INVESTIGATIONS INTO MODULATION OF BRAIN OXIDATIVE STRESS BY VARIOUS INTERVENTIONS

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INVESTIGATIONS INTO MODULATION OF BRAIN OXIDATIVE STRESS BY VARIOUS INTERVENTIONS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Arts and Sciences at the University of Kentucky

By

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2012

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ABSTRACT OF THESIS

INVESTIGATIONS INTO MODULATION OF BRAIN OXIDATIVE STRESS BY VARIOUS INTERVENTIONS

In this thesis study we examined glycogen synthase kinase-3β (GSK-3β) and its effects over Nrf2 and Pin 1 as it relates to Alzheimer’s disease (AD). AD is a neurodegenerative disease characterized by a prolonged high oxidative environment. Transcription factor Nrf2 is vital in the brain’s defense against oxidative insults through its up-regulation of over 100 antioxidants. Depletion of the brain’s antioxidant defense system results in intolerance to an oxidative environment, contributing to the progression of AD. The regulatory Pin 1 protein promotes cellular homeostasis, and when down-regulated results in increased deposits of neurofibrillary tangles (NFTs) and amyloid-β (Aβ) plaques, the two pathological hallmarks of AD.

Using aged SAMP8 mice treated with antisense oligonucleotide (AO) directed at GSK-3β and random AO, the data presented here demonstrate decreased oxidative stress and increased Nrf2 transcriptional activity and Pin 1 levels as a result of the down-regulation of GSK-3β. Collectively, these results implicate GSK-3β activity in the increased oxidative stress of AD and support its inhibition as a possible therapeutic treatment for the disease. Further, we elucidate a possible mechanism connecting GSK-3β to the loss of tolerance to an oxidative environment and increased deposits of NFTs and Aβ plaques observed in AD.

KEYWORDS: Alzheimer’s disease (AD), oxidative stress, glycogen synthase kinase-3β (GSK-3β), nuclear factor-E2-related factor 2 (Nrf2), Pin 1

Jessica Lynn Harris

11/30/2012
INVESTIGATIONS INTO MODULATION OF BRAIN OXIDATIVE STRESS BY VARIOUS INTERVENTIONS

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12/05/12
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To my mom, Diane
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Chapter 1: Introduction

This thesis study was conducted with the intent of gaining further insight into the effects glycogen synthase kinase-3β (GSK-3β) activity have over the brain cellular oxidative status in association with the activity of antioxidant transcription factor nuclear factor-E2-related factor 2 (Nrf2) and the level of regulatory Pin 1 protein as it relates to Alzheimer’s disease (AD). Currently, there are 5.4 million Americans living with AD, a number Alz.org predicts to increase to 16 million by 2050. Phenotypically, AD begins with the decline in memory and eventually progresses to dementia and extremely compromised quality of life. Biochemically, AD is characterized by the two pathological hallmarks: neurofibrillary tangles (NFTs), composed of irregularly hyperphosphorylated tau, and amyloid-β (Aβ) plaques, generated from amyloid precursor proteins (APP). In addition, a prolonged high oxidative environment that contributes to the increased oxidative stress and eventual neurodegeneration associated with the disease.

Under normal conditions, there exists an antioxidant system within the brain that works to maintain a low oxidative status. When this vital antioxidant system is depleted, from either decreased antioxidant expression or increased oxidant production, cells undergo oxidative stress that if prolonged can lead to subsequent neurodegeneration. Vital to the brain’s defense against oxidative insults is the antioxidant transcription factor Nrf2, considered a master regulator of redox homeostasis. Under basal conditions Nrf2 is found at low levels in the brain, which is regulated by its high turnover rate maintained by the ubiquitin-proteasome system. During times of stress Nrf2 expression rises and translocates to the nucleus, where it induces the transcription of over 100 genes a part of the antioxidant response system, thereby protecting the brain from oxidative damage (Phiel, Wilson et al. 2003; Rojo, Rada et al. 2008; Espada, Ortega et al. 2010; Zhang, An et al. 2012). GSK-3β is a negative regulator of Nrf2 activity, known to decrease the transcription factor’s nuclear localization. Recent studies have demonstrated GSK-3β involvement in targeting Nrf2.
for degradation by the ubiquitine-proteasome system (Chowdhry, Zhang et al. 2012; Rada, Rojo et al. 2012). In AD brain, GSK-3β and Nrf2 have been reported at high and low levels respectively, suggesting a role of the kinase’s negative regulatory activity over the antioxidant transcription factor in AD (Lucas, Hernandez et al. 2001; Ramsey, Glass et al. 2007).

For decades, the post translational modification, phophorylation, has been known as a common cellular regulatory mechanism over the activity of a given protein and its associated upstream and downstream pathways (Manning, Plowman et al. 2002; Pawson and Scott 2005). Pin 1 is a regulatory protein that binds a phosphoylated Ser/Thr-Pro motif of its target protein and cataylzes the isomerization of the peptide bond to a cis or trans confirmation. The cis or trans confirmation of a target protein’s Ser/Thr-Pro motif is specifically recognized by kinases and/or phosphatases, thereby controlling the phosphorylation/dephosphorylation of their substrate and regulating its function. Tau and APP are two target proteins of Pin 1 and are also phosphorlated by GSK-3β. The activities of Pin 1 and GSK-3β reportedly have opposite effects over APP processing and tau hyperphosphorylation, but the details of this inverse relationship remains to be elucidated.

In summary, this thesis research has used aged SAMP8 mice, as an AD model, treated with antisense oligonucleotide (AO) directed at GSK-3β (gAO) and random AO (rAO) to address the following hypotheses:

1. Supression of GSK-3β in gAO SAMP8 mice significantly decreases oxidative stress through increased antioxidant transcription.
2. Decreased oxidative stress as a result of GSK-3β decreases the level of Pin 1.
Chapter 2: Background

2.1 Oxidative Stress

2.1.1 Overview of Oxidative Stress

Within cells there exist antioxidant systems that serve to maintain a normal cellular redox balance by keeping oxidant levels low. When this redox balance is disrupted, through depletion of antioxidants or increased generation of free radicals, a cell undergoes oxidative stress in which its cellular components, such as lipids and proteins, become oxidatively modified and damaged (Finkel and Holbrook 2000). Free radical oxidative stress has been implicated in a large variety of neurodegenerative disorders including Alzheimer’s disease, Huntington’s disease, and amyotrophic lateral sclerosis (Butterfield and Lauderback 2002; Contestabile 2011; Lee, Kosaras et al. 2011).

Free radicals are highly reactive molecules that contain at least one unpaired electron which readily reacts with less reactive species nearby and subsequently produce a new free radical, often, triggering a chain reaction. When a free radical interacts with molecular oxygen, a reactive oxygen species (ROS) is produced, for example hydroxyl radicals (HO·) and superoxide anions (O₂⁻). While low levels of cellular ROS can be specifically used to initiate signaling cascades and maintain homeostasis, high levels can be detrimental for a cell by causing oxidative stress. Through the use of enzymatic and non-enzymatic antioxidants, such as glutathione and glutathione peroxidase (Gpx) respectively, a cell can maintain low oxidant levels (Pocernich, Cardin et al. 2001; Joshi, Hardas et al. 2007).

While all proteins and cell types are at risk of oxidative stress, some are considerably more susceptible than others. The brain is highly susceptible to oxidative stress due to high levels of polyunsaturated fatty acids (PUFAs), brain areas rich in redox-active transition metal ions, high consumption of oxygen, and relatively low antioxidant capacity.
2.1.2 Production of Reactive Oxygen Species

As mentioned above, ROS are generated from molecular oxygen and can be exogenous or endogenous. ROS production can be triggered by extracellular sources including ultraviolet light, chemotherapeutics and environmental toxins (Heck, Vetrano et al. 2003; Chen, Jungsuwadee et al. 2007). The majority of endogenous ROS comes from the mitochondria and can be rationalized by its high consumption of molecular oxygen coupled with the flow of electrons through the electron transport chain. Of the total molecular oxygen consumed by the mitochondria, 1-2% is converted into superoxide anions by complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome C reductase) of the electron transport chain (Boveris and Chance 1973; Turrens 1997). Under normal metabolic conditions, the Q-cycle of complex III is primarily responsible for the leak of mitochondrial ROS in which the reactive intermediate semiquinone radical (·QH) is generated during the two electron transfer from ubiquinol (QH₂) to cytochrome c. When the semiquinone radical encounters molecular oxygen, which is highly concentrated in the mitochondria, it is readily reduced by the reactive intermediate to superoxide (Turrens 1997). The leaked ROS leaves mitochondrial DNA (mtDNA), proteins, and lipids vulnerable to oxidative damage, which can lead to the disruption of mitochondrial functionality and a subsequent increase of ROS generation.

Among the enzymes that protect mtDNA and other cellular components against O₂·⁻ induced oxidative damage, are cytosolic Cu,Zn-superoxide dismutase (SOD) and mitochondrial Mn-superoxide dismutase, which generate hydrogen peroxide (H₂O₂) (reaction 1).

\[
2O_2^- + 2H^+ \rightarrow 2H_2O_2 + O_2 \quad \text{(reaction 1)}
\]

Hydrogen peroxide can then be metabolized to water and molecular oxygen by antioxidant enzyme glutathione peroxidase (reaction 2).

\[
2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(reaction 2)}
\]
Highly unstable ROS, like superoxide, are normally enclosed in the compartment they are produced, putting only nearby components in danger of oxidative damage. Unlike superoxide, the less reactive hydrogen peroxide can freely diffuse across cellular membranes and damage distant components, making it more toxic than the former. When a cell’s antioxidant system is depleted, hydrogen peroxide can begin to accumulate and lead to the generation of highly reactive hydroxyl radical (HO·) and hypochlorous acid (HOCl). Through hydrogen peroxide, the hydroxyl radical can be generated either spontaneously through the Haber-Weiss reaction with superoxide (reaction 3) or catalytically through protein- or other molecularly-bound metal ions, for example Fenton chemistry (reactions 4-5).

\[ 2H_2O_2 + 2O_2^- \rightarrow OH^- + O_2 + \cdot OH \]  

(reaction 3)

\[ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \]  

(reaction 4)

\[ Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot OOH + H^+ \]  

(reaction 5)

In Fenton chemistry the oxidation of ferrous iron (II) can reduce hydrogen peroxide to the hydroxyl radical and anion (reaction 4). The newly formed ferric iron (III) ion can then be reduced back to ferrous iron (II) by the same hydrogen peroxide (reaction 5) or vitamin C. The oxidation and regeneration of ferrous iron (II), and concomitant decomposition of hydrogen peroxide forms a chain reaction resulting in high levels of hydroxyl radical generation from only small amounts of ferrous iron (II) ions. It is through Fenton chemistry in which iron rich areas contribute to the oxidative vulnerability of the brain.
2.1.3 Lipid Peroxidation

Lipid peroxidation is characterized by the oxidative degradation of lipids within the cellular membrane by free radicals, which lead to their fragmentation or oxidation. The primary substrate of lipid peroxidation is PUFAs, rationalized by the electron-rich hydrogens adjacent to their electron-donating double bonds (Frankel 1984). PUFAs are a major component of biological membranes located on the β-chain of glycerophospholipids, which forms the lipid bilayer. High levels of PUFAs put the brain at risk for lipid peroxidation. The production of a single hydroxyl radical can lead to a cascade of lipid peroxidation that, if prolonged, can lead to the destabilization of the cellular membrane.

There are three main steps of lipid peroxidation: initiation, propagation, and termination. Initiation is driven by ROS, such as the hydroxyl radical, which attacks allylic hydrogens of an electron-donating double bond in PUFA systems, thus generating a carbon centered radical. This lipid carbon radical then rapidly reacts with molecular oxygen to generate a peroxyl radical. The newly generated peroxyl radical then attacks allylic hydrogen of nearby PUFAs generating lipid hydroperoxide and a new carbon-centered lipid radical, beginning the chain reaction of the propagation step (Frankel 1984). The propagation step continues until two lipid radicals meet and form a dimer, thus terminating the chain reaction of lipid peroxidation (Figure 2.1). α,β-Unsaturated aldehydes are some of the products of lipid peroxidation, including 4-hydroxy-2-trans-nonenal (HNE) and acrolein, both found at increased levels in AD brain (Markesbery and Lovell 1998; Lovell, Xie et al. 2001)
Initiation: \[ LH + R \cdot \rightarrow L \cdot + RH \]

Propagation: \[ L \cdot + O_2 \rightarrow LOO \cdot \]

\[ LOO \cdot + LH \rightarrow LOOH + L \cdot \]

Termination: \[ L \cdot + L \cdot \rightarrow L - L \]

Figure 2.1: General Lipid Peroxidation Reactions

HNE is one of the most important products of lipid peroxidation and is generated primarily from arachidonic acid (AA), but also docosahexaenoic acid (DHA) as well (Figure 2.2) (Esterbauer, Schaur et al. 1991). Because HNE is much more stable than the free radicals it was generated from, it can diffuse throughout a cell and cause more widespread damage by oxidizing cellular proteins at cysteine, histidine and lysine moieties (Uchida and Stadtman 1992; Neely, Sidell et al. 1999; Uchida 2003).
Figure 2.2: HNE formation from arachidonic acid. Adapted from Butterfield, Bader Lange et al. (2010)
2.1.4 Protein Carbonylation

During oxidative stress, proteins can undergo oxidative modifications to either their backbone or side-chains resulting in the change of protein conformation and functionality (Hensley, Carney et al. 1994). One way to quantify oxidative damage of proteins is to measure their carbonyl levels. Heightened levels of carbonyl species have been observed in the brain of those affected by a variety of neurodegenerative diseases including Alzheimer’s disease (Hensley, Hall et al. 1995; Markesbery and Lovell 1998; Butterfield, Drake et al. 2001; Lovell, Xie et al. 2001).

Oxidatively-induced protein carbonylation can occur through either the direct reaction with a free radical or covalent reaction with a reactive carbonyl. The extremely unstable hydroxyl radical can directly oxidize the side chains of threonine, arginine, proline and lysine through the addition of a carbonyl group (Amici, Levine et al. 1989). Reactive carbonyls are the product of oxidatively damaged lipids, sugars and amino acids that have the capability to alkylate and crosslink proteins. The lipid peroxidation product HNE is an example of a reactive carbonyl. Through Michael addition, lysine, cysteine, and histidine residues of essential cellular proteins can covalently bind through their electron-rich sites to the highly electrophilic β-carbon of HNE (Figure 2.3) (Uchida and Stadtman 1993; Nadkarni and Sayre 1995), resulting in the change of protein conformation and subsequent activity. The aldehyde of protein-bound HNE increases the level of carbonyl groups in a given protein. Due to the polarity, addition of carbonyl groups to a given protein can result in exposure of hydrophobic residues and conformational changes resulting in protein aggregation and change in function (Subramaniam, Roediger et al. 1997).
Figure 2.3: HNE Adducts of Histidine, Cysteine, and Lysine. Adapted from Butterfield, Bader Lange et al. (2010).
2.2 Glutathione

2.2.1 Glutathione Functions and Metabolism

The γ-glutamyl-cysteinyl-glycine tripeptide, glutathione (GSH), is one of the primary antioxidants of the brain responsible for maintaining the essential redox balance by keeping oxidant levels low. Through spontaneous and catalytic reactions, GSH reduces oxidants through the electron-donating thiol of its cysteine residue (Butterfield, Castegna et al. 2002; Butterfield, Castegna et al. 2002; Drake, Kanski et al. 2002). GSH can either donate a reducing equivalent or complex with reactive oxidants to protect the cell from potential oxidative stress.

When a reduced GSH encounters ROS, the antioxidant can reduce the radical to a more stable species via hydrogen donation of its cysteinyl moiety. Through GSH-dependent reduction of reactive hydroxyl (Figure 2.4a) (Pocernich, La Fontaine et al. 2000) or lipid hydroperoxide radicals, GSH can prevent the initiation and/or propagation phase of lipid peroxidation. Oxidized GSH then readily forms a disulfide bond with other oxidized GSH to form glutathione disulfide (GSSG), which is converted back to its reduced form by the NADPH dependent enzyme glutathione reductase (Figure 2.4b). If oxidized GSSG is not recycled back to its reduced GSH form, this can cause the increase of oxidant levels from the decrease of the antioxidant defense system and subsequent oxidative stress.
Figure 2.4: GSH Functions. a.) GSH-Mediated Hydroxyl Radical Reduction
b.) Recycling of GSH Adapted from Pocernich and Butterfield (2012).
Through spontaneous and catalytic reactions, GSH complexes with metals and oxidants to further protect cells from oxidative stress. Iron is among the metals GSH complexes with, preventing hydroxyl radical generation via Fenton chemistry (Hammond, Lee et al. 2001; Butterfield, Castegna et al. 2002). GSH can also complex with HNE for export from the CNS, protecting the brain from its toxic oxidative effects (Alin, Danielson et al. 1985).

### 2.2.2 Glutathione S-Transferase

GSH is a co-enzyme for many cell defense enzymes of the brain, including glutathione s-transferase (GST). GST functions to keep HNE brain levels low by catalyzing its conjugation to GSH (Alin, Danielson et al. 1985). The generated GSH-HNE conjugate can then be exported from the brain via membrane-bound multi-drug resistance protein-1 (MRP-1) transporter, which helps protect the brain from oxidative damage caused by HNE (Morrow, Smitherman et al. 1998). Decreased GST activity has been reported in the brain of Alzheimer’s patients (Lovell, Xie et al. 1998), which could result in the increase of the oxidatively damaging effects of HNE in brain. Increased levels of bound HNE to α-GST and MRP1 have also been reported in AD hippocampal tissue compared to control brain, suggesting depletion of the GSH antioxidant system (Sultana and Butterfield 2004).
2.3 Glycogen Synthase Kinase-3β

2.3.1 Overview of Glycogen Synthase Kinase-3β

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase for over forty different proteins including transcriptional factors, metabolic enzymes and structural proteins. Through the post translational modification of phosphorylation, GSK-3 tends to negatively affect the activity of its substrates highlighting the need of tight regulation over the kinase’s activity. GSK-3 exists in two isoforms, GSK-3α and GSK-3β, both of which are ubiquitously expressed and constitutively active. Although GSK-3α and GSK-3β share 85% homology, different genes are responsible for encoding the two isoforms (Woodgett 1990).

GSK-3β, the smaller of the two isoforms, is most abundant in the brain with greater expression in neurons than astrocytes (Takahashi, Tomizawa et al. 1994; Takahashi, Tomizawa et al. 2000). Nuclear and mitochondrial activity of GSK-3β is reportedly greater than in the cytosol (Bijur and Jope 2003). Some roles of GSK-3β include regulation of glycogen synthase, a rate-limiting enzyme for glycogen biosynthesis, and tau, a microtubule-associated protein. GSK-3β has been implemented in the pathogenesis of Alzheimer’s disease, where its deregulation contributes to the hyperphosphorylation of tau and subsequent neurofibrillary tangle (NFT) aggregation (Lovestone, Reynolds et al. 1994; Yamaguchi, Ishiguro et al. 1996; Hong, Chen et al. 1997; 2003; Lovell, Xiong et al. 2004).
2.3.2 GSK-3β Regulation

Because GSK-3β is a constitutively active kinase that primarily inhibits the activity of its associated substrates, including pro-survival transcriptional factors and metabolic enzymes, its tight regulation is essential. GSK-3β is normally down-regulated directly through the phosphorylation of Ser9 in the pseudosubstrate domain, which can inhibit its activity or binding strength to a substrate. Some common inhibitors include PKA, p90Rsk, and Akt (PKB) (Grimes and Jope 2001). When GSK-3 activity is inhibited this promotes the de-phosphorylation and subsequent activation of its substrates.

The pro-survival pathway governed by phosphatidylinositol-3 kinase (PI3K) and its downstream effector Akt (PKB) negatively regulates GSK-3β through Ser9 phosphorylation (van Weeren, de Bruyn et al. 1998; Tomobe, Shinozuka et al. 2012). The PI3K/Akt pathway is reportedly activated by reactive oxygen species (ROS)-generating agents and H₂O₂ (Martin, Salinas et al. 2001; Tang, Okada et al. 2001). However, prolonged exposure to H₂O₂ and amyloid β-peptide (Aβ) reportedly inhibits the PI3K/Akt pathway, promoting the de-phosphorylation of Ser9 and subsequent GSK-3β up-regulation (Takashima, Noguchi et al. 1996; Martin, Salinas et al. 2001; Rojo, Sagarra et al. 2008).

2.4 Nuclear Factor-E2-Related Factor 2 (Nrf2)

Transcription factor nuclear factor-E2-related factor 2 (Nrf2) is a substrate of GSK-3β responsible for both basal and induced expression of antioxidant phase II genes such as heme oxygenase-1, glutathione S-transferases, glutathione peroxidases, and γ-glutamylcysteine ligase. The Nrf2 pathway is the primary mechanism in the induction of GSH biosynthesis, consequently playing a key role in maintaining a cell’s redox homeostasis and reportedly protects neurons from oxidative stress (Shih, Johnson et al. 2003). (Wild, Moinova et al. 1999)
It has been recently suggested that cellular localization of Nrf2 may be a key part of its regulation. In the absence of oxidative injury Nrf2 is sequestered in the cytoplasm though association with the chaperone protein Keap1, which promotes Cul3-Rok1 complex-mediated ubiquitination of Nrf2 and subsequent proteasomal degradation (Kobayashi, Kang et al. 2004; Lo, Li et al. 2006; Tong, Katoh et al. 2006). During oxidative conditions Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to antioxidant response elements (AREs) and stimulates the transcription of phase II genes to protect the cell from oxidative stress (Katsuoka, Motohashi et al. 2005). GSK-3β activity, which is higher in the nucleus compared to the cytosol, has been reported to directly promote nuclear exclusion of Nrf2, thereby decreasing its pro-survival transcriptional activity (Bijur and Jope 2003; Salazar, Rojo et al. 2006). Recent studies have demonstrated GSK-3β involvement in targeting Nrf2 for degradation by the ubiquitine-proteasome system (Chowdhry, Zhang et al. 2012; Rada, Rojo et al. 2012).

2.5 Alzheimer’s Disease

2.5.1 Alzheimer’s Disease Overview

Alzheimer’s disease (AD) is a neurodegenerative disorder that affects over 5 million Americans today and is characterized by cognitive impairment and progressive memory loss of individuals affected by the disease. The two pathological hallmarks of AD include neurofibrillary tangles (NFTs), consisting of paired helical filaments of hyper-phosphorylated tau proteins, and Aβ plaques, consisting of aggregated Aβ surrounded by dystrophic neuritis. Aβ formed from the proteolytic cleavage of β-amyloid precursor protein (APP). One of the most commonly accepted AD hypotheses today is the amyloid cascade hypothesis, which postulates that the over-production of Aβ plaques leads to tau hyper-phosphorylation and subsequent NFT generation and neurodegeneration.
Tau is a soluble microtubule-associated structural protein which functions to improve microtubule stabilization through binding to tubulin and thereby promoting tubulin polymerization and maintaining the neuronal cytoskeleton. The binding of Tau to tubulin is regulated through its phosphorylation state, which is controlled by some kinases including cyclin-dependent kinase 5 (Cdk5) and GSK-3β and phosphatases such as protein phosphatase 2A (PP2A). Phosphorylation of Tau at key residues leads to its dissociation from tubulin and consequential instability of microtubule organization. When tau becomes hyper-phosphorylated NFT formation and neurodegeneration can result (Lee and Tsai 2003; Lu, Liou et al. 2003).

APP is an integral membrane glycoprotein containing a single transmembrane domain along with a large extracellular N terminal and small C terminal domain. Within the bilayer, APP can be cleaved at its transmembrane domain by β-secretase (BACE) and γ-secretase to form the Aβ peptide (Glenner and Wong 1984; Glenner, Eanes et al. 1988). Aβ(1-40) and Aβ(1-42) are the two most common forms of the peptide associated with human AD and the latter shown in AD models to be more toxic than the other (Selkoe 1996; Butterfield and Boyd-Kimball 2004; Butterfield and Boyd-Kimball 2005; Mohmmad Abdul, Sultana et al. 2006). Aβ peptides can aggregate and form Aβ plaques.

2.5.2 GSK-3β Involvement

GSK-3β has been tied to AD through its contribution to NFT generation and up-regulation via Aβ plaques. As mentioned earlier, one role of GSK-3β is to regulate tau proteins through phosphorylation. When tau becomes hyper-phosphorylated it detaches from microtubules and aggregates, forming NFTs. Hyper-phosphorylation of tau and neurodegeneration has been reported in GSK-3β transgenic mice (Lucas, Hernandez et al. 2001). Using double transgenic mice with over-expression of GSK-3β and tau treated with the GSK-3β inhibitor chronic lithium, tau hyper-phosphorylation and NFT formation was prevented (Engel, Goni-Oliver et al. 2006). Aβ plaques
reportedly up-regulates GSK-3β activity through the inhibition of its associated negative regulatory pathway, PI3K/Akt (Takashima, Noguchi et al. 1996). When GSK-3β activity or expression were decreased, Aβ-induced neurodegeneration was inhibited (Takashima, Noguchi et al. 1993; Alvarez, Munoz-Montano et al. 1999).

2.6 Pin 1

The Pin 1 protein, belonging to the peptidyl-prolyl cis/trans isomerase (PPIase) family, regulates the activity of its associated target proteins by binding to a pSer/pThr moiety located on the N-terminal side of proline and catalyzes the conformational change of the peptide bond to the cis or trans conformation. Pin 1 is composed of two domains: the PPIase catalytic and WW binding domains. The PPIase catalytic domain is responsible for catalyzing the cis-trans conformational change of the peptide bond of pSer/Thr-Pro of the target protein and the WW domain recognizes and links Pin 1 to the specific motif (Ranganathan, Lu et al. 1997; Yaffe, Schutkowski et al. 1997; Lu, Zhou et al. 1999). The regulatory functions of Pin 1 are diverse including regulation of proteins involved in the cell-cycle, transcription, apoptosis, and DNA damage response (Lu, Hanes et al. 1996; Lu, Liou et al. 2003). Some key target proteins of Pin 1 include tau and APP.

As mentioned earlier, tau plays an important role in maintenance of microtubule stability through binding to tubulin and promoting tubulin assembly. The phosphorylation status of tau dictates whether the structural protein binds to and stabilizes microtubules or dissociates from tubulin thereby promoting instability of microtubule organization. When activities of tau-associated kinases and phosphatases become unbalanced tau hyper-phosphorylation and subsequent NFT formation can result (Wang, Gong et al. 1995). Pin 1 regulates the phosphorylation status of tau through the activity of the conformation-specific phosphatase, PP2A, which catalyzes the de-phosphorylation of trans pSer/pThr-Pro sites of tau. After the Ser/Thr-Pro sites of tau become phosphorylated, Pin 1 can bind to the pSer/pThr-Pro sites and either positively or negatively affect PP2A activity by catalyzing trans or cis conformational
changes of the peptide bond respectively (Lu, Wulf et al. 1999; Zhou, Kops et al. 2000). The Pin 1-mediated cis confirmation of tau inhibits PP2A activity, thereby increasing the risk of tau hyperphosphorylation though the activity of its associated kinases, including GSK-3β and Cdk-5.

The amyloidogenic cleavage of APP by β- and γ-secretases results in the generation of Aβ peptide. Pin 1 has been linked to Aβ peptide formation through its isomerization activity of the pThr668-Pro APP motif. The cis pThr668-Pro conformation reportedly promotes amyloidogenic APP cleavage, while the trans pThr668-Pro conformation promotes non-amyloidogenic APP cleavage (Pastorino, Sun et al. 2006).

Through its regulation of tau phosphorylation/de-phosphorylation and Aβ generation, Pin 1 is heavily implicated in AD pathogenesis (Hamdane, Smet et al. 2002; Liou, Sun et al. 2003). In AD brain, Pin 1 activity and expression is reportedly reduced in the presence of oxidative stress (Sultana, Boyd-Kimball et al. 2006). Other studies have shown Pin 1 to be oxidatively modified in hippocampus from mild cognitive impairment (MCI) and AD subjects (Butterfield, Poon et al. 2006; Sultana, Boyd-Kimball et al. 2006).

2.7 Hypotheses

Normally increased activity of GSK-3β inhibits Nrf2-ARE activity, thereby decreasing antioxidant production and increasing oxidative stress, as shown in Figure 3.1a (Salazar, Rojo et al. 2006; Rojo, Sagarra et al. 2008). In AD brain the level of GSK-3β is reportedly high, which leads to the hypotheses that suppression of GSK-3β in SAMP8 mice, an AD model, will increase the levels of Pin 1, nuclear Nrf2 and GST, and decrease the level of 3-HNE, shown in Figure 3.1b (Leroy, Yilmaz et al. 2007).
Figure 3.1: Hypotheses tested.  

a.) Normal regulatory Gsk-3β activity.  

b.) Hypothesized effect of Gsk-3β suppression in SAMP8 mice.
Chapter 3: Materials and Experimental Procedures

3.1 Chemicals and Materials

All chemicals used were of the highest purity and purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. 2,4-dinitrophenylhydrazine (DNPH), primary Rb x DNP polyclonal and secondary anti-rabbit IgG antibodies were purchased from Chemicon (Temecula, CA). Primary anti-HNE, anti-GST, and anti-Nrf2 antibodies were obtained from Alpha Diagnostic (San Antonio, TX), Epitomics (Burlingame, CA), and Enzo Life Sciences (Farmingdale, NY) respectively. Nitrocellulose membranes, polyacrylamide gels, XT MES electrophoresis running buffer, and Precision Plus Protein™ All Blue Standards were purchased from Biorad (Hercules, CA). Primary anti-GSK-3β was purchased from Cell Signaling (Danvers, MA) and primary anti-Pin 1 [H-123] was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Cydye3 and cydye5 were purchased from GE healthcare (Pittsburgh, PA).

3.2 Animals and Treatments

In this study, cortex brain regions were collected from aged senescence-accelerated mice prone 8 (SAMP8) mice treated with antisense oligonucleotide (AO) against GSK-3β (n = 9) and random AO (n = 7) as previous dictated (Kumar, Farr et al. 2000). Brain samples from such treated mice were provided by Dr. Susan A. Farr, St. Louis University.
3.3 Sample Preparation

Using a Wheaton tissue homogenizer, brain samples were briefly homogenized in ice-cold lysis buffer (pH 7.4; 320 mM sucrose, 1% mM Tris-HCl (pH 8.8), 0.098 mM MgCl₂, 0.076 mM EDTA, proteinase inhibitors leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), aprotinin (0.5 mg/mL) and PMSF (40 μg/mL), and phosphatase inhibitor cocktail (Sigma-Aldrich)), and then diluted 2X with lysis buffer. After homogenization, a small aliquot of homogenized samples were sonicated for 10 seconds at 20% power with a Fisher 550 Sonic Dismembrator (Pittsburgh, PA), then saved for measurement of oxidative stress, and to run western blots for GST and Pin-1 detection (described later). The remaining homogenate was centrifuged at 3000 g for 5 min and the supernatant cytosolic and membranous fractions were transferred out into another set of tubes. Following the addition of 400 μL of lysis buffer, the remaining pellet nuclear fraction was centrifuged at 3,000 g for 5 min and supernatant removed. The pellet was suspended in 20 μL of homogenization buffer and inhibitor. The supernatant cytosolic and membranous fractions were centrifuged at 10,000 g for 10 min, and the resulting supernatant cytosolic fraction was transferred out into another set of tubes leaving the pellet membranous fraction. All fractions were stored at -70 °C until used for further experiments. Resulting sample solutions’ protein concentrations were measured through Pierce Bicinchoninic Acid (BCA) method (Smith, Krohn et al. 1985).

3.4 Protein Concentration

Through the use of bicinchoninic acid (BCA) assay and bovine serum albumin (BSA) used as a standard, protein concentrations were determined. The BCA assay is a two part mixture made up of reagent A, composed of BCA, sodium carbonate, and sodium tartrate in 0.1 M sodium hydroxide, and reagent B, composed of a 4% cupric sulfate pentahydrate solution. In a 96-well microplate, samples (5 μl) are pipetted in duplicates and diluted with BCA solutions (95 μl) alongside the BSA standard of known concentrations. During the 15 min incubation period at 37°C, the sample protein
peptide bonds (4-6) reduce cupric ions (Cu\(^{2+}\)) of reagent B to cuprous ions (Cu\(^{1+}\)), in which BCA of reagent A then complexes with. The newly generated BCA- Cu\(^{1+}\) complex produces a purple color with a strong absorbance at 562 nm, and its intensity correlates with the relative amounts of certain amino acids in each protein sample, incubation time, and temperature (Olson and Markwell 2007). After incubation, a UV-Vis instrument (uQuant plate reader by BIO-TEK Instruments, Inc.) was used to determine color intensity at 562 nm. Through use of the Lambert-Beer law and known bovine serum albumin (BSA) concentrations, a BSA standard curve was generated and used to determine the sample protein concentration.

### 3.5 Protein Carbonyl Determination

As described in Chapter 2, the protein carbonyl level is an important marker of protein oxidation. The slot blot technique was used to measure carbonyl levels of protein samples.

Five μl of sample, 5 μl of 12% sodium dodecyl sulfate (SDS), and 10 μl of 2,4-dinitrophenylhydrazine (DNPH) were incubated at room temperature for 20 min. After incubation, 7.5 μl of neutralization solution (2 M Tris in 30% glycerol) were added to each sample and diluted to 100 μl with 1xPBS (phosphate buffer solution containing sodium chloride, mono, and dibasic sodium phosphate). Following derivatization samples were diluted to 1 μg/mL. The corresponding sample solution (250 μl) was loaded as duplicates into the wells of a slot blot apparatus, located directly above a nitrocellulose membrane. Through water vacuum pressure, proteins were rapidly loaded on the membrane and then blocked with BSA in wash blot (35.2 g sodium chloride, 1.77 g monobasic sodium phosphate, 9.61 g dibasic sodium phosphate, 1.6 mL TWEEN, diluted to 4 L with deionized water) for 90 min. The membrane was then incubated with a 1:100 dilution of RbxDNP polyclonal primary antibody in wash blot for 2 h. After three 5 min washes with fresh wash blot, the membrane was then incubated with 1:8000 dilution of anti-rabbit IgG alkaline phosphatase polyclonal secondary
antibody for 1 hour and washed with fresh wash blot in three increments of 5, 10 and 10 min. After washing, 20 mL of developing solution (66 μl BCIP and 133 μl NBT diluted with DI water to 20 mL) was added to each blot. After development, blots were dried and scanned (CanoScan8800F scanner) using Adobe Photoshop and analyzed using Scion Image software.

3.6 Protein-Bound 4-hydroxy-2-trans-nonenal (HNE) Determination

As described in Chapter 2, the protein-bound HNE level is an important marker for lipid peroxidation. Through the slot blot technique, protein-bound HNE levels of homogenized brain samples were immunochemically detected.

Five μl of 12% sodium dodecyl sulfate (SDS), and 10 μl of Laemmli buffer were combined with 5 μl of sample and incubated at room temperature for 20 min. As described earlier for protein carbonyl, following incubation sample solutions were diluted and loaded in duplicates on nitrocellulose membrane, then blocked with BSA in wash blot (35.2 g sodium chloride, 1.77 g monobasic sodium phosphate, 9.61 g dibasic sodium phosphate, 1.6 mL TWEEN, diluted to 4 L with deionized water) for 90 min. The membrane was then incubated with a 1:5000 dilution of anti-HNE polyclonal primary antibody in wash blot for 2 h. After three 5 min washes with fresh wash blot, the membrane was then incubated with a 1:8000 dilution of anti-rabbit IgG alkaline phosphatase polyclonal secondary antibody for 1 hour and washed with fresh wash blot in three increments of 5, 10 and 10 min. After washing, 20 mL of developing solution (66 μl BCIP and 133 μl NBT diluted with DI water to 20 mL) was added to each blot. After development, blots were dried and scanned (CanoScan8800F scanner) using Adobe Photoshop and analyzed using Scion Image software.
3.7 TCA Precipitation

The cytosolic fraction proteins were enriched by precipitating with trichloroacetic acid (TCA). Ice-cold 100% TCA was added to the sample proteins to obtain a final concentration of 15%, and then placed on ice for 10 min. Precipitates were centrifuged at 16,000 g for 2 min. The resulting pellets were then washed four times with an ethanol/ethyl acetate solution (1:1) and centrifuged after each wash at 16,000 g for 2 min. The final protein pellets were then suspended in 20 μL of DI water.

3.8 Western Blotting

The Western blot technique was used to measure protein levels of GSK-3β, Nrf2, GST and Pin1. In this method, sample proteins were subjected to an SDS-containing buffer and denatured. SDS is a detergent that coats proteins and imparts a negative charge as a result of the sulfate moiety; this new negative charge evenly distributed across a protein helps maintain its denatured state through electrostatic repulsion. The denatured sample proteins were then separated through gel electrophoresis by their electrophoretic mobility (often related to molecular weight) which gave varying migration rates, in a bis-tris-polyacrylamide gel at a specific voltage (SDS-PAGE). Smaller proteins have a faster migration rate through polyacrylamide mesh towards the positively charged electrode compared to larger proteins. The resulting separated proteins on the electrophoresis gel were then transferred onto a nitrocellulose membrane (Western blotting) through the application of a voltage at a 90 degree angle to the gel. The negative charges on the denatured proteins, imparted by SDS, are attracted to the positively charged nitrocellulose membrane causing the proteins to move out of the gel and onto the membrane. The resulting protein-bound membrane was then blocked and probed with specific antibodies corresponding to target proteins.

In this project prior to gel loading, sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] was added to either 30 μg or 50 μg of sample protein and then denatured in boiling water for 5 min.
and cooled on ice. Sample proteins and Precision Plus Protein™ All Blue Standards were electrophoresed on a 4-12% bis-tris polyacrylamide gel at room temperature using a Criterion Cell™ vertical electrophoresis buffer tank filled with 1X XT MES running buffer. To ensure proper protein stacking, the voltage was initially set at 80 V for ~10 min, and then increased to 120 V for the remaining ~130 min of the electrophoretic run. Resolved proteins were then transferred to nitrocellulose membrane at 1.0 A/gel for 30 min using a Trans-Blot® Turbo™ transfer system SD semi-Dry Transfer Cell (Bio-Rad). The protein transfer was checked using ponceau S, a reversible protein stain. The blots were blocked for 90 min at room temperature with 750 mg BSA in 25 mL of wash blot [35.2 g sodium chloride, 1.77 g monobasic sodium phosphate, 9.61 g dibasic sodium phosphate, 1.6 mL TWEEN, diluted to 4 L with deionized water]. Dilutions of primary anti-GSK-3β (1:2000), anti-Nrf2 (1:1000), anti-GST (1:1000) and anti-Pin1 (1:1000) were prepared in wash blot, then washed three times with fresh wash blot. The blots were incubated with ECL Plex CyDye conjugated secondary antibodies for 1 h in dark at room temperature, then washed again with fresh wash blot three times. Bands were visualized using a fluorescent laser Typhoon™ FLA9500 (GE Healthcare, Pittsburgh, PA) scanner and quantified using Scion Image software. For loading control, the blots were probed with anti-β-actin or anti-histone 2B antibodies raised in mouse, followed by incubation with anti-mouse secondary antibody (Cy3).

3.9 Statistical Analysis

A Mann-Whitney test was employed to assess statistical significance in comparing protein carbonyl, protein-bound HNE, GSK-3β, Nrf2, GST and Pin1 levels in protein samples between control and experimental data sets. Significant differences were set at P < 0.05.
Chapter 4: Results and Discussion

4.1 Introduction

Aged SAMP8 mice, a model of AD, exhibit age-related declines in learning and memory, and increased oxidative stress and Aβ deposits in their CNS (Flood and Morley 1993; Butterfield, Howard et al. 1997; Farr, Poon et al. 2003; Poon, Castegna et al. 2004; Butterfield and Poon 2005; Del Valle, Duran-Vilaregut et al. 2010). GSK-3β levels were measured to insure a down-regulation of the kinase in brain isolates of SAMP8 mice treated with antisense oligonucleotide (AO) directed at GSK-3β (GAO) compared to random AO (RAO). Levels of oxidative stress markers, Nrf2, GST, and Pin 1 were determined in brain isolated from SAMP8 GAO and RAO mice.
4.2 Protein carbonyl level decreased in SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

Figure 4.1 illustrates protein carbonyl levels in brain isolated from GAO and RAO SAMP8 mice. GAO SAMP8 mice exhibit a significant decrease in the level of protein carbonyl compared to SAMP8 RAO mice. Protein carboxyls are an important marker for protein oxidation.

![Protein Carbonyl Levels](image)

Figure 4.1: Protein carbonyl level in GAO compared to RAO SAMP8 mice. Protein Carbonyl level decreased in SAMP8 mice treated with AO directed at GSK-3β (N=9) compared to that of SAMP8 mice treated with random AO (N=7). Data are represented as % control, and shown as mean ± SEM with *P<0.02.
4.3 Protein-bound HNE level decreased in SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

SAMP8 cAO mice show a significant decrease in the protein-bound HNE level compared to rAO SAMP8 mice, as presented in Figure 4.2. HNE is an important biomarker for lipid peroxidation and its protein-association generally leads to changes in the conformation and functionality of the given protein (Subramaniam, Roediger et al. 1997; Reed, Perluigi et al. 2008).

![Protein-Bound HNE Levels](image)

Figure 4.2: Protein-bound HNE level in cAO compared to rAO SAMP8 mice. Protein-bound HNE level decreased in SAMP8 mice treated with AO directed at GSK-3β (N=9) compared to that of SAMP8 mice treated with random AO (N=6). Data are represented as % control, and shown as mean ± SEM with *P<0.0008.
4.4 GSK-3β levels decreased in cytosolic and nuclear fractions of SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

As expected, Figure 4.3 and Figure 4.4 show that the AO reduced the levels of GSK-3β in cytosolic and nuclear fractions, respectively, from SAMP8 cAO mice compared to rAO-treated SAMP8 mice. Actin and histone 2B were loading controls for the cytosolic and nuclear fractions, respectively. Nuclear (30 μg) and cytosolic (50 μg) fractions of sample proteins were resolved on polyacrylamide gel via SDS-PAGE and through the Western blot technique transferred to a nitrocellulose membrane.

Figure 4.3: Cytosolic GSK-3β level in cAO compared to rAO SAMP8 mice. Level of GSK-3β decreased in the cytosolic fraction of SAMP8 mice treated with AO directed at GSK-3β (cAO) compared to that of SAMP8 mice treated with random AO (cAO). Data are represented as % control, and shown as mean ± SEM with *P<0.05. Total number of animals used in each group are: cAO=8 and rAO=7.
Figure 4.4: Nuclear GSK-3β level in _G_AO compared to _R_AO SAMP8 mice. Level of GSK-3β decreased in the nuclear fraction of SAMP8 mice treated with AO directed at GSK-3β (G_AO) compared to that of SAMP8 mice treated with random AO (R_AO). Data are represented as % control, and shown as mean ± SEM with *P<0.04. Total number of animals used in each group are: G_AO=5 and R_AO=5.
4.5 Nrf2 levels decrease in the cytosolic and increase in the nuclear fractions of SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

Since Nrf2 translocation to the nucleus depends in part on its decreased phosphorylation via GSK-3β, 50 μg of the nuclear and cytosolic fractions of sample protein were determined using the Western blot method. The level of Nrf2 decreased in the cytosol and increased in the nucleus, respectively, for \(_G^O\)-AO-treated SAMP8 mice compared to \(_R^O\)-AO-treated SAMP8 mice, illustrated in Figure 4.4 and Figure 4.5. Actin and histone 2B were loading controls for the cytosolic and nuclear fractions, respectively.

Figure 4.5: Cytosolic Nrf2 level in \(_G^O\)AO compared to \(_R^O\)AO SAMP8 mice. Reduced expression of Nrf2 in the cytosolic fraction of SAMP8 mice treated with AO directed at GSK-3β (\(_G^O\)AO) compared to that of SAMP8 mice treated with random AO (\(_R^O\)AO). Data are represented as % control, and shown as mean ± SEM with *P<0.02. Total number of animals used in each group are: \(_G^O\)AO=9 and \(_R^O\)AO=5.
Figure 4.6: Nuclear Nrf2 level in GAO compared to RAO SAMP8 mice. The level of Nrf2 increased in the nuclear fraction of SAMP8 mice treated with AO directed at GSK-3β (GAO) compared to that of SAMP8 mice treated with random AO (RAO). Data are represented as % control, and shown as mean ± SEM with *P<0.04. Total number of animals used in each group are: GAO=5 and RAO=5.
4.6 GST level increased in the homogenized brain samples of SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

As a means of determining Nrf2 transcriptional activity, the level of GST was measured. Homogenized (30 μg) sample proteins were resolved on polyacrylamide gel via SDS-PAGE and through the Western blot technique transferred to a nitrocellulose membrane. Figure 4.7 shows an increase in the GST level of SAMP8 GAO compared to RAO SAMP8 mice.

Figure 4.7: GST level in GAO compared to RAO SAMP8 mice. The level of GST increased in the homogenized samples of SAMP8 mice treated with AO directed at GSK-3β (GAO) compared to that of SAMP8 mice treated with random AO (RAO). Data are represented as % control, and shown as mean ± SEM with *P<0.03. Total number of animals used in each group are: GAO=7 and RAO=6.
4.7 Pin1 level increased in the homogenized brain samples of SAMP8 mice treated with AO directed at GSK-3β compared to random AO treated SAMP8 mice

Figure 4.8 demonstrates an increased trend in the level of Pin 1 in SAMP8 \( g \)AO compared to \( r \)AO SAMP8 mice, although insignificant. Homogenized (30 \( \mu \)g) sample proteins were resolved on polyacrylamide gel via SDS-PAGE and through the Western blot technique transferred to a nitrocellulose membrane.

![Bar graph showing Pin 1/β-Actin levels](image)

**Figure 4.8:** Pin 1 level in \( g \)AO compared to \( r \)AO SAMP8 mice. The level of Pin 1 increased in the homogenized samples of SAMP8 mice treated with AO directed at GSK-3β \( (gAO) \) compared to that of SAMP8 mice treated with random AO \( (rAO) \). Data are represented as % control, and shown as mean ± SEM with *\( P<0.05 \). Total number of animals used in each group are: \( gAO=5 \) and \( rAO=5 \).
4.8 Discussion

There are 5.4 million Americans today living with AD. The direct cost of caring for these individuals is estimated to be $200 billion in 2012, which, according to the Alzheimer’s association website (Alz.org), could rise to $1.1 trillion by 2050 if something is not done to slow the increasing number of individuals affected by AD. With the cause of the disease yet to be determined, the search for a strong therapeutic treatment for AD has proven difficult, despite intense research efforts. AD phenotypically begins with declines in memory, which later progresses to dementia and extreme compromised quality of life. On a biochemical level, patients with AD exhibit increased levels of NFTs and Aβ plaques, the two principle pathological hallmarks of the disease, as well as a heightened oxidative environment in brain and consequential neurodegeneration. Reducing the oxidative stress associated with AD, through either increasing the antioxidant defense system or decreasing oxidant production, is likely a promising therapeutic strategy.

Nrf2, a master regulator of redox homeostasis, plays a key role in the protection of neurons against oxidative insults through the controlled expression of over 100 genes of the antioxidant response system (Phiel, Wilson et al. 2003; Rojo, Rada et al. 2008; Espada, Ortega et al. 2010; Zhang, An et al. 2012). Nrf2 has a low basal activity maintained through phosphorylation of key sites on the transcription factor. This post translational modification of Nrf2 promotes nuclear exclusion and ubiquitin-proteasome dependent degradation. Under normal conditions, oxidative insults stimulate the de-phosphorylation and translocation of Nrf2 to the nucleus where it binds to ARE sites and induces the transcription of many antioxidant and detoxification genes. Many studies have demonstrated the neuroprotective effects of Nrf2, including its protection against Aβ pathology found in AD and increased oxidative stress measured in hippocampal slices of Nrf2 knockout mice (Lee, Shih et al. 2003; Kanninen, Malm et al. 2008; Rojo, Rada et al. 2008; Zhang, An et al. 2012). In AD brain, suppressed levels of Nrf2 were reported, despite the high oxidative environment characteristic of the disease, suggesting a down-regulation of the Nrf2-ARE pathway (Ramsey, Glass et al. 2007).
Protein kinase GSK-3β is a negative regulator of Nrf2-ARE activity and is found at reported high levels in AD brain (Pei, Tanaka et al. 1997; Leroy, Yilmaz et al. 2007). In this study, brain samples of aged SAMP8 mice, a model of AD, were treated with either AO targeted at GSK-3β (GAO) or random AO (RAO), and decreased expression of GSK-3β was confirmed in GAO compared to RAO SAMP8 mice. We found brain levels of carbonyl and HNE, parameters of oxidative damage, significantly decrease in GAO compared to RAO SAMP8 mice. Suggestive that the characteristically high oxidative environment of AD brain may be due impart to the activity of GSK-3β. Supporting this idea are several studies linking GSK-3β to oxidative stress and neurodegeneration, as well as the two pathological hallmarks of AD (Lucas, Hernandez et al. 2001; Phiel, Wilson et al. 2003; Schafer, Goodenough et al. 2004). Over expression of GSK-3β has resulted in reported memory deficits similarly found in AD (Hernandez, Borrell et al. 2002).

One aim of this thesis study was to examine the involvement of GSK-3β and its negative regulation of the antioxidant transcription factor Nrf2 in AD. To determine if the decreased oxidative stress observed in GAO compared to RAO SAMP8 mice was associated with increased Nrf2 activity, cytoplasmic and nuclear levels of the transcription factor were measured. Nrf2 levels decreased in the cytoplasm and increased in the nucleus of GAO compared to RAO SAMP8 mice, suggesting higher antioxidant transcriptional activity. Further supporting this notion is the heightened levels of GST, an antioxidant transcribed by Nrf2, measured in GAO compared to RAO SAMP8 mice. GST helps maintain low amounts of HNE in the brain by catalyzing GSH-HNE conjugation for MRP-1 transporter-dependent export form the brain. The data presented in the thesis study, demonstrating increased nuclear localization of Nrf2 and levels of GST in SAMP8 GAO mice compared to RAO SAMP8 mice, implicates the deregulation and subsequent increased inhibitory activity of GSK-3β over the Nrf2-ARE pathway in AD.
One regulator of GSK-3β activity is the PI3K/Akt pathway, which inhibits its activity via phosphorylation of the constitutively active kinase at residue Ser9 of its pseudosubstrate domain (Cross, Alessi et al. 1995; Woodgett 2005). Under normal conditions, oxidative insults up-regulate the PI3K/Akt pathway resulting in the inhibition of GSK-3β, thus, allowing Nrf2/ARE activity to work and maintain cellular homeostasis (Shaw, Cohen et al. 1998; Martin, Salinas et al. 2002). Consistent with the findings in one study in which neuroblastoma cells were subjected to short term hydrogen peroxide exposure, up-regulation of the PI3K/Akt pathway, suppression of GSK-3β, and increased Nrf2 nuclear localization resulted. However, in the same study, after long term hydrogen peroxide exposure, the PI3K/Akt pathway was down-regulated, with increased GSK-3β activity and decreased Nrf2 nuclear localization (Rojo, Sagarra et al. 2008). This is particularly important in AD, a chronic neurodegenerative disorder with prolonged oxidative stress. It is possible that a similar suppression of the PI3K/Akt pathway, as reported in neuroblastoma cells, is taking place in aged SAMP8 mice as a result of increased oxidative stress. The data presented in this thesis study support the proposed suppression of the PI3K/Akt pathway in SAMP8 mice, which would result in elevated GSK-3β activity and subsequent suppression of Nrf2-ARE pathway shown in Figure 4.9; this taken together further supports a possible mechanism involved in AD explaining the loss of tolerance to an oxidative environment. Further, these considerations strengthen the notion that inhibiting GSK-3β may be a viable therapeutic approach to treat this disorder.
Another aim of this study was to examine the effect GSK-3β has over the level of Pin 1, a member of the PPIase family. Through catalyzing the cis-trans isomerization of pThr-Pro or pSer-Pro residues of target proteins, Pin 1 regulates the activity of these proteins, including tau and APP. Here, although not significant, we observed a trend of increased levels of Pin 1 from cAO compared to rAO SAMP8 mice, which is in agreement with reports of down-regulation and decreased activity of Pin 1 in AD and MCI brain (Butterfield, Poon et al. 2006; Sultana, Boyd-Kimball et al. 2006). This observed trend of
up-regulation of Pin 1 may be a result of the decreased oxidative status measured in this same study, possibly implicating Gsk-3β-induced oxidative stress in the down-regulation of Pin 1. Further supporting this idea is the increased level of oxidized Pin 1 measured in MCI and AD hippocampal tissue and subsequent decline in activity of the regulatory protein (Boyd-Kimball, Poon et al. 2006; Sultana, Boyd-Kimball et al. 2006). Under normal conditions, Pin 1 regulates the de-phosphorylation of tau, preventing hyper-phosphorylation of the structural protein found in NFT, and the amyloidogenic cleavage of APP (Lee, Kao et al. 2003; Liou, Sun et al. 2003; Pastorino, Sun et al. 2006). Overexpression of Pin 1 reportedly suppresses tauopathy and Aβ secretion (Hsiao, Chapman et al. 1996; Lim, Balastik et al. 2008). This, taken together, suggests that the oxidative inactivation of Pin 1 could subsequently increase the two primary pathological hallmarks of AD: NFT and Aβ plaques.

In closing, this thesis research is supportive of the notion that increased activity of GSK-3β contributes to the increased oxidative stress and the accumulation of NFT and Aβ plaques observed in AD brain. We postulate that the characteristic prolonged oxidative stress of AD may result in the down-regulation of the PI3K/Akt regulatory pathway of GSK-3β, causing the loss of regulation over the constitutively active kinase and its subsequent inhibition of the antioxidant transcription factor Nrf2. This suppression over Nrf2-ARE activity, supported by this study, may lead to the depletion of the vital antioxidant system, essential to maintaining low oxidant levels, and contribute to the loss of tolerance to an oxidative environment in brain observed in AD. Further, this study supports an inverse relationship between levels of oxidants and Pin1. The increased oxidative stress observed in AD to which we posit the over-activity of GSK-3β contributes, decreases the activity of Pin 1. This suppression of Pin 1 level and activity can lead to a potential increase in NFT and Aβ plaques, implicating GSK-3β in the generation of the two primary pathological hallmarks of AD. Together, the data presented in this thesis research support GSK-3β inhibitors as a possible therapeutic treatment of AD, which may serve to increase the cellular antioxidant defense system.
and suppress the accumulation of NFT and Aβ plaques associated with this devastating neurodegenerative disease.
Appendix I: The Effects of Antioxidant Diets Over Protein Oxidation Levels in Diabetic Mice

A.1 Overview

In 2010 a reported 25.8 million American citizens were affected by diabetes, according to the U.S. Centers for Disease Control and Prevention. Diabetes is a chronic disease characterized by the body’s inability to maintain homeostasis between glucose and insulin, resulting in hyperglycemia. Prolonged diabetes may lead to complications including retinopathy, neuropathy, and nephropathy. Hyperglycemia has been linked to free radical production and subsequent oxidative stress, both contributing factors to the progression of diabetes and its associated complications (Giacco and Brownlee 2010; Pitocco, Zaccardi et al. 2010; Rains and Jain 2011). Diabetic subjects have been reported to have comparatively higher oxidative environments, including increased oxidative stress markers (Jain 1989; Jain, Levine et al. 1990; Bloch-Damti and Bashan 2005). Because oxidative stress plays such strong role in diabetes, antioxidants are a main therapeutic candidate researched in clinical studies today as possible treatments. The aim of this study was to examine the oxidative effects of various antioxidant diets in the brains of diabetic mice to determine any possible benefits the dietary supplements may have in treating the disease.

A.2 Background

Oxidants can be beneficial or harmful to a cell depending on their levels. Low levels of oxidants can be used to initiate signaling cascades and maintain cellular homeostasis, serving as a pre-conditioning phase to up-regulate anti-oxidant systems. For example, low levels of oxidants can up-regulate transcription factor NF-E2-related factor 2 (Nrf2), as opposed to prolong and/or high levels which can inhibit its transcriptional role (Katsuoka, Motohashi et al. 2005; Rojo, Sagarra et al. 2008). Nrf2 plays a vital role in the maintenance of cellular homeostasis through the transcription of a large variety of antioxidants. Alternatively, high oxidant levels can be detrimental for
a cell, inducing cellular oxidative stress and subsequent apoptosis. In order for a cell to maintain a more reducing environment, antioxidant systems work to keep oxidant levels low, thereby maintaining a cell’s vital redox balance. A decrease in antioxidant systems or increase in oxidant production are the two ways to trigger cellular oxidative stress.

Reactive oxygen species (ROS) are an example of oxidants and include the superoxide anion (\(O_2^-\)) and the extremely reactive hydroxyl radical (\(HO^\cdot\)). The mitochondrion is the primary source of the endogenous ROS superoxide, and this notion is rationalized by its high consumption of molecular oxygen, high electron flow, and the reactive intermediate semiquinone radical (\(^\cdot QH\)) generated during the two electron transfer from ubiquinol (QH\(_2\)) to cytochrome c (Boveris 1977; Turrens 1997). In the presence of antioxidants Cu, Zn-superoxide (SOD) and glutathione peroxidase (Gpx), superoxide can be converted first to the less reactive hydrogen peroxide (H\(_2\)O\(_2\)) and then to water respectively (reactions 1-2).

\[
2O_2^- + 2H^+ \xrightarrow{\text{Cu,Zn-SOD}} 2H_2O_2 + O_2 \quad \text{(reaction 1)}
\]

\[
2H_2O_2 \xrightarrow{\text{Gpx}} 2H_2O + O_2 \quad \text{(reaction 2)}
\]

However, as hydrogen peroxide accumulates and/or Gpx activity diminishes, the increased generation of the more reactive hydroxyl radical can occur via the Haber-Weiss reaction (reactions 3).

\[
2H_2O_2 + 2O_2^- \rightarrow OH^- + O_2 + ^\cdot O \quad \text{(reaction 3)}
\]

The hydroxyl radical is among the most toxic ROS leading to indirect and direct oxidative-carbonylation of key cellular proteins. The highly reactive hydroxyl radicals can trigger the initiation and subsequent propagation of lipid peroxidation, leading to the generation of reactive \(\alpha,\beta\)-unsaturated aldehydes such as 4-hydroxy-2-trans-nonenal (HNE). Because HNE is much more stable than its free radical substrates, it can diffuse to distant parts of a cell before forming adducts with the protein moieties cysteine, histidine and lysine (Uchida and Stadtman 1992; Neely, Sidell et al. 1999; Uchida 2003). The hydroxyl radical can directly oxidize threonine, arginine, proline and
lysine side chains of closely located proteins (Nadkarni and Sayre 1995). These oxidative-additions of polar carbonyl groups can lead to a change in a protein’s confirmation and subsequent functionality.

ROS play a key role in the origin and/or progression of a large variety of diseases and disorders, leaving antioxidants as popular therapeutic candidates. However, not all antioxidants behave the same and should be heavily researched before therapeutically used (Valdecantos, Perez-Matute et al. 2010). High doses of antioxidants do not always lead to more benefits and can become toxic. Lipoic acid (LA) is a common antioxidant found in supplements marketed to improve weight loss or provide energy, and clinically studied to treat a variety of diseases and conditions including chronic liver disease, hepatic coma and diabetes. LA is an essential cofactor of two mitochondrial enzyme complexes and not only has an antioxidant role, but also functions as a pro-oxidant as well (Moini, Packer et al. 2002; Smith, Shenvi et al. 2004).

Depending on conditions, including dose amount, incubation time, physiological circumstances and type of oxidant stress, LA can behave as an antioxidant or pro-oxidant (Cakatay 2006; Coleman, Williams et al. 2006). Multiple studies have shown LA treatments to decrease levels of ROS, protein oxidation, and lipid peroxidation (Humphries and Szweda 1998; Poon, Farr et al. 2005). In one study, cortical neuronal cells were treated with LA and showed a decrease in HNE induced neurotoxicity and oxidative stress (Abdul and Butterfield 2007). LA-treated SAMP8 mice also exhibited a decrease in HNE levels (Farr, Poon et al. 2003). The pro-oxidant role of LA has been tied to glucose transport via stimulation of the insulin-signaling pathway (Estrada, Ewart et al. 1996; Packer, Kraemer et al. 2001; Moini, Packer et al. 2002; Cho, Moini et al. 2003). In one study, 3T3-L1 adipocytes were pre-treated with LA, and levels of oxidants, antioxidant glutathione (GSH) and glucose uptake were monitored. LA-Pre-treated adipocytes exhibited a maximal increase of intercellular oxidant levels at six hours and a decrease in the oxidant levels and rate of glucose uptake, while increasing GST levels after twelve hours (Moini, Packer et al. 2002). LA is hypothesized to initially shift the
intercellular redox status towards a more oxidizing environment to stimulate the insulin-signaling pathway, then, at a later point in time, work to re-establish more reducing conditions. The pro-oxidant role of LA is not well characterized and serves as an example of why caution should be used before using high doses of antioxidants to treat various diseases and disorders.

A.3 Materials and Methods

A.3.1 Chemicals and Materials

Left brain regions collected from 70 mice provided by Dr. Susan A. Farr, St. Louis University. Brain regions were briefly homogenized in ice-cold Media 1 lysis buffer (pH 7.4; 320 mM sucrose, 1% mM Tris-HCl (pH 8.8), 0.098 mM MgCl₂, 0.076 mM EDTA, proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 mg/ml) and PMSF (40 μg/ml), and phosphatase inhibitor cocktail (Sigma-Aldrich)) using a Wheaton tissue homogenizer. The homogenized samples were then diluted 2X with Media 1. All chemicals used were of the highest purity and obtain mostly from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. The Rb x DNP polyclonal primary and anti-rabbit IgG antibodies were purchased from Chemicon (Temecula, CA). Anti-HNE antibody obtained from Alpha Diagnostic (San Antonio, TX).

A.3.2 Protein Estimation by Bicinchoninic Acid (BCA)

Protein concentrations were determined through the use of bicinchoninic acid (BCA) assay involving the protein-mediated reduction of cupric ion (Cu²⁺) to the cuprous ion (Cu¹⁺) under basic conditions. Protein peptide bonds are responsible for the reduction of cupric ions, and the sum of Cu¹⁺ is proportional to the total protein concentration of a sample. The BCA assay is a mixture of two reagents, reagent A and B, which are combined immediately prior to being added to protein samples. BCA reagent A is an alkaline solution made up of BCA, sodium carbonate, and sodium tartrate in 0.1
M sodium hydroxide. Reagent B is a 4% solution of cupric sulfate pentahydrate. BCA consists of two carboxylated quinolone rings that can chelate with Cu\(^{+1}\), forming a purple water soluble complex. This BCA-Cu\(^{+1}\) complex has a strong absorbance at 562nm wavelength and its intensity in color correlates with the relative amounts of certain amino acids in each protein sample, incubation time, and temperature (Olson and Markwell 2007).

Known concentrations of bovine serum albumin (BSA) were used to generate a standard curve (Figure 1a). In a 96-well microplate, BSA is pipetted in duplicate amounts of 0, 5, 10, 15, 20, 25, and 30 µg and diluted with BCA reagent mixture to a total volume of 100 µl. Protein samples were pipetted in duplicates of 5 µl and diluted with BCA reagent mixture to a total volume of 100 µl each. Once the BCA mixture is added to the samples, the proteins reduce cupric ions of reagent B to cuprous ions during the 15 minute incubation at 37°C. Two BCA molecules of reagent A then chelate with one cuprous ion generated from the protein-mediated reduction, producing a purple color. After incubation, the color intensity was measured through the use of a UV-Vis instrument (uQuant plate reader by BIO-TEK Instruments, Inc.) at 562nm and the Lambert-Beer law was applied using the BSA standard to determine the sample protein concentration (Figure 1b).
Figure 1. a.) BCA standard curve. Blank562 vs. protein concentration graph. b.) BCA standard curve fitting results.
A.3.3 Protein Carbonyls

5 μl of 12% sodium dodecyl sulfate (SDS) and 10 μl of 2,4-dinitrophenylhydrazine (DNPH) were added to 5 μl of each sample and incubated at room temperature for 20 minutes. After incubation, samples were neutralized with 7.5 μl of neutralization solution (2 M Tris in 30% glycerol) and diluted with 1xPBS (phosphate buffer solution containing sodium chloride, mono, and dibasic sodium phosphate) to 100 μl. Following derivatization samples were diluted to get a protein concentration of 1 μg/1 ml. 250 μg of the resulting sample solution was loaded per well into a slot blot apparatus and onto a nitrocellulose membrane under water vacuum pressure. The resulting membrane was then blocked with 750 mg BSA in 25 ml of wash blot (35.2 g sodium chloride, 1.77 g monobasic sodium phosphate, 9.61 g dibasic sodium phosphate, 1.6 mL TWEEN, diluted to 4 L with deionized water) for 90 minutes. The blocking solution was then reduced to 20 ml and 100 μl of Rb x DNP polyclonal primary antibody was added and incubated for 2 hours. The blots were then washed three times with fresh wash blot for 5 minutes each. After washing, 2.5 μl of anti-rabbit IgG alkaline phosphatase polyclonal secondary antibody was added to 20 ml of fresh wash blot and incubated for 1 hour. The membrane was then washed with fresh wash blot in three increments of 5, 10 and 10 minutes. After washing, 20 ml of developing solution (66 μl BCIP and 133 μl NBT diluted with DI water to 20 mL) was added to each blot. After development, bolts were dried and scanned (CanoScan8800F scanner) using Adobe Photoshop and analyzed using Scion Image software.

A.3.4 Protein-Bound 4-Hydroxynonenal (HNE)

5 μl of protein, 5 μl 12% sodium dodecyl sulfate (SDS) and 10 μl of a Laemmli buffer (containing 0.125 M tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) glycerol) were combined and incubated at room temperature for twenty minutes. The resulting sample solution was then diluted and loaded on a nitrocellulose membrane as described earlier for protein carbonyl. The resulting protein-bound membrane was blocked with
750 mg BSA in 25 ml of wash blot for 90 minutes. The blocking solution was the reduced to 20 ml with 4 μl of anti-HNE polyclonal primary antibody added and incubated for 1 hour. After incubation, the membrane was washed three times with fresh wash blot for 5 minutes each, then incubated in 20 ml of wash blot containing 2.5 μl of anti-rabbit IgG alkaline phosphatase polyclonal secondary antibody for one hour. After three intervals of 5, 10 and 10 minutes washes with wash blot, the blot was then dried, developed, and analyzed the same as carbonyl above.

Figure 2. Developed sample slot blot membrane.
A.4 Results

A Student’s t-test was employed to assess statistical significance in comparing protein carbonyl and protein-bound HNE levels in protein samples between control and experimental data sets. Significant differences were set at P<0.05.

Protein carbonyl and HNE levels were determined via slot blot analysis in diabetic and non-diabetic mice that were fed a normal or antioxidant diet (Figure 3). When comparing diabetic (P and E) to non-diabetic (C) mice, all on normal diets, an insignificant 14% and significant 19% increase were observed in protein carbonyl and HNE levels respectively; agreeing with reports of heightened oxidative environments found in diabetic subjects. Diabetic mice sets treated with varying antioxidant diets (L, M, N and O) exhibited a significant increase of protein carbonyl levels compared to non-diabetic mice under a normal diet (Figure 3a). HNE levels of these diabetic mice under antioxidant diets were also increased, although insignificantly (Figure 3b).
Figure 3. Protein oxidation determined by elevated levels of: a.) Protein Carbonyl  b.) Protein-bound HNE. *P<0.05. Data is represented as % control, and shown as mean ± SEM. Total number of animals used in this study for each group are: L=9, M=9, N=12, O=13, P=7, E=10, and C=10.
A.5 Discussion

Diabetes mellitus is a chronic disease characterized by elevated blood sugar levels, known as hyperglycemia, resulting from either lack of insulin production or insulin resistance. Hyperglycemia is a major contributor to the generation of ROS, which aids in the progression of diabetes and its associated complications including neuropathy and retinopathy (Giacco and Brownlee 2010; Pitocco, Zaccardi et al. 2010; Rains and Jain 2011). Hyperglycemic-induced ROS generation leads to cellular oxidative stress including protein oxidation and lipid peroxidation (Jain, Levine et al. 1990; Pennathur, Wagner et al. 2001; Pennathur, Ido et al. 2005; Susztak, Raff et al. 2006). There have been many reports of diabetic patients having internal environments under more oxidized conditions compared to that of non-diabetic subjects (Jain 1989; Gokulakrishnan, Mohanavalli et al. 2009; Piwowar, Knapik-Kordecka et al. 2009). In this study, protein carbonyl and HNE levels, both indices of oxidative stress, were elevated in diabetic mice compared to the non-diabetic control (Figure 3), which supports findings of higher oxidative internal environments of diabetic subjects. Hyperglycemic-induced ROS generation and its effects on the progression of diabetes and its associated complications has made antioxidants, such as lipoic acid, a popular candidate for therapeutic treatment of the chronic disease. In this study, the oxidative effects of varying antioxidant diets fed to diabetic mice were examined and shown to surprisingly increase protein oxidation (Figure 3).

The increase of protein carbonyl and protein-bound HNE levels observed in diabetic mice fed antioxidant diets could be the result of a protective pre-conditioning phase or other dual pro-oxidant roles of the antioxidants. It is possible that the increased protein carbonyl and protein-bound HNE levels exhibited could be the result of a pre-conditioning phase to up-regulate homeostatic pathways to protect against cellular oxidative stress. Oxidant production at low levels is known to up regulate protective cellular pathways, for example leading to the increased activity of the antioxidant transcription factor Nrf2, thus up regulating its associated antioxidants (Katsuoka, Motohashi et al. 2005; Olson and Markwell 2007). Additional pro-oxidant
roles of the antioxidants could also be the cause of the increased protein carbonyl and protein-bound HNE levels. As mentioned earlier, lipoic acid is known to have both pro-oxidant and antioxidant roles which vary depending on intracellular environmental conditions. Pro-oxidant roles of lipoic acid have been linked to the up-regulation of insulin signaling, although currently not well understood (Estrada, Ewart et al. 1996; Moini, Packer et al. 2002). This study demonstrates the importance of fully understanding the roles of antioxidants before using them as supplements to treat various diseases.
Appendix II: Data to Supplement Figures

**Figure 4.1 Protein Carbonyl Levels**

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**Figure 4.2 Protein-Bound HNE Levels**

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Figure 4.3 Cytosolic GSK/Actin Levels

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### Figure 4.4 Nuclear GSK/Histone 2B Levels

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### Figure 4.5 Cytosolic Nrf2 /Actin Levels

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### Figure 4.6 Nuclear Nrf2/Histone-2B Levels

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SEM: 15.21, 18.67
**Figure 4.7 GST/Actin Levels**

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References


Chowdhry, S., Y. Zhang, et al. (2012). "Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity." Oncogene.


Vita

Jessica Lynn Harris was born on November 18th, 1986 in Bloomington, IN. She obtained her bachelor’s degree in chemistry with a minor in business from Indiana University in May 2010. She came to the University of Kentucky and enrolled for her graduate studies in August 2010. During her time at the University of Kentucky she was honored with the Charles H. H. Griffith outstanding general chemistry teaching assistant award. This thesis work was completed under the guidance of Dr. D. Allan Butterfield.

Publications:
