THESIS

PROTECTIVE EFFECTS OF SULFORAPHANE ON NITRIC OXIDE INDUCED MITOCHONDRIAL DYSFUNCTION

Submitted by

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ABSTRACT

PROTECTIVE EFFECTS OF SULFORAPHANE ON NITRIC OXIDE INDUCED MITOCHONDRIAL DYSFUNCTION

Sulforaphane (SFN), an isothiocyanate compound that is formed in the breakdown process of cruciferous vegetables, has demonstrated the ability to interfere with dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. The present study investigated whether SFN can protect cells exhibiting persistent mitochondrial fission induced by nitrosative stress (S-nitrosoglutathione; GSNO), and shed light on the mechanism by which this occurs. Results show that SFN (5 μM) prevents decreases in the rate of mitochondrial oxidative phosphorylation (ATP production) in SH-SY5Y neuroblastoma cells treated with 200-600 μM GSNO, which was associated with significant improvements in cell viability at all doses. Based upon the understood activation mechanism of Drp1, we further explored the possibility that SFN interferes with phosphorylation of Drp1 at serine residue 616 (pDrp1-Ser616). Indeed, SFN significantly reduced GSNO-mediated increases in pDrp1-Ser616, suggesting a possible mechanism of cytoprotection. However, due to the various reported targets of SFN, it remains unclear if SFN interferes directly with Drp1 phosphorylation or with other targets upstream of this event.

ACKNOWLEDGEMENTS

I would first like to acknowledge my partner and best friend, Elizabeth Glover. Your selfless personality causes you to place my needs before all others, even your own. You always find ways to soothe my frustrations and focus my mind. I look forward to many more years together and what wonderful things the future has in store for us.

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Lastly, I would like to acknowledge and state a very special thanks to Dr. William Hanneman and Dr. Marie Legare. Both of you took a special interest in me and afforded me the means to pursue my dreams. I placed a special importance on everything you both have provided me. I know that without your mentorship and invaluable guidance, I would not be where I am today -- let alone be the person I am today. You both are true miracle workers and I look forward to our future endeavors, friendship, and your continued mentorship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1:

HYPOTHESIS AND AIMS

1.1 Chapter Introduction

Age-related diseases are often defined by the dysfunction of a biological system leading to the ultimate inability of an organism to survive. The process of aging as it relates to the imbalance between mitochondrial dynamics, including mitochondrial fission and fusion, has been linked to various neurodegenerative disease pathologies. Recent reports investigating Alzheimer's (AD), Huntington's (HD), and Parkinson's disease (PD) show that the unregulated production of the signaling molecule nitric oxide (NO) is an important driving factor in the development or progression of the disease states (Cho et al., 2009; Haun et al., 2013; Zhang, V. L. Dawson, & Dawson et al., 2006). Specifically, high levels of NO have demonstrated the ability to activate the known fission factor, Dynamin-Related Protein 1 (Drp1), through post translational modifications (Barsoum et al., 2006; Cho et al., 2009). Mitochondrial fission is an integral element in the remodeling process of the mitochondrial network within the cell and is necessary for normal cellular functions; however, continuous mitochondrial breakdown is associated with the prolonged fragmentation of the mitochondrial network ultimately resulting in the disruption of energy homeostasis and the programmed death of the affected cells (Barsoum et al., 2006; Schmitt et al., 2018).

Sulforaphane (SFN), an isothiocyanate compound derived from cruciferous vegetables, has recently been demonstrated to have the ability to modulate the mitochondrial fusion-fission dynamics within a cell (O'Mealey, Berry, & Plafker, 2017). Although the precise mechanism by which SFN exerts these effects remains unknown, current research infers that SFN acts upon

Drp1 to impair mitochondrial fission and consequently promote hyper-fusion. The study hypothesis is: NO-induced mitochondrial fission resulting in programmed cellular death, as seen in age-related neurodegenerative diseases, may be attenuated through treatment of neuronal cells with sulforaphane. To test this hypothesis, this project will focus on two specific aims.

1.2 Specific Aim 1

Specific Aim 1: To determine if SFN can attenuate the detrimental effects of NO-induced excessive mitochondrial fission.

This study will utilize S-nitrosoglutathione (GSNO), a NO-donor on a neuronal cell line to induce excessive levels of mitochondrial fission. SFN will be applied as a pre-treatment to NO in all experiments in order to determine any protective effects of SFN on mitochondrial injury. First, the effects of SFN on cell viability in response to NO treatment will be assessed through the use of a reduction-based cell viability assay. To further characterize any protective effect of SFN on NO-induced mitochondrial dysfunction, mitochondrial bioenergetics, and mitochondrial ATP production will be assessed using an Oroboros Oxygraph-2k (O2K).

1.3 Specific Aim 2

Specific Aim 2: Determine if SFN exerts an effect on post-translational modification of Drp1 by NO-induced phosphorylation.

This aim is designed to shed insight into the underlying mechanism behind SFN's ability to interfere with the activation of Drp1. Previous research has yet to fully understand the mechanism by which NO-mediated activation of Drp1 occurs; however, the current understanding is that it occurs through a downstream phosphorylation event after NO exposure. This study will utilize a western blot analysis to determine the relative abundance of phosphorylated Drp1 at specific serine residue 616 (pDrp1-Ser 616) between treatment groups.

1.4 Overview of Results

The findings suggest that SFN is effective at attenuating the decline in ADP phosphorylation associated with persistent mitochondrial fission. Moreover, SFN provides protection against the occurrence of cellular death after a 24-hour exposure to GSNO. Additionally, the data collected demonstrates that a pre-treatment of cells with SFN prior to a 2-hour treatment with GSNO significantly reduced levels of pDrp1-Ser616.

1.5 Thesis Organization

This thesis will consist of eight chapters, beginning with this first one, which will generally describe the research and the organization of the work. Chapter 2 will present important background information necessary to understanding the research on mitochondrial dynamics and its subsequent phytochemical modulation. The methods employed to explore each specific aim will be presented in Chapter 3, while Chapters 4 and 5 will present the detailed methodology and the resulting outcomes of each aim. Chapter 6 will discuss areas for future exploration and further research. The results and conclusions will be presented in Chapter 7. Lastly, Chapter 8 will describe the original contributions of this research and discuss any publications in progress.

CHAPTER 2:

BACKGROUND

2.1 Mitochondrial Dynamics

The primary function of mitochondria has traditionally been understood to be to meet the energy demands of the cell. However, more recently it is thought that mitochondria play an important role in numerous physiologically functions and therefore an equally important role in the pathology of numerous age-related diseases. To meet the energy demands of its biological processes, a cell undergoes a continuous remodeling of the mitochondrial network through the dynamic processes known as fission and fusion. Fission permits daughter cell inheritance of mitochondria during cellular division and the removal of damaged mitochondria through the process of mitophagy (Ashrafi, Schlehe, LaVoie, & Schwarz,, 2013; Mishra & Chan 2014). Conversely, fusion is the process that supports the mitochondrial network through the promotion of proper localization of the fused mitochondrion and the redistribution of functional mitochondrial components throughout the cell (Chen et al., 2007, Legros et al., 2002).

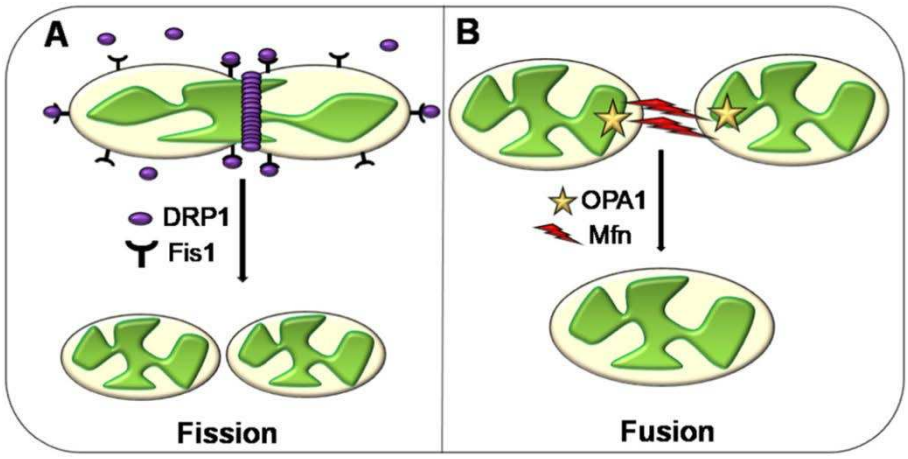

Figure 2.1. Schematic Diagram Depicting Mitochondrial Dynamics (Fission and Fusion) General outline of the dynamic processes of mitochondrial fission and fusion, which includes key proteins of both pathways. From "Mitochondrial Dynamics, a Key Executioner in Neurodegenerative Diseases," by K. Panchal & A. K. Tiwari, 2018 *in press, Mitochondrion*. Copyright 2018 Elsevier B.V. and Mitochondria Research Society. Reprinted with permission.

Both mitochondrial fission and fusion are controlled by large GTPases, all known to be a part of the dynamin family of proteins. Proteins involved in fusion include mitofusin 1 (MFN1), and optic atrophy 1 (OPA1) whereas, the breakdown of the network is largely controlled by the GTPase, dynamin related protein 1 (Drp1) and its mitochondrial surface bound protein, mitochondrial fission protein 1 (Fis1) (see Figure 2.1.) (Otera et al., 2010; Panchal & Tiwari, 2018; Smirnova, Griparic, Shurland, & van der Bliek, 2001; Yoon, Krueger, Oswald, & McNiven, 2003).

2.2 Effects of Nitric Oxide on Mitochondrial Fission

Research has demonstrated that the processes of mitochondrial fission and fusion are equally important, and that any continuous disruption of the balance between the two can result in impaired energy homeostasis and the programmed death of the affected cells (see Figure 2.2) (Barsoum et al., 2006; Bossy et al., 2010, Knott, & Bossy-Wetzel, 2008).

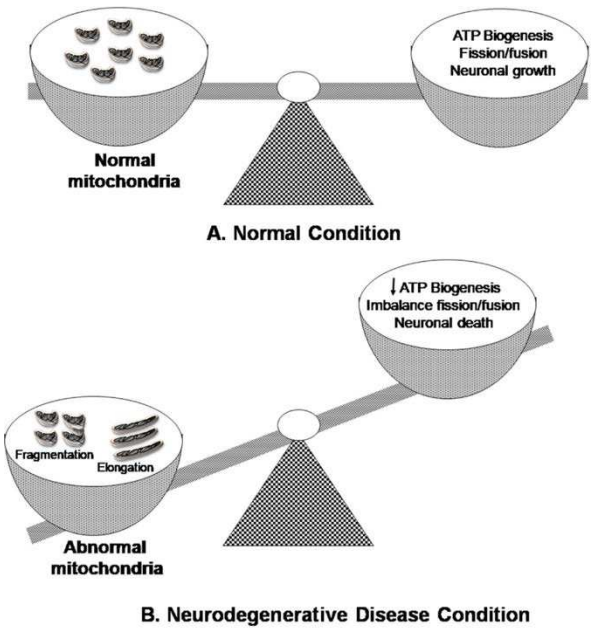

Figure 2.2. Mitochondrial Physiology in Normal and Neurodegenerative Disease Condition The balance between mitochondrial fission and fusion is vital to normal physiology and any imbalance can result in decreased ATP biogenesis and neuronal cell death. From "Mitochondrial Dynamics, a Key Executioner in Neurodegenerative Diseases," by K. Panchal & A. K. Tiwari, 2018 *in press, Mitochondrion*. Copyright 2018 Elsevier B.V. and Mitochondria Research Society. Reprinted with permission.

This is of significance as the death of neurons results in irreversible neurodegenerative tissue damage. Nitric oxide, an endogenously produced signaling molecule ubiquitously found in the human body, is involved in numerous physiological functions; however, some disease states, such as AD, PD, and HD, can be marked by significantly elevated levels of endogenous NO (Akama, Albanese, Pestell, & Van Eldik; Cho et al., 2009; Haun et al., 2013; Zhang et al., 2006). Excessive NO production has been shown result in the activation of Drp1, an important protein required for mitochondrial fission. Consequently, persistent mitochondrial fission has been reported to result in both significant decreases in ATP levels and subsequent neuronal cell death (Barsoum et al., 2006; Cho et al., 2009).

Although the mechanism by which NO acts to induce mitochondrial fission is not fully understood, it is thought to occur through post-translational conformational changes of various

upstream kinases that in turn phosphorylate Drp1 (Bossy et al., 2010). The first proposed mechanism by which this may occur is through NO's potential to result in the phosphorylation of protein kinase B at serine residue 473. When Akt is phosphorylated, it can induce the phosphorylation of many cytosolic proteins including Drp1at specific serine residue 616 (Kim et al., 2016). Alternatively, NO has also been shown to s-nitrosylate — a process analogous to phosphorylation — cyclin dependent kinase 5 (Cdk5). This in turn can result in the downstream phosphorylation of Drp-Ser616 (Qu et al., 2011; Strack, Wilson, & Cribbs, 2013). When phosphorylated at serine 616, Drp1 can then translocate from the cytosol to mitochondrial fission receptors (Mff) and assemble into large oligomeric spiral structures around specific scission sites on the mitochondrial network. Upon assembly, the Drp1 oligomer will constrict in a GTPase-dependent mechanism resulting in the complete cleavage along the mitochondrial membrane, which ultimately results in two independent mitochondria (Ingerman et al., 2005).

2.3 Modular Effects of Sulforaphane on Mitochondrial Dynamics

SFN, an isothiocyanate compound derived from glucoraphanin, is a compound found in cruciferous vegetables that has been extensively researched for its well-defined chemopreventive effects (Kensler et al., 2013). The established chemopreventive effects are thought to occur due to SFN's ability to target cysteine residue 151 on the kelch-like ECH-associated protein 1 (Keap1). This results in the dissociation of Keap1 from nuclear factor erythroid 2–related factor 2 (Nrf2) and the subsequent upregulation of various phase-2 anti-oxidants (Zhang et al., 2013).

However, researchers have recently demonstrated that SFN possesses the novel ability to act independently of the Nrf2 pathway to modulate mitochondrial dynamics. In 2017, O'Mealey et al. (2017) discovered that SFN promotes mitochondrial hyper-fusion by acting upon Drp1 to inhibit mitochondrial fission. The researchers determined that SFN impairs the natural ability of

Drp1 to relocate to the scission regions on the mitochondrial network, limiting the process of mitochondrial fission; however, the exact mechanism by which this occurs has yet to be elucidated (O'Mealey et al., 2017). SFN has previously demonstrated the ability to target numerous proteins by which it may act to modulate various biological pathways (Myzak & Dashwood, 2008). Particularly of interest is that SFN has demonstrated anti-cancer properties via inhibiting phosphorylation of Ser-473 on Akt, a protein kinase important in cell proliferation and apoptosis that has been shown to phosphorylate Drp1 (Chaudhuri, Orsulic, & Ashok, 2008). Since SFN impairs the ability of Drp1 to relocate to the mitochondrial network, and because SFN can interact with numerous proteins, it is plausible that SFN is exerting its effect upstream perhaps by inhibiting the post-translational modification of Drp1 (see Figure 2.3). Additionally, the discovery of a compound that could selectively inhibit pDrp1-Ser616 could be of particular pharmacological interest for its ability to attenuate the deleterious effects of aberrant NO-induced mitochondrial dynamics.

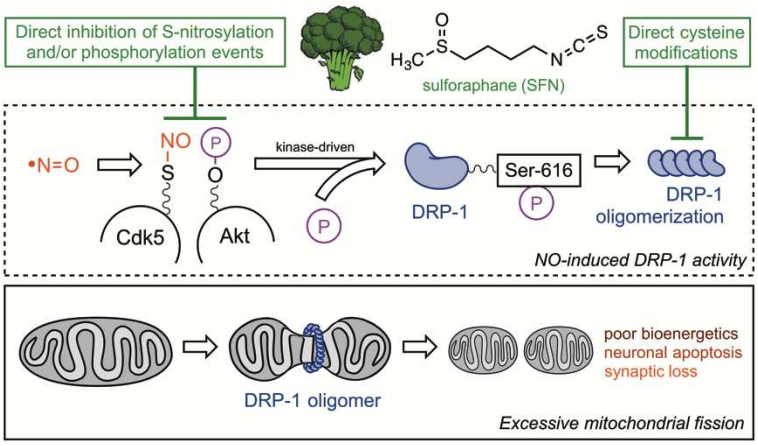

Figure 2.3. Dual Illustration of Proposed Possible Mechanisms of Both GSNO-Dependent Activation of Drp1 and SFN-Dependent Inhibition of Drp1 Activity. Illustration depicting the possible mechanism by which NO phosphorylates Drp1 and the downstream effects of excessive mitochondrial fission. Additionally, this illustration demonstrates the proposed mechanisms by which SFN may act to interfere with Drp1 phosphorylation.

2.4 *In vitro* Model

For the purposes of this research, the ideal immortal cell line for *in vitro* experimentation investigating neurodegeneration is one that closely resembles the functioning cells of the human brain. For this reason, the cell line chosen was the SH-SY5Y neuroblastoma cell line. The SH-SY5Y cell line was derived through subcloning an original bone marrow biopsy from a four-year-old female with a metastatic form of the cancer. The SH-SY5Y line has been used in numerous previous studies to serve as an *in vitro* model to investigate various aspects of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases. The reason SH-SY5Y cells are extensively used in neuroscience experimentation is that undifferentiated SH-SY5Y cells share similar biological properties to immature neurons. Furthermore, SH-SY5Y cells possess the ability to be differentiated to cells that closely resemble adult primary neuronal cells, if desired (Krishna et al., 2014). The use this specific cell line in

thesis research will allow for the analysis of the ability of SFN to beneficially modulate mitochondrial dynamics in a neuronal-like cell line model.

2.5 Chapter Summary

The present study aims to further characterize how SFN may rescue cells exhibiting persistent mitochondrial fission and to shed light on the mechanism by which this occurs. First, this research assessed the ability of SFN to attenuate decreased ATP production in SH-SY5Y cells undergoing persistent mitochondrial fission. Next, the study determined whether SFN could provide protection by way of viability to SH-SY5Y cells undergoing persistent mitochondrial fission. Lastly, relative levels of pDrp1-Ser616 were analyzed to determine if SFN provides these benefits by reducing Drp1-depedent mitochondrial fission.

CHAPTER 3:

MATERIALS AND METHODS

3.1 Chapter Introduction

This chapter will provide the general materials and methods designed to test each of the aims of this thesis. This will include *in vitro* cell line maintenance and reagents utilized for experimentation. Specific methodology per experiment will be located within their respective chapters.

3.2 Reagents, Treatments, and Antibodies

SH-SY5Y human neuroblastoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Culture media known as Dulbecco Modified Eagle Medium (DMEM) for cell subculturing was acquired from Mediatech Inc. (Manassas, VA) and fetal bovine serum (FBS) was purchased from Peak Serum (Wellington, CO). Antibodies for pDrp1-Ser616, β-actin, and the HRP-linked secondary antibody and western blot reagents were purchased from Cell Signaling Technology (Danvers, MA). NO-Donor, S-nitrosoglutathione (GSNO), was obtained from EMD Millipore Corporation (Temecula, CA), and SFN was purchased from Sigma-Aldrich (now Millipore-Sigma, St. Louis, MO). PrestoBlue cell viability reagent was purchased from Invitrogen-Life Technologies (Grand Island, NY).

3.3 *In vitro* Maintenance

SH-SY5Y neuroblastoma cell line culture and maintenance was performed in accordance to the ATCC protocol. Cells were grown in DMEM (Hyclone, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Peak Serum, Wellington, CO) and 1% Penicillin/Streptomycin Solution. Cells were subcultured in 25cm^2 culture flasks and kept in an

incubator at 37 ℃ with 5% CO_2. Cell media was replaced with fresh media as needed and cells were passed based upon cell confluency.

Subculturing involved the aspiration of all media from the cell culture flask followed by the addition of 1 mL of a 0.25% trypsin, 0.53 mM EDTA solution. Trypsinization was performed at room temperature for 2 to 5 minutes or until cells become unadhered. Mechanical disruption by hand was used to help cells become dislodged when necessary. When cells became unadhered to the flask, trypsin was neutralized through the addition of complete DMEM and cells were subcultured in a ratio of 1:2 or 1:5, depending on the experimental timeline. Prior to all experimentation, media and cells were collected and suspended in a 15 mL conical vial and centrifuged for 6 minutes at 125 x g. Following centrifugation, the supernatant was then removed and cells were resuspended in complete DMEM and counted manually using a hemocytometer and microscope. All counts were performed in duplicate and the average of the counts were used to calculate the cell count. All cell culture was performed in a Class II Biosafety Laminar Flow Hood.

3.4 Oroboros O2K Oxygraph

All respirometry and ATP data collection was performed on an Oroboros O2K Oxygraph equipped with DatLab analysis software.

3.5 Western Blot

All western blotting imaging was performed on a Bio-Rad ChemiDoc MP System running Image Lab Software. Further densitometry analysis was performed utilizing Imagej software.

3.6 PrestoBlue Cell Viability

All cell viability data were collected measuring light absorbance at 570 nm with a reference wavelength set at 600 nm using a VersaMax microplate reader equipped with SpectraMax M2 software.

CHAPTER 4:

SPECIFIC AIM 1

4.1 Chapter Introduction

The primary objective of this aim was to determine if treating neuron-like cells with SFN prior to treating the cells with NO, a known inducer of mitochondrial fission, would attenuate the deleterious downstream effects of persistent mitochondrial fission. To accomplish this, a technique to measure cell viability was first established and optimized. Data collected from this assay helped to determine the proper doses of GSNO and SFN to use in addition to determining the ability of SFN to provide cytoprotection in response to GSNO cytotoxicity. To further explore the downstream effects of persistent mitochondrial fission and the ability of SFN to attenuate these effects, mitochondrial ATP production and respiration data was collected using an Oroboros O2K Oxygraph.

4.2 Nitric Oxide Donor (S-nitrosoglutathione)

NO is an important bio-regulatory signaling molecule that can have profound effects on the cardiovascular and nervous systems. The upregulation of neuronal nitric oxide synthase in cell culture to produce endogenous NO can be both costly and result in unexpected collateral experimental outcomes. To ensure there are not unintended consequences, it was decided that a NO release through a drug would be utilized (Miller & Megson, 2009). Various NO-donors that release NO exist; however, the modus by which NO is released and potential downstream effects can vary dependent upon the selected NO-donor. Therefore, careful consideration in choosing the correct NO-donor was applied in the decision-making process. S-nitrosoglutathione (GSNO) was selected for its ability to both act as an NO-donor and subsequently act as a S-nitrosylating

agent. Additionally, GSNO has been used in previous literature to induce mitochondrial fission in neurodegenerative cell culture models (Qu et al., 2011).

Researchers have previously demonstrated that NO-donors induce mitochondrial fission through the S-nitrosylation of Drp1; however, it was later discovered that although this did occur, the S-nitrosylation of Drp1 was not responsible for the activation of Drp1-dependent mitochondrial fission (Bossy et al., 2010; Cho et al., 2009). The activation of NO-induced Drp1-dependent mitochondrial fission is likely due to an indirect phosphorylation event in which NO activates upstream kinases such as Cdk5 (Qu et al., 2011).

GSNO is not readily transported into cells upon addition to cell culture, instead, it is understood that NO is first liberated from GSNO through the homolytic or reductive cleavage of the S-N bond. NO can then passively diffuse across cellular membranes gaining entry to cells. Upon entry, NO can either act independently, as a biological signaling molecule, or spontaneously reform an S-nitrosothiol such as GSNO, through interactions with thiyl radicals from Glutathione (GSH) (Broniowska, Diers, & Hogg, 2013). Furthermore, all NO-donors possess different stabilities and will liberate NO at different rates when added to physiological conditions such as cell culture. GSNO is known to be more stable than its similar NO-donor option S-nitrosocysteine. It is known that 50–200 μM concentrations of S-nitrosocysteine have been demonstrated to liberate approximately 1.5–5 μM of NO over the course of 20 minutes, therefore it is expected that similar concentrations of GSNO will release the same amount of NO over a slightly longer period (Brorson, Schumacker, & Zhang, 1999).

4.3 NO-Induced Cytotoxicity Protocol Development

Previous researchers have determined that NO-induced mitochondrial fission will not result in necrotic cellular death but instead in the programmed cellular death of otherwise healthy

neuronal cells. Future studies by others may require a deeper analysis of cytotoxicity that incorporates a more quantitative approach to differentiate the mode of cellular death; however, due to time constraints and the scope of the proposed study, it was determined that a cell viability assay is suitable. Dose cytotoxicity and cell viability can be performed through a variety of methods, the most cost and time effective method available employs the PrestoBlue cell viability reagent. The PrestoBlue assay utilizes a Resazurin-based dye that is rapidly taken up into living cells. Upon entry, the Resazurin-based dye is rapidly reduced in the healthy cellular environment to result in a solution color change from blue to red. The color change can be then directly measured using an absorbance or fluorescence-based spectrophotometry detection method (Lall, Henley-Smith, De Canha, Oosthuizen, & Berrington, 2013). Utilizing the manufacturer's protocol, the PrestoBlue assay was optimized using SH-SY5Y neuroblastoma cells. The first step involved evaluating the ability of the assay to determine cell densities. To do this, SH-SY5Y cells were plated at various densities (20,000, 60,000, and/or 100,000 cells per well) in 96-well plates. Five wells were designated to each cell density and the absorbance of each of the five wells was averaged to obtain a final value per cellular density. Plated cells were incubated at 37 °C with 5% CO_2 for 24 hours to allow cells to adhere to the well surface. Ten µL of PrestoBlue reagent was added to each well after 24 hours and allowed an additional 2 hours to incubate. This resulted in a linear R^2 value of 0.9908, which verified that the method is effective at quantifying cell viability (see Figure 4.1.).

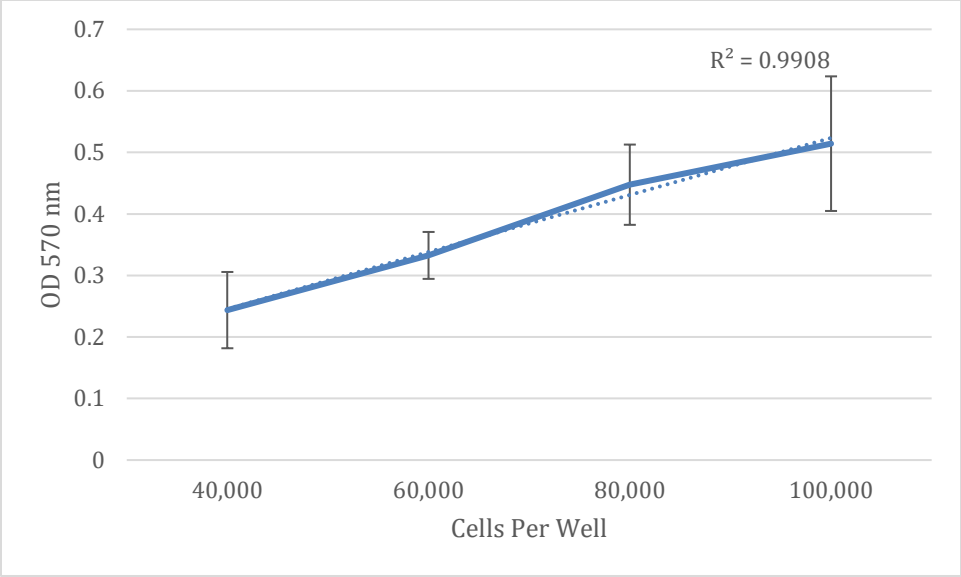

Figure 4.1. Analysis of Various Cell Densities: 20,000 – 100,000 Cells per Well. Each point represents the mean of two individual experiments with five technical replicates on each plate.

4.4 Materials and Methods: Effects of SFN on GSNO-Induced Cytotoxicity

SFN was solubilized in 100% dimethyl sulfoxide. Due to the potential cytotoxic effects of dimethyl sulfoxide reported in mammalian cells, the concentrated stock solution of SFN in 100% DMSO was diluted down so that the final 5 µM contained only 0.03% dimethyl sulfoxide. Alternatively, GSNO required solubilization in a 1X phosphate buffered saline solution, which has reported minimal cytotoxic effects when used in short treatment durations. Due to the unstable nature of solubilized GSNO even when frozen, for all experimentation throughout this study, all GSNO was prepared and solubilized the day that experimentation was performed. However, because of the use of vehicles for drug solubilization, we included dimethyl sulfoxide and 1X phosphate buffered saline solution treatment groups in our cell viability assay to demonstrate that neither of the final concentrations used in the SFN and GSNO treatment groups had any effect on the experimental outcomes. SH-SY5Y cells utilized in this experiment were all obtained from the same culturing passage number. Cells were harvested from the 25cm^2 culture flasks using the cell culture passage method found in Section 3.3 and suspended in 8 mL of fresh

DMEM culture media. Utilizing 10 µL of suspended cells, a cell count using a hemocytometer was performed twice and the calculations were averaged. Upon obtaining the total cell count in each of the 25cm^2 culture flasks; cells were plated at 50,000 cells per well on 96-well plates in 100 µL of DMEM. The experiment consisted of four individual 96-well plates that served as biological replicates. Furthermore, each individual 96-well plate included five technical replicates for each treatment group.

While adhered to 96-well plates, cells were assigned to one of 10 treatment groups: (a) no treatment (control); (b) dimethyl sulfoxide treatment (0.03%); (c) phosphate buffered saline solution (in accordance to the 600 µM GSNO treatment); (d) SFN treatment at 5 µM for 30 minutes; (e) GSNO treatment at 200 µM for 24 hours; (f) GSNO treatment at 400 µM for 24 hours; (h) GSNO treatment at 600 µM for 24 hours; (i) SFN pre-treatment at 5 µM for 30 minutes followed by GSNO treatment at 200 µM for 24 hours; (j) SFN pre-treatment at 5 µM for 30 minutes followed by GSNO treatment at 400 µM for 24 hours; and, (k) SFN pre-treatment at 5 µM for 30 minutes followed by GSNO treatment at 600 µM for 24 hours. Lastly, each plate included one row of five wells that served as a blank group that consisted of only DMEM culture media.

All treatment durations occurred at 37 ℃ with 5% CO_2, cells from all treatment groups were washed once with 1X Phosphate-buffered saline prior to and after all treatments. Following the duration of all treatments, 10 µL of PrestoBlue reagent was added to each well on all plates and allowed to incubate at 37 ℃ with 5% CO_2 for 2 hours. Upon completion of PrestoBlue incubation, absorption of 570 nm light was assessed and standardized to 600 nm for confirmation as described in the manufacturer's protocol. Percent cell viability was then determined from these values by setting the control value to 1 and standardizing all other treatment groups to a

percent of this value. Statistical analysis was performed in Graphpad Prism software. A two-way analysis of variance (ANOVA) with Sidak post hoc analysis was performed. Significance was set at p-value < 0.05.

4.5 Results: Effects of SFN on GSNO-induced Cytotoxicity

Upon quantification of cell viability data between treatment groups, the potential of SFN to protect the integrity of cells after NO treatment was determined. The excipients utilized to solubilize both SFN and GSNO did not have any effect on the cell viability (see Figure 4.2).

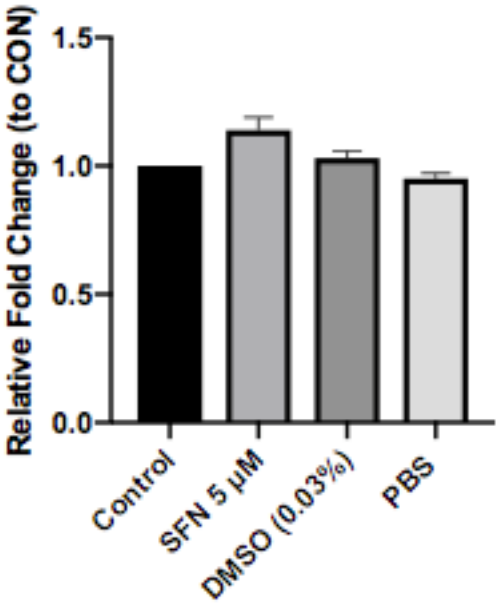

Figure 4.2. Effect of Solubilizing Excipients and SFN on Cell Viability. DMSO and PBS were used to solubilize SFN and GSNO respectively. Results are presented as the mean +/- S.E.M. of four independent experiments. Results demonstrate no statistical difference between either vehicle in comparison to the control or SFN in comparison to the control.

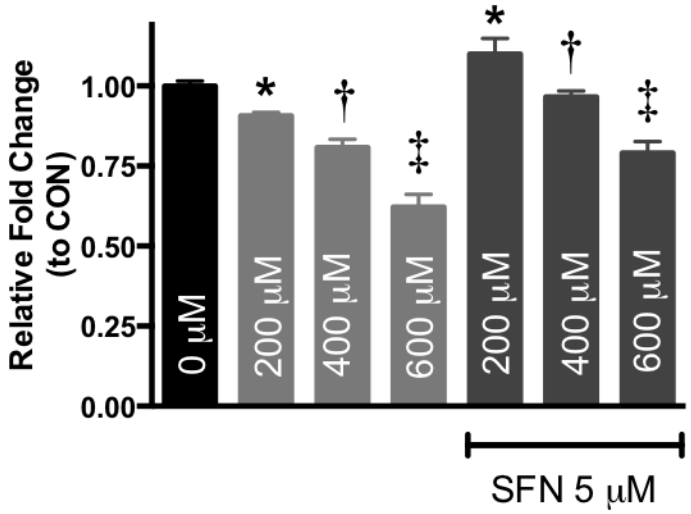

Figure 4.3. The Protective Effect of SFN on Cell Viability. SFN attenuated reductions in cell viability of SH-SY5Y cells insulted with GSNO at varying concentrations. SH-SY5Y cells were treated with increasing doses of GSNO (200 µM, 400 µM, 600 µM) for 24 h with or without an accompanying pre-treatment of 5 µM SFN for 30 min. Cell viability was measured using the PrestoBlue cell viability assay. Results are presented here as the means ± S.E.M.s of four independent experiments. A two-way ANOVA, with Sidak post hoc analysis, was utilized to compare each GSNO treatment against its SFN + GSNO treatment counterpart *, †, ‡ and $p < 0.05$.

The results demonstrate that treatment with 200 µM GSNO reduced cell viability to 90.7 ± 1.0% of control; 400 µM GSNO reduced cell viability to 80.7 ± 2.5% of control; and 600 µM GSNO reduced cell viability to 62.1 ± 3.9% of control. Pretreatment with 5 µM SFN for 30 minutes prior to a 200 µM GSNO insult attenuated cell viability to 109.9 ± 4.8% of control; 400 µM GSNO insult attenuated cell viability to 96.5 ± 1.8% of control; and 600 µM GSNO insult attenuated viability to 79.1 ± 3.5% of control (see Figure 4.3).

4.6 Discussion: Effects of SFN on GSNO-Induced Cytotoxicity

Previous research has demonstrated that 200 µM of S-nitrosocysteine, a similar NO-Donor to GSNO, results in excessive mitochondrial fission after just one hour upon treatments to cell culture. Neuronal cell death began to occur after 6 hours and continued up to 16 hours after treatments resulting in 80% neuronal cell death (Barsoum et al., 2006). As expected, this

experimentation demonstrated that cell viability decreased in a dose-dependent manner as doses of GSNO increased (from 200 µM to 400 µM to 600 µM). The goal of this experiment was to determine if a pre-treatment with SFN to these increasing concentrations of GSNO could provide protection to cells that would otherwise not survive. Of interest, when a 30-minute pre-treatment of SFN was applied to the increasing doses of GSNO, a significant attenuation in the decline in cell viability was observed. These results demonstrate that SFN has a protective effect against NO-based cytotoxicity. Because GSNO is known to promote excessive mitochondrial fission, the results may be indicative that SFN is providing protection by reducing mitochondrial fission. Confirmation of this hypothesis will require the analysis of the collective results of both Chapters 4 and 5.

4.7 Materials and Methods: Effect of SFN on ATP Production and Cellular Respiration in GSNO Treated Cells

SH-SY5Y cells were prepared for experimentation as described in Chapter 3.3. Cells utilized in the Oroboros O2K Oxygraph are required to be in suspension and thus no re-plating of cells was required. However, optimization of the experimentation protocol determined that the best reading on the Oxygraph were obtained when two 75% confluent flasks were combined. Therefore, for all further Oxygraph experimentation, two 75% confluent flasks of cells in a collective suspension constituted one treatment group and were injected into the Oxygraph to obtain results. All data collected from the Oxygraph were standardized to 8 million cells as two flasks at 75% confluency produced varying concentration of cells in suspension. Cell treatment groups were as follows: (a) Control (no treatment), (b) 200 µM GSNO treatment for 2 hours, and (c) a 30-minute pre-treatment with 5 µM SFN prior to 200 µM GSNO for 2 hours. Prior to injection into the Oxygraph, complete media were aspirated and cells were re-suspended in

mitochondrial respiration medium (MiR05) that contained the following components (in mM): 0.5 EGTA, 3 MgCl2, 60 Lactobionic Acid, 20 Taurine, 10 KH2PO4, 20 HEPES, and 110 D-Sucrose. Cells were re-suspended by mechanically flowing MiR05 over the pellet with the use of a 1000 µL pipette. The entire cell suspension was added to a 2 mL chamber in an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria), as previously described (Chicco et al., 2018). Oxygen flux (JO_2) was measured through examining the alterations in the negative time derivative of the oxygen concentration readings of the individual chambers normalized to 8 million cells. The O2K chambers were set to emulate a hyper oxygenated environment (400-150 µM O_2) and were calibrated to maintained a specific temperature of 37 °C. ROUTINE (respiration of intact cells on endogenous substrates) JO_2 was measured prior to addition of Digitonin, a selective plasma membrane permeabilizer, to a final concentration of 10-15 µg/mL, to allow substrate access to mitochondria. Mitochondrial respiratory flux was stimulated using TCA intermediates (final concentration in mM): 1 Malate, 5 Pyruvate, 2.5 Mg2+ -free ADP, 10 Glutamate, and 10 Succinate. ADP phosphorylation, ATP-ADP exchange from mitochondrial matrix to chamber, was measured following magnesium green (MgG) fluorescences using excitation and emission wavelengths at 503 and 530 nm, respectively (Goo et al., 2013). The rate reported herein is the rate of disappearance of MgG fluorescence, which is the inverse of ATP appearance in the chamber.

4.8 Results: Effect of SFN on ATP Production and Cellular Respiration in GSNO Treated Cells

Following results from the cell viability assays, it was important to determine if cells treated with GSNO were consequently experiencing drastic decreases in ATP production as consistent with excessive mitochondrial fission. The goal of this assay was to determine if SFN

pre-treatment could rescue any declines in ATP production and cellular respiration experienced by GSNO-treated cells. ROUTINE respiration of SH-SY5Y cells on endogenous substrates after treatment of GSNO or SFN-GSNO was assessed prior to permeabilization by digitonin and showed no difference in JO_2 between any of the groups (see Figure 4.4a). After permeabilization and addition of TCA intermediates, maximum JO_2 was obtained for the control and was found to be 124.5 ± 7.9 pmol O_2 per 8 million cell per sec. After treatment of GSNO, maximum JO_2 was significantly reduced to 90.9 ± 5.6 pmol O_2 per 8 million cells per sec ($p < 0.05$). GSNO-treated cells that were preincubated with SFN obtained a maximum JO_2 of 115.7 ± 11.0 pmol O_2 per 8 million cells per sec, this was not statistically different than either CON- or GSNO-treated cells (Figure 4.4a). Rate of ATP appearance was measured by recording the rate of disappearance of MgG in the medium. GSNO-treated cells had a 32% lower ATP appearance rate (0.129 ± 0.008) compared to CON (0.191 ± 0.018) ($p < 0.05$). This effect was attenuated by SFN pretreatment, which maintained ATP appearance rate to that of CON (0.198 ± 0.016) (see Figure 4.4b). Given differences in JO_2 after stimulation by TCA intermediates, ATP appearance rates was presented relative to JO_2 (ATP/O). GSNO-treated cells had a 13% lower ATP/O ratio (0.139 ± 0.001) compared to CON (0.156 ± 0.005), and this was attenuated with SFN treatment (0.1728 ± 0.007) ($p < 0.05$) (see Figure 4.4c). Statistical analysis was performed in Graphpad Prism software through a one-way ANOVA with statistical significance set at $p < 0.05$.

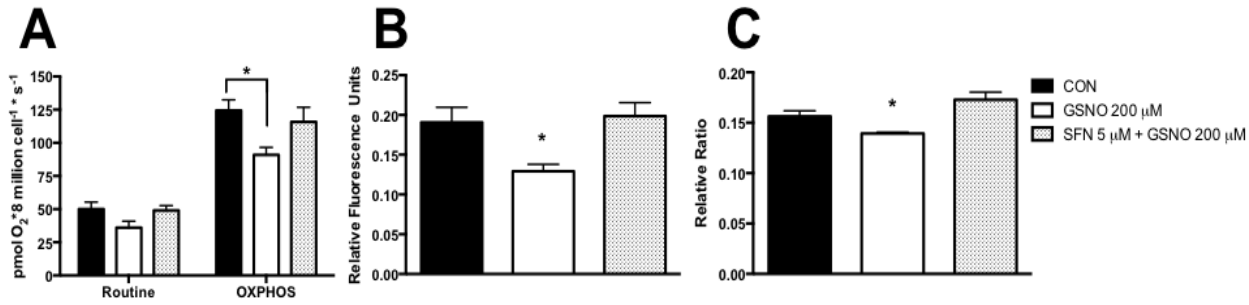

Figure 4.4. SFN Pre-Treatment Attenuates Suppressed JO_2 and ADP-Phosphorylation from GSNO Insult. (a) ROUTINE JO_2 and maximum JO_2 measurements of experimental groups; (b) ADP phosphorylation rates determined by changes in MgG fluorescence; (c) Ratio of ADP phosphorylation rate by maximum JO_2. One-way ANOVA * $p < 0.05$.

4.9 Discussion: Effect of SFN on ATP Production and Cellular Respiration in GSNO Treated Cells

ATP energy is vital for all cellular processes and therefore, the inability of a cell to produce adequate levels of ATP for these required processes will result in the cessation of these cellular processes and ultimately cellular death. Persistent mitochondrial fission results in mitochondrial instability and the permeabilization of the outer mitochondrial membrane, which can lead to reduction in cellular respiration, ATP production, and the induction of apoptosis. As expected, our results demonstrate that 200 μM of GSNO for 2 hours resulted in significant reduction in cellular respiration and the cell's ability to phosphorylate ADP to ATP. Interestingly, a 5 μM SFN pre-treatment for 30 minutes did not provide protection to cellular respiration; however, in accordance to our hypothesis, SFN did attenuate declines to ATP production significantly. These results further support the idea that SFN may be providing protection to cells undergoing excessive mitochondrial fission through interfering with the activation of Drp1.

4.10 Chapter Summary

Chapter 4 presented data collected from two assays that served two purposes. First, these experiments aided in confirming the effective doses of SFN and GSNO to utilize for further work. Second, these studies helped characterize both the detrimental effects of NO-induced mitochondrial fission on cellular physiology and the protective effects that SFN may evoke to mitigate these effects. Chapter 4 began with the optimization of the PrestoBlue cell viability assay in SH-SY5Y cells. The effects of GSNO and a pre-treatment of SFN on cell viability were then explored employing this protocol. Utilizing data from the cell viability assay, we then assessed the ability of 200 μM of GSNO to reduce ADP phosphorylation and decrease cellular respiration; and, the ability of 5 μM of SFN to attenuate these responses. Collectively, the results support the idea that SFN may be acting to reduce the activation of Drp1 to result in less mitochondrial fission to rescue cells from undergoing ATP depletion and apoptosis.

CHAPTER 5:

SPECIFIC AIM 2

5.1 Chapter Introduction

This chapter will build upon the results obtained in the previous chapter by investigating a possible mechanism of action by which SFN attenuates the effects of NO-induced mitochondrial fission. To accomplish this, the relative levels of phosphorylated Drp1 at specific serine residue 616 were examined in response to GSNO with and without a 30-minute pre-treatment of SFN. To obtain these results, a technique to identify specific proteins based upon their molecular weight known as a "western blot" was utilized.

5.2 Materials and Methods: Effect of SFN on Phosphorylation of Drp1 Ser616

SFN treatment groups utilized for the western blot experimentation in this chapter utilized the same stock solution and dilutions for SFN treatments as used in the previous chapter. As before, due to the unstable nature of solubilized GSNO even when frozen, GSNO solubilization occurred the same day as treatments for this experiment, to maintain the integrity of the compound. Treatment groups and treatment times for all western blot analysis are consistent with the treatments utilized for ATP and respiration as seen in the previous chapter. Cell treatment groups were as follows: (a) Control (no treatment); (b) 200 μM GSNO treatment for 2 hours; and, (c) a 30-minute pre-treatment with 5 μM SFN prior to 200 μM GSNO for 2 hours (see Table 5.1).

Table 5.1

Treatment Groups Utilized for all Western Blot Analyses

Group Name		**µM**	**Time (hrs/mins)**
(a)	Control	No treatment	N/A
(b)		GSNO 200 µM	2 hours
(c)		SFN 5 µM (pre-treatment) GSNO 200 µM	30 minutes 2 hours

To analyze the relative amounts of proteins within a cell such as phosphorylated Drp1 Serine 616 through the western blot analysis technique, protein isolation from cells is first required. After the completion of GSNO and SFN treatments, cells were lysed utilizing a lysis buffer containing phosphatase inhibitors. Because phosphorylation of proteins is a transient event by nature, phosphatase inhibitors are required to ensure that the integrity of any phosphorylation events is maintained during the protein isolation and freezing processes. A BCA protein analysis was then preformed to determine the relative amounts of total protein that was acquired from the protein isolation. The ability of the BCA to determine the relative amounts of protein was validated through linear regression with an obtained R^2 value of 0.99 as seen in Figure 5.1.

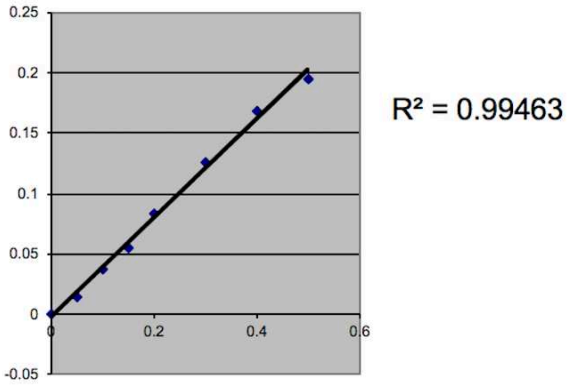

Figure 5.1. BCA Linear Regression for Protein Quantification

After protein quantification, proteins were resolved through 12.5% sodium-dodecyl sulfate polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) electrophoresis and immediately transferred to a nitrocellulose membrane. Blocking was performed using a tris-buffered saline (TBS) solution that contained 0.05% tween-20, and 5% (weight/volume) bovine serum albumin for 1 hour. Incubation of the membrane after the addition of the primary pDrp1-ser616 antibodies occurred overnight at 4 ℃. Secondary antibodies were added and incubated for 1 hour at room temperature. Desired proteins were then visualized through use of chemiluminescence visualization. ImageJ was utilized for all densitometric analysis on immunoblots to further quantify results.

5.3 Results: Effect of SFN on Phosphorylation of Drp1 Ser616

To determine the effects of SFN on NO-induced mitochondrial fission, relative levels of cytosolic phosphorylated Drp1 at serine residue 616 were analyzed through western blot analysis. The results revealed that a 2-hour treatment of cells with 200 μM GSNO significantly increased the amount of cytosolic pDrp1-ser616 to 162 ± 4.3% of control. Furthermore, the results demonstrated that a 30-minute pre-treatment with 5 μM of SFN prior to a 2-hour treatment of 200 μM GSNO, significantly attenuated cytosolic pDrp1-ser616 to 128.3 ± 8.4% of control (see Figure 5.2).

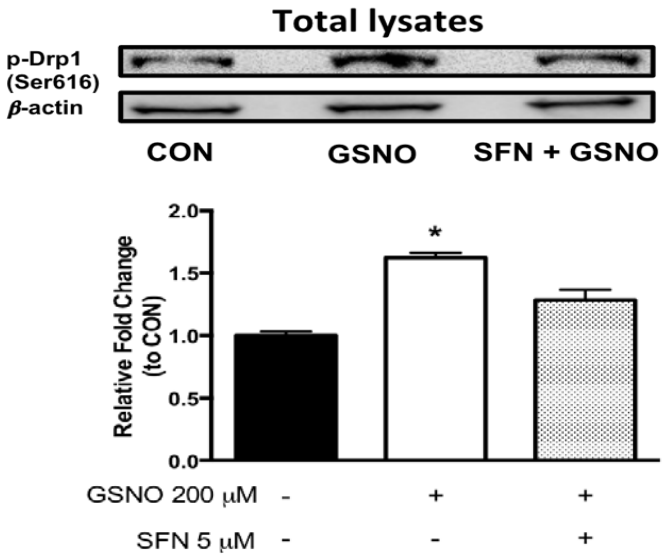

Figure 5.2. Effects of SFN on GSNO-induced pDrp1 Ser616. SH-SY5Y cells were treated with 200 μM GSNO for 2 hours to induce mitochondrial fission. Select treatment groups were pre-treated with 5 μM SFN for 30 minutes. Relative levels of pDrp1-ser616 between treatment groups were determined through western blot analysis and protein densitometry. Amounts of the protein of interest are represented as the ratio of pDrp1-ser616 to β-actin loading protein. The results are expressed as the mean ± S.E.M. of three individual experiments. One-way ANOVA showed GSNO treatment increased p-DRP1 compared to CON, whereas SFN pretreatment attenuated p-Drp1. *P < 0.05

5.4 Discussion: Effect of SFN on Phosphorylation of Drp1 Ser616

The above results help provide mechanistic insight into how SFN may act to inhibit the action of mitochondrial fission. A 2-hour treatment of SH-SY5Y cells with GSNO demonstrated the clear ability of NO to result in the phosphorylation of Drp1 at specific serine residue 616. Furthermore, the results indicate that SFN does reduce the levels of pDrp1-ser616; however, the means by which SFN accomplishes this are yet to be determined. Nonetheless, important inferences can be made. Research by O'Mealey et al. (2016) demonstrated that SFN acts in an Nrf2-independent manner; therefore, it is unlikely that any downstream effects of Nrf2 activation by SFN fully accounts for the protective effects of SFN on NO-induced mitochondrial fission. Additionally, experimentation by Li et al. (2009) demonstrated that endogenous NO can equally induce Nrf2 activity, which would further support the idea that any role SFN has in reducing the

phosphorylation of Drp1 is not likely related to Nrf2 activity (Li et al., 2009). In light of these observations, and those denoting SFN's various molecular targets referred to in Chapter 2, it appears more likely that SFN acts directly (i.e., via Drp1 serine residues) or indirectly (i.e., via upstream kinases) to interfere with the phosphorylation of Drp1-ser616, as presented on Figure 2.3 (shown below for ease of reference).

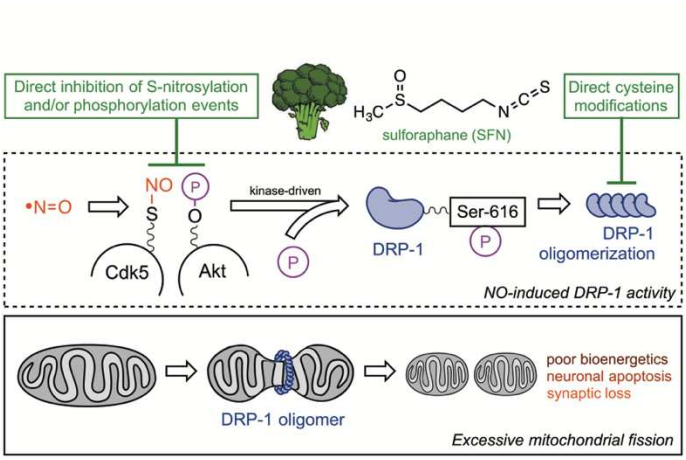

Figure 2.3. Dual Illustration of Proposed Possible Mechanisms of Both GSNO-Dependent Activation of Drp1 and SFN-Dependent Inhibition of Drp1 Activity. Illustration depicting the possible mechanism by which NO phosphorylates Drp1 and the downstream effects of excessive mitochondrial fission. Additionally, this illustration demonstrates the proposed mechanisms by which SFN may act to interfere with Drp1 phosphorylation.

5.5 Chapter Summary

This chapter explored the mechanistic action through which SFN may act to attenuate the results obtained in Chapter 4. The activation of mitochondrial fission largely occurs due to the activation of mitochondrial fission factor Drp1. Therefore, specifically, this chapter investigated the ability of SFN to interfere with the phosphorylation of Drp1 at specific serine residue 616. Results demonstrated that SFN does in fact reduce the levels of phosphorylation between treatment groups implying that SFN may possess the ability to modulate aberrant mitochondrial dynamics as seen in some disease states. Precisely how this occurs remains unknown and merits additional investigation by others.

CHAPTER 6:

FUTURE WORK

6.1 Chapter Introduction

Although this research has helped to elucidate various aspects of mitochondrial dysfunction and the novel properties of SFN as a therapeutic, more questions were generated in the process than answers. The purpose of this chapter is to help address the important questions that remain unknown and to help focus the design of potential future work.

6.2 Future Exploration

This study found that SFN could attenuate the detrimental effects of NO-induced mitochondrial fission. Additionally, this research found that SFN may have been acting to reduce these effects by interfering with the phosphorylation of Drp1 at specific serine residue 616. However, mitochondrial fission has also been reported to occur through the de-phosphorylation of Drp1 at specific serine residue 637; therefore, more studies to detail SFN's ability to act upon Drp1 serine residue 637 could be of importance (Cereghetti et al., 2008). Furthermore, as outlined in Chapter 2's Figure 2.3, there are numerous possible pathways by which NO can activate Drp1. These pathways (see Figure 2.3) should be further explored to determine the precise mechanism by which SFN may act.

6.3 Replication and Enhancement of the Study

While results obtained during this study are both significant and impactful, the ability of SFN to act upon Drp1 activation is an emerging area of research and so a confirmational study would be useful. Of interest, the phosphorylation of Drp1 at Serine 616 was detected through western blot analysis, however, a confirmational study performed utilizing another method, such

as flow cytometry, may provide more quantitative assessment of SFN's potential. Our laboratory began exploring this possibility, however, due to time constraints the optimization of the protocol was never completed.

6.4 Effectiveness of SFN on Various Methods of Inducing Mitochondrial Fission

This experimentation utilized GSNO, an NO-donor, to promote excessive mitochondrial fission and mimic a hallmark of specific neurodegenerative disease pathologies. However, numerous methods to induce mitochondrial fission exist, and the effectiveness of SFN to inhibit mitochondrial fission that is induced through a variety of methods could be of interest when attempting to fully understand the potential of SFN as a therapeutic.

CHAPTER 7:

CONCLUSION

Previous research demonstrated that SFN was acting independently of the Keap1-Nrf2 pathway to decrease mitochondrial fission and subsequently promote mitochondrial fusion. It was found that SFN may have been acting to inhibit the translocation of known mitochondrial fission factor Drp1 from the cytosol to the mitochondrial matrix, which is required for fission to occur; however, the mechanism by which this occurs remains unclear. The intent of this research was two-fold. Our laboratory first set out to further understand the capabilities of SFN to modulate mitochondrial dynamics by exploring the ability of SFN to attenuate the detrimental effects associated with excessive mitochondrial fission. Second, we aimed to provide insight into how SFN might do this by investigating the ability of SFN to interfere with the activation of Drp1 specifically through the phosphorylation of Drp1 at specific serine residue 616.

It was our hypothesis that NO-induced mitochondrial fission resulting in the programmed death of neuronal cells, as seen in age-related onset of neurodegenerative diseases (e.g., Huntingdon's, Alzheimer's, and Parkinson's diseases), may be attenuated through treatment of neuronal cells with SFN. While the data collected does support the original hypothesis, the potential of SFN to inhibit excessive mitochondrial fission as it relates to neurodegeneration requires a more detailed experimental approach. Highlighted findings of this research include:

- GSNO significantly decreased ATP production as determined through ADP phosphorylation.
- In contradiction to previous literature and our expectations, GSNO did not have any significant effect on mitochondrial respiration.

- SFN significantly attenuated both reduced levels of ATP production and declines seen in cell viability of GSNO-treated cells.
- Through western blot analysis, this research determined that SFN reduces levels of phosphorylated Drp1 at specific serine residue 616, although the precise mechanism by which SFN accomplishes this remains unclear.

The obtained results provide both detail surrounding the properties of SFN and insight into the potential for its use in combatting disease states that exhibit excessive mitochondrial fission. Continued research to explore the potential of SFN to modulate mitochondrial dynamics is imperative to determine its efficacy as treatment in human cases of age-related neurodegeneration.

CHAPTER 8:

PUBLICATIONS IN PROGRESS

One publication is anticipated to result from the covered research material. The collected data included in this thesis is expected to be submitted for peer review and publication in March of 2019.

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