.

Bradford Assay and Western Blotting

Bradford assays were conducted to quantify protein levels in each cell lysate sample. Cell lysates were run neat in duplicate on a 96-well plate. 5 uL of sample or standard were mixed with 250 uL of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) in the appropriate wells. The plates were incubated in the dark for 40 minutes before reading the absorbance at 595 nm on a FluoStar Omega plate reader (BMG Labtech, Cary, NC). The analyzed protein concentrations were used to prepare the samples for Western blotting.

Western blots were used to semi-quantify Nrf2 and HO-1 protein levels in the samples, with β-actin as the loading control. Samples were mixed with MPER and Laemmli sample buffer and boiled at 100 ºC for 5 minutes. Samples were briefly centrifuged at 10,000 rpm. 30 µL sample or 8 µL of standard ladder (Bio-Rad Laboratories, Hercules, CA) were added to BioRad 4-15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA). The gels were run in Tris-glycine running buffer for one hour at 110 V, 300 watts, and 3.0 amps. After completion, the gels, PVDF membranes (Immobilon-P Transfer Membrane; Sigma-Aldrich, St. Louis, MO), and filter paper were submerged in cold Towbin buffer for 30 minutes. Semi-dry transfers were run at 18 V and 0.3 amps for 30 minutes on a Trans-Blot SD transfer cell (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in fish gelatin (Sigma-Aldrich, St. Louis, MO) for 2-4 hours, rocking at room temperature. Rabbit anti-mouse (Cell Signaling Technology, Danvers, MA) and mouse anti-mouse (Santa Cruz Technology, Dallas, TX) polyclonal primary antibodies were used to detect Nrf2. Rabbit anti-rabbit polyclonal primary antibodies were used to detect HO-1 (Cell Signaling Technology, Danvers, MA) and β-actin (Proteintech Group Inc, Rosemont, IL). Primary antibodies were diluted in fish gelatin at varying concentrations (Figure 4) and left shaking overnight at 4 ºC. Primary antibodies were then removed, and the membranes were washed with TBST for one hour. HRP-tagged goat-anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were added in TBST at varying concentrations (Figure 4) for 1.5 hours. The membranes were again washed with TBST for 1 hour, treated with Super Signal West Pico chemiluminescent reagent (Thermo Scientific, Rockford, IL), and imaged using Syngene GeneSys Image Acquisition Software (Bangalore, India). Band pixel density was calculated by dividing the band of interest by the β-actin control

band. The ratios were used to semi-quantify changes in the protein of interest. Band density was computed using ImageJ software (37).

Protein Target	1º Antibody Concentration	2º Antibody Concentration
Nrf2	1:1,000	1:10,000
HO-1	1:10,000	1:10,000
β-actin	1:25,000	1:75,000

Figure 4. Antibody concentrations used for Western blot analysis.

RESULTS

Increasing Levels of Nrf2 and HO-1 Following L2 Treatment

To assess the therapeutic potential of L2, RAW 264.7 macrophage cells were treated with 1, 10, or 50 μM of L2 for increasing durations (0, 1, 3, 6, 12, and 24 hours). Nrf2 and HO-1 levels were analyzed using Western blot analysis. More specifically, the mouse anti-mouse primary antibody was used to analyze Nrf2 levels. When treated with 1 μM (Figure 5), 10 μM (Figure 6), and 50 μM (Figure 7) of L2, there was a general increase in Nrf2 and HO-1 protein levels. Replicates of these experiments have not been completed, so no statistical analysis can be conducted at this time.

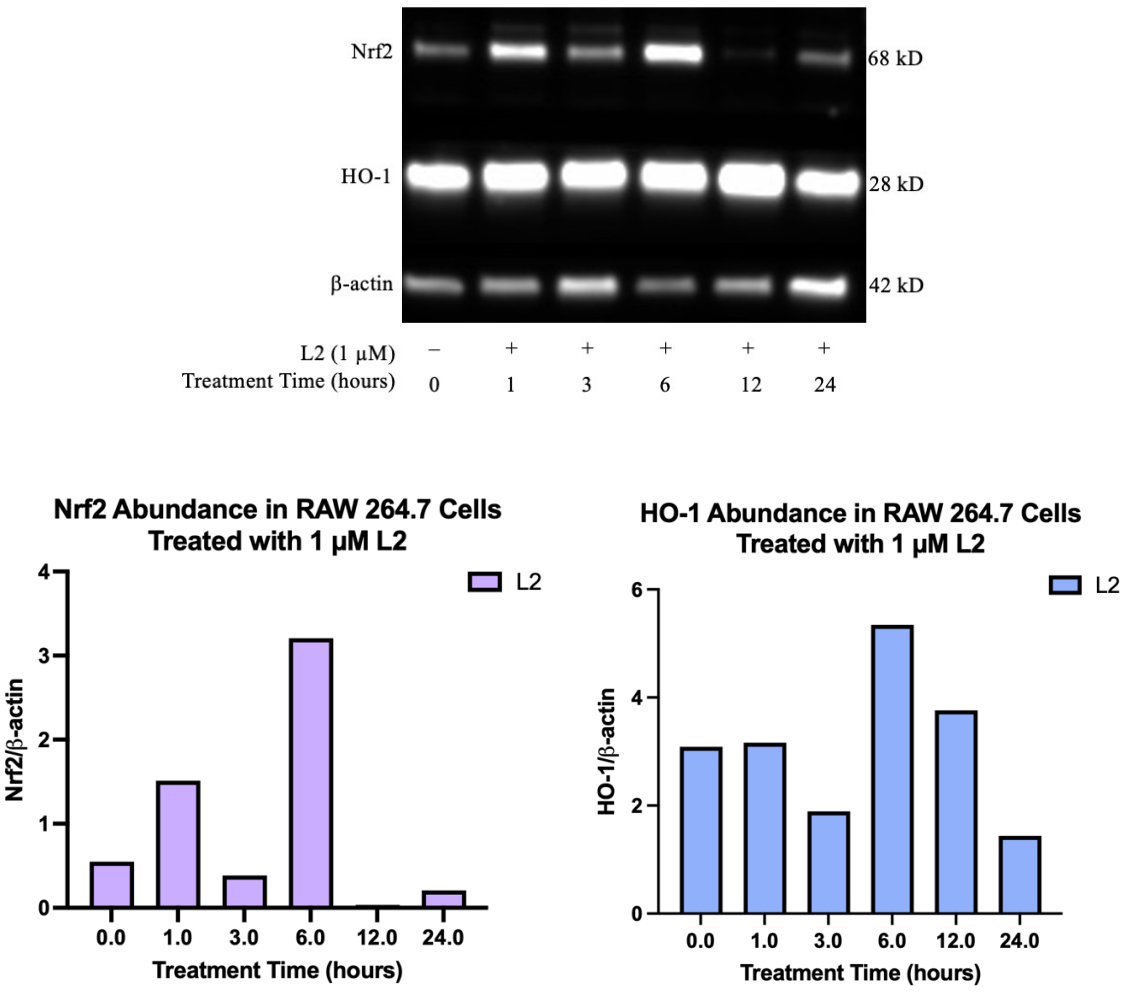

Figure 5. Evaluation of Nrf2 and HO-1 levels. RAW 264.7 cells were treated with 1 μM L2 for 0, 1, 3, 6, 12, or 24 hours. Western blots were used to analyze the concentration of Nrf2 and HO-1. Densitometric estimation was conducted using β-actin as an internal loading control.

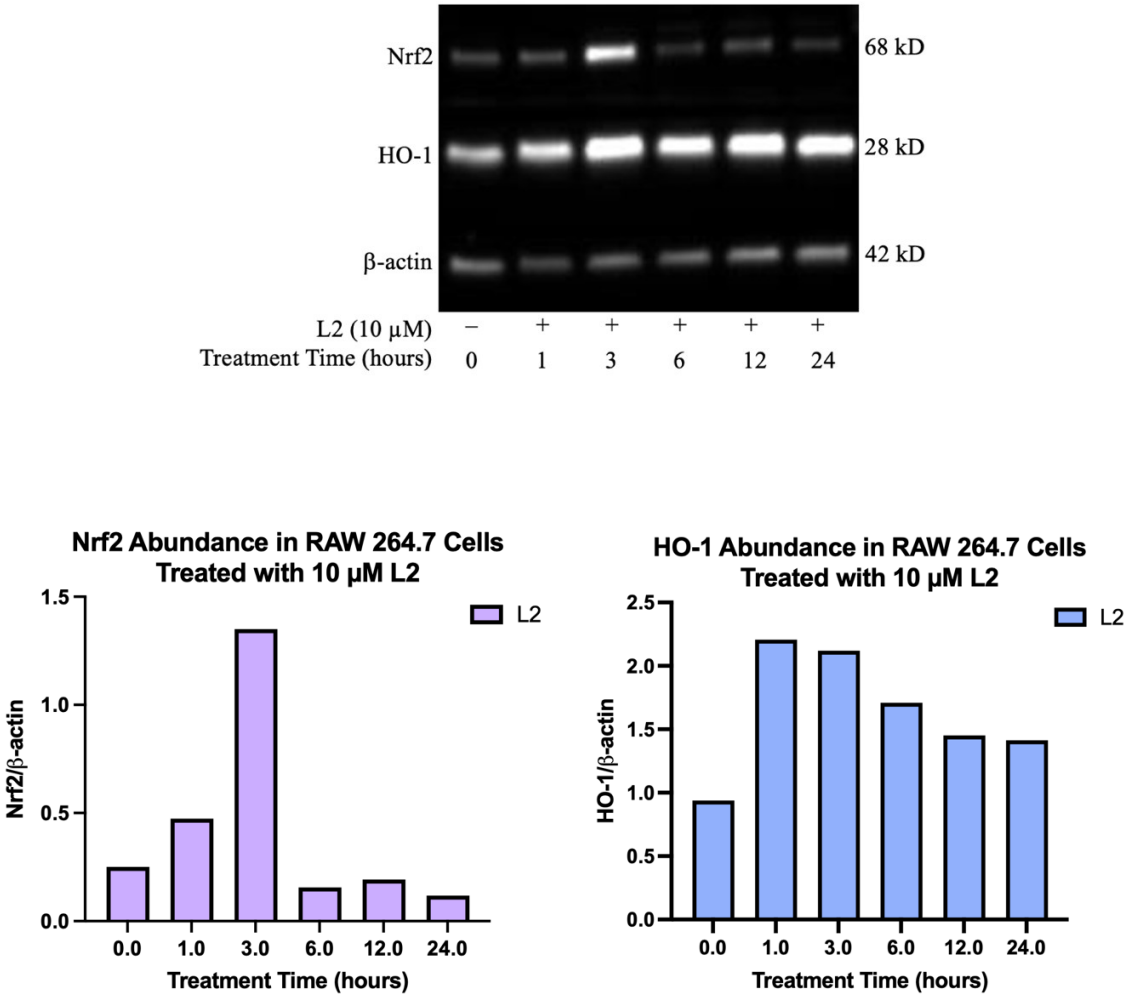

Figure 6. Evaluation of Nrf2 and HO-1 levels. RAW 264.7 cells were treated with 10 µM L2 for 0, 1, 3, 6, 12, or 24 hours. Western blots were used to analyze the concentration of Nrf2 and HO-1. Densitometric estimation was conducted using β-actin as an internal loading control.

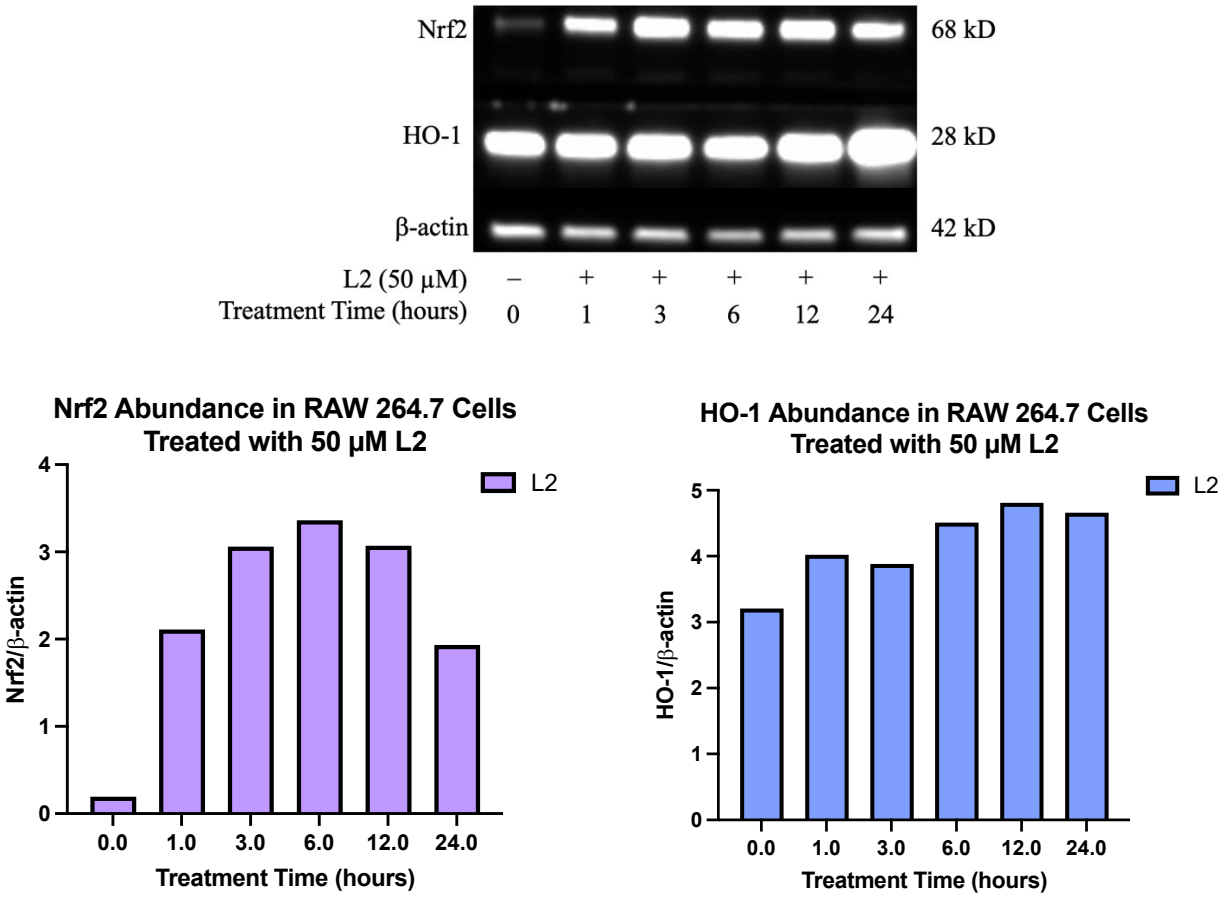

Figure 7. Evaluation of Nrf2 and HO-1 levels. RAW 264.7 cells were treated with 50 μM L2 for 0, 1, 3, 6, 12, or 24 hours. Western blots were used to analyze the concentration of Nrf2 and HO-1. Densitometric estimation was conducted using β-actin as an internal loading control.

Native and Ubiquitinated Nrf2 Analysis Following L2 Treatment

To expand on the previous findings, we looked to replicate the results in both RAW 264.7 macrophages and BV2 microglial cells. Rabbit anti-mouse Nrf2 primary antibody was used to detect Nrf2 at two different molecular weights, approximately 68 kD and 100 kD. In these experiments, RAW 264.7 and BV2 cells were treated with 10 μM of L2 for 0, 1, 2, 3, 4, or 5 hours. Western blots were conducted to analyze native Nrf2 and ubiquitinated Nrf2 (UB-Nrf2) levels. In both cell lines, we found a general trend of increasing native Nrf2 and decreasing Ub-Nrf2 following L2 treatment (Figure 8).

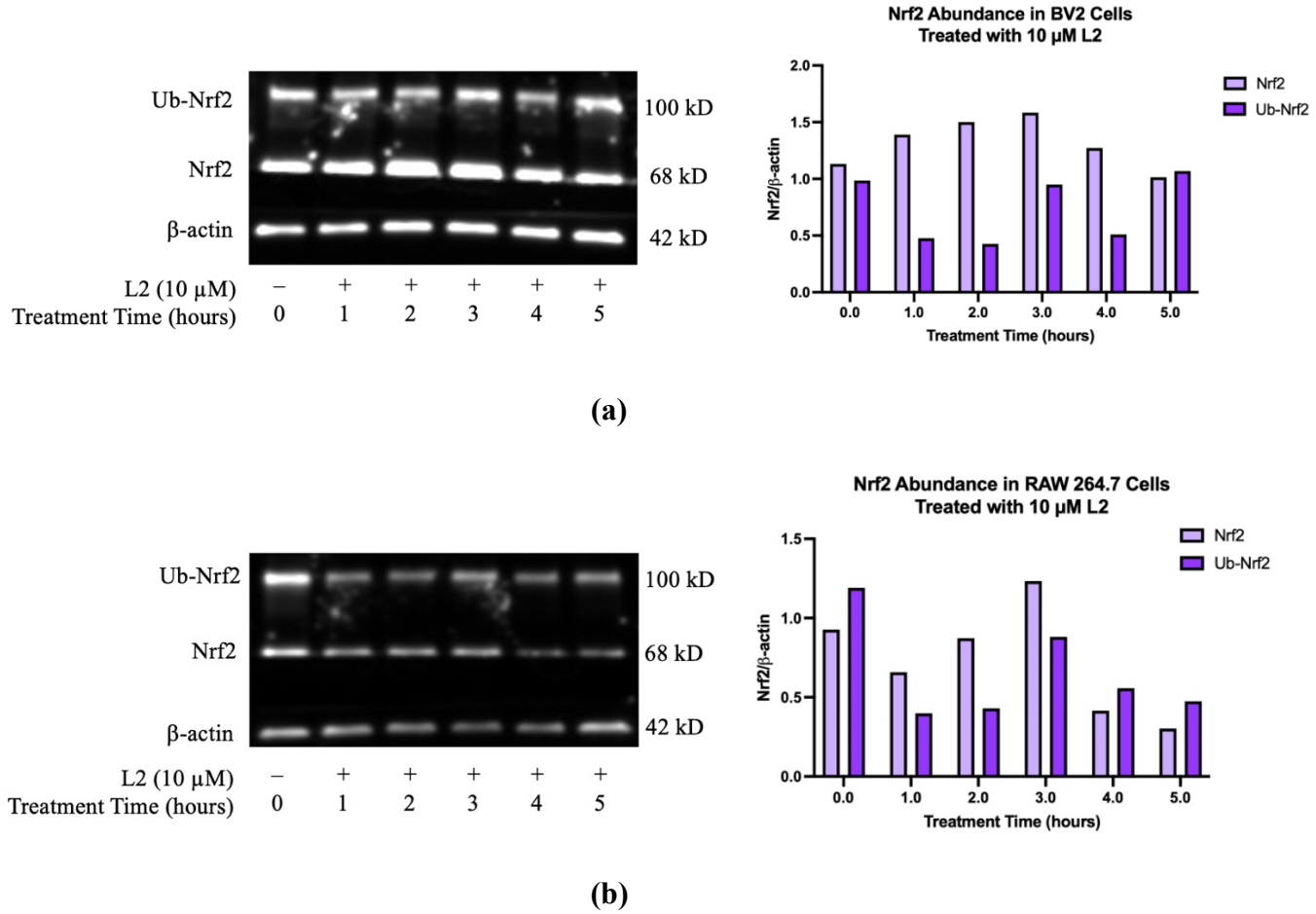

Figure 8. Evaluation of native Nrf2 and ubiquitinated Nrf2 levels in (**a**) BV2 and (**b**) RAW 264.7 cells. Western blots were used to analyze Nrf2 concentration and densitometric estimation was conducted using β-actin as a loading control.

DISCUSSION

When cells were treated with 1, 10, or 50 μM of L2, we saw a general increase in Nrf2 and HO-1. However, for the 50 μM L2 treatment, it is likely that this dosage induced cytotoxicity. Cytotoxic conditions induce ROS production, activating the Nrf2 pathway by means other than L2 disruption and therefore no sound conclusions about L2 can be drawn from this trial.

In the experiment treating with 10 μM L2 for 0, 1, 2, 3, 4, or 5 hours, we used the rabbit anti-mouse primary antibody to analyze two different Nrf2 levels. In these experiments, we observed a general increase in native Nrf2 levels and a decrease in ubiquitinated Nrf2 (UB-Nrf2) levels following L2 treatment. The bands that appeared at 68 kD are native Nrf2, which was blotted for in the previous experiments using the mouse anti-mouse primary Nrf2 antibody. However, the bands at approximately 100 kD are likely UB-Nrf2. When a protein is ubiquitinated, as is the case of Nrf2 when regulated by the Keap1 complex, four to five ubiquitin molecules are tagged onto the protein before being degraded. Each ubiquitin molecule weighs 8 kD, so adding the weight of four ubiquitin molecules to the weight of the native Nrf2 protein would total about 100 kD. In the future, we will look to confirm our UB-Nrf2 theory through immunoprecipitation of ubiquitin. After, we would blot for Nrf2 to check that we still observe bands at approximately 100 kD.

Overall, we observed a general increase in native Nrf2 and HO-1 levels and a decrease in UB-Nrf2 levels following L2 treatment. All these experiments will be replicated to run statistical analysis to confirm the observed trends. From these preliminary results, we propose two hypotheses for L2's disruption of Nrf2 levels.

First, L2 may disrupt the Keap1 regulation of Nrf2, mimicking natural regulatory mechanisms. As previously discussed, ROS alter the Keap1-Nrf2 binding conformation to prevent Nrf2 ubiquitination. It is possible that L2 mimics a similar disruption, decreasing Nrf2 ubiquitination rates. This could explain the observed experimental decrease in UB-Nrf2 levels, partnered with the assumption that the basal ubiquitinated levels formed prior to L2 treatment would still be degraded by the proteasome. Building on this, non-ubiquitinated Nrf2 would occupy the Keap1 binding site, limiting Keap1's availability to regulate Nrf2 levels in the

cytoplasm. Consequently, more newly synthesized Nrf2 would be free to translocate to the ARE and increase the expression of target genes, including HO-1. The other mechanism of Nrf2 regulation discussed was p-p62, a competitive inhibitor with higher affinity for the Keap1 binding site than Nrf2. If L2 similarly competitively inhibits Nrf2 regulation, this would increase the levels of unbound Nrf2 and potentially upregulate antioxidant production.

To test these hypotheses, we can run immunoprecipitation assays. This assay isolates Keap1 and any bound, associated proteins. From there, we would conduct a Western blot to analyze Nrf2 levels to analyze if L2 disrupts Keap1-Nrf2 binding rates. Another future direction would be to distinguish between cytoplasmic and nuclear levels of Nrf2. If Keap1 cytoplasmic regulation is inhibited, that does not necessarily mean that Nrf2 translocates into the nucleus. Isolating nuclear extracts and analyzing Nrf2 levels via Western blots could determine whether increased Nrf2 levels correlate with increased translocation.

Our second hypothesis is that L2 increases the expression of Nrf2 protein. If the transcription and translation of Nrf2 is increased, its abundance may overwhelm basal levels of Keap1 so that an increased proportion of Nrf2 translocates to the nucleus. This would support the observed trend of increasing Nrf2 and HO-1 levels following L2 treatment. To further test this hypothesis, we will conduct reverse transcriptase polymerase chain reaction (rtPCR) assays to quantify the mRNA levels of Nrf2 and target genes.

This research could provide preliminary results regarding the therapeutic potential of L2 in the treatment of oxidative stress diseases. Currently, our experiments have only been conducted on phagocytic immune cells and we're looking to expand into other cell lines to look at cells more impacted by oxidative stress diseases. For example, we are looking to replicate these experiments in Caco-2 colon epithelial cells and HT-22 immortalized mouse hippocampal

neurons. Further relating to disease states, we can induce disease-related oxidative stress to look at the therapeutic effect of L2. Regarding Alzheimer's Disease, previous studies have found that therapeutic compounds can protect HT-22 cells from amyloid beta-induced oxidative stress and cytotoxic cell death (38). Therefore, we could expand on these experiments using L2 to look at potential protective effects.

Our studies can also expand to explore the connection between oxidative stress and inflammatory pathways. Previous studies have identified numerous points of cross-talk between the Nrf2 antioxidant pathway and nuclear factor-κB (NF-κB) inflammatory pathway (Figure 9). NF-κB is a transcription factor activated in response to inflammatory triggers, such as bacterial lipopolysaccharide (LPS). The NF-κB pathway upregulates expression of proinflammatory soluble factors, including inducible nitric oxide synthase (iNOS) and proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β). Expression of these targets promotes further inflammation, inducing a self-perpetuating cycle and disease progression. One specific point of cross-talk between these two pathways is HO-1 involvement in NF-κB inhibition. One study in endothelial cells found an association between increased HO-1 activation and decreased NF-κB-mediated transcription (39). In relation to our project, we could look at whether L2 protects against inflammatory responses by analyzing pro-inflammatory cytokine levels and NF-κB activation.

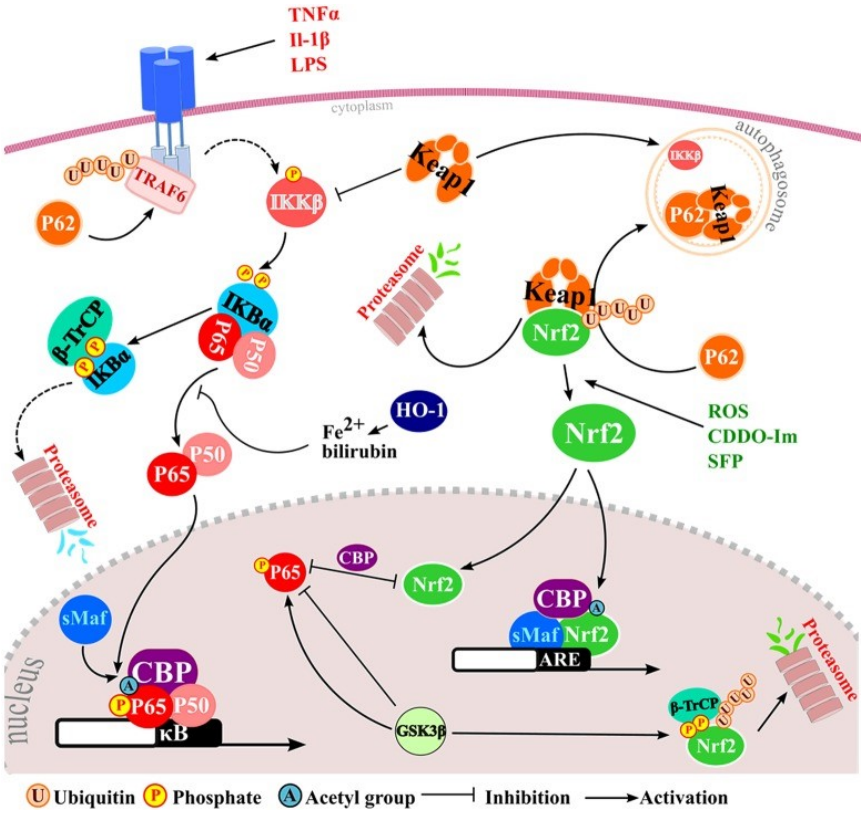

Figure 9. Cross-talk pathways between Nrf2 antioxidant pathway and NF-κB inflammation pathway. Figure obtained from Wardyn et al., 2015.

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