NEUROPROTECTIVE EFFECTS OF CINNAMON (*CINNAMOMUM VERUM*) VIA MODULATION OF STRESS RESPONSE SIGNALING

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Yuki Gomada B.S.

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Committee Members Approved:

_____

Dhiraj A. Vattem, Chair

_____

C. Reed Richardson

_____

Emmanouil Apostolidis

Approved:

_____

J. Michael Willoughby
Dean of the Graduate College

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LIST OF ABBRIBIATION

INS/ILS= Insulin and insulin like signaling pathway, **IGFR**=Insulin growth factor receptors, **IGF**=Insulin like growth factors, **IRS-1**= Insulin receptor substrate 1, **PI_3K**=Phosphoinositide 3-kinase, **PIP_2**=Phosphatidylinositol 4,5-bisphosphate, **PIP_3**=Phophatidylinositol-3, 4, 5-trisphosphate , **PDK-1**=3'-phosphoinositide dependent kinase-1, **AKT**= v-Akt Murine Thymoma Viral Oncogene, **PKB**= Protein Kinase-B, **FOXO**=forkhead box, class O, **CAT**= Catalase, **SOD**= superoxide dismutase, **ROS**= reactive oxygen species, **GLUT**= Glucose transporter, **mTOR**= Mammalian target of rampamycin, **4E-BP**=4E binding protein, **SGK**=Serum glucocorticoid-responsive kinase, **PTEN**= Phosphatase and Tensin Homolog Deleted on Chromosome 10, **PP2A**= Protein phosphatase 2A, **TGF**=Transforming growth factor, **NF-κB**=Nuclear factor-kappa B, **AD**=Alzheimer's disease, **PD**=Parkinson's disease, **ROCK-1**=Rho-activated protein kinase-1, **HIF**=Hypoxia-inducible factors, **TLR**=Toll-like receptors, **IRF-3**=Interferon regulatory factor-3, **COX**=Cyclooxygenase, **NOS**= Nitric oxide synthase, **ACE**=Aqueous cinnamon extract, **AGE**=Advanced glycation end product, **CE**= cinnamon extract, **VEGF**= Vascular endothelial growth factor, **VEGFR**= Vascular endothelial growth factor receptor, **EGF**= Epidermal growth factor, **AP-1**= Activator protein-1, **5XFAD**=five FAD mutations, **Aβ**=Amyloid beta, **GFP**=Green fluorescent protein, **NGM**=Nematode growth media, **RF**=Relative fluorescence, **SE**=Standard error, **ER**= Endoplasmic reticulum, **Nrf-2**= Nuclear respiratory factor-2, **HSF**=heat shock factor, **UPR**=unfolded protein response, **MAPK**= Mitogen-activated protein kinase, **Keap-1**= Kelch-like ECH-associated protein-1, **GSK-3**= Glycogen synthase kinase, **ARE**= antioxidant response elements, **GSH**=Glutathione, **GST**= Glutathione S-transferase, **GSS**= glutathione synthetase, **GSR**= Gutathione disulfide reductase, **GSSG**=Oxidized glutathione, **DATS**=Diallyl trisulfide, **TRX**=Thioredoxin, **TRXR**=Thioredoxin reductase, **HRE**= Hypoxia responsive element, **PHD**= prolyl hydroxylases, **FIH**= Factor inhibiting HIF, **VHL**= von Hippel Lindau E3 ubiquitin ligase, **ADM**=adrenomedullin, **RhoG**= Ras homology Growth-related, **AhR**=aryl hydrocarbon receptor, **AHRE**= Aryl hydrocarbon response element, **CAD**=C-terminal activation domain, **HSP**=Heat shock protein, **PERK**= PKR-related ER kinase, **eIF2**=eukaryotic initiation factor 2 initiation factor 2α, ATF-6= Activating transcription factor-6, **BIP**=Immunoglobulin binding, **TIR**= Toll/interleukin-1 receptor, **JNK**= c-Jun N-terminal kinases, **APP**= Amyloid precursor protein, **AE**=aqueous extract, **PA**=polar acidic, **PB**=polar basic, **PN**=polar neutral, **LG**=L-glutamate, **NDMA**= N-Nitrosodimethylamine, **NDMAR**=NDMA receptor, **AMPA**= α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, **AMPAR**=AMPA receptor, **L-DOPA**= L-3,4-dihydroxyphenylalanine, **SNpc**= substantia nigra pars compacta, **TH**= tyrosine hydroxylase, **HO-1**= Heme oxygenase-1, **LPS**=Lipopoly-saccharide, **MPTP**=1-methyl-4-phenyl-1 ,2,3,6-tetrahydropyridine, **MPP^+**= 1-methyl-4-phenylpyridinium, **NaB**=Sodium benzoate, **PPAR**=Peroxisome proliferator-activated receptor, **6-OHDA**=6-hydroxydopamine.

CHAPTER I

DIETARY SPICE CINNAMON AS *IN VIVO* MODULATORS OF INSULIN SIGNALING AND IMPACT ON DISEASE

Introduction

Insulin and Insulin like signaling pathway (INS/ILS)

The insulin/insulin like growth factors signaling (INS/ILS) pathway modulates many cellular functions, such as cell growth, metabolism, stress responses and apoptosis (Baur & Sinclair, 2006; Lant & Storey, 2010; Prahlad & Morimoto, 2009). The flux through the ILS is regulated by metabolic (glucose, energy) and mitogenic (growth factors) signals, and thus is a primary cellular nutrient and growth sensing pathway (Cersosimo et al., 2012; Towler & Hardie, 2007). Abundance of nutrients, such as elevation in post-prandial glucose levels are sensed via the synthesis and secretion of insulin that serves as activating ligand to the insulin growth factor receptors (IGFR) on cell membranes, resulting in many metabolic effects including, activation of lipid biosynthesis, protein biosynthesis, glycogenesis, and inhibition of glycolysis, gluconeogenesis, and lipid oxidation (Bibollet-Bahena & Almazan, 2009; Buzzai et al., 2005; Dubois et al., 2009; Z. Liu et al., 2009; McManus et al., 2005; Sekine et al., 2007). Similarly, activation of ILS by Insulin like growth factors (IGF's) results in activation of cell replication, differentiation survival, and inhibition of cell death (apoptosis) (Amcheslavsky et al., 2009; Rattan et al., 2005; Wilsbacher et al., 2008).

Decreased insulin signaling, by caloric restriction or biochemical agents activates ergogenic metabolic pathways including glycolysis and lipid oxidation (Bruss et al., 2010; Hipkiss, 2006). Low flux through the ILS also decreases DNA replication, macromolecular biosynthesis, cell growth and replication (Bocchetta et al., 2008; Burhans & Weinberger, 2007). Additionally, increased oxidative stress response, extension of cellular and organismal lifespan have also been linked to downregulation of insulin signaling (Barbieri et al., 2003; Baur & Sinclair, 2006; Richardson et al., 2004).

The insulin signaling pathway is highly conserved in eukaryote (Barbieri et al., 2003). In animals, binding of insulin/IGF-1 to transmembrane insulin like growth factor-1 receptor (IGF-1R) results in conformation change induced activation of an intrinsic tyrosine kinase (Kato et al., 1993). Phosphorylation of IRS-1 by the tyrosine kinase results facilitates the recruitment and phosphorylation and of PI_3K that catalyzes the conversion of PIP_2 to phophatidylinositol-3, 4, 5-trisphosphate (PIP_3) on the plasma membrane (Niswender et al., 2003). PIP_3 is a secondary messenger that binds to the 3'-phosphoinositide dependent kinase-1 (PDK1) at the PH domain resulting in its activation (Alessi & Downes, 1998). Active PI_3K and PDK-1 can independently phosphorylate and activate v-Akt Murine Thymoma Viral Oncogene (AKT), which is also called Protein Kinase-B (PKB) (Vanhaesebroeck & Alessi, 2000). In the cytoplasm AKT phosphorylates several proteins resulting in the metabolic and mitogenic effects of insulin. Metabolic effects of ILS are mediated by AKT via the phosphorylation of an important FOXO (forkhead box, class O) (Stitt et al., 2004). Phosphorylation of FOXO prevents nuclear translocation and transcriptional activation of genes regulating glycolysis and lipid oxidation. FOXO also regulates transcription of several antioxidant

enzymes like catalase (CAT) and *sod-2* (iron/manganese superoxide dismutase), DNA repair enzymes and proteins that activate apoptosis and attenuate cell cycle (Nakae et al., 2001; Rathbone et al., 2008; Sim & Denlinger, 2011; Stahl et al., 2002). Phosphorylation of AKT also increases glucose uptake by upregulating translocation of glucose transporter 4 (GLUT-4) to the cell membrane (Russell et al., 1999). Phosphorylation of AKT also regulates gluconeogenesis by repressing transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6pase) (J. Liao et al., 1998).

AKT also regulates several mitogenic functions, such as cell growth, cell division, cell proliferation and protein synthesis. Phosphorylation of the mammalian target of rampamycin (mTOR) by AKT results in inactivation of eukaryotic translation initiation factor 4E binding proteins (4E-BPs) to promotes mRNA translation on ribosome (Dowling et al., 2010). In *Drosophila* inhibition of 4E-BPs upregualtes both cell growth and proliferation (Lachance et al., 2002; Montagne et al., 1999), whereas in mammalian cells inhibition of 4E-BPs upregualte only cell proliferation (Dowling et al., 2010). Both mTOR and PDK-1 can independently phosphorylate Serum glucocorticoid-responsive kinase (SGK) (Biondi et al., 2001; Hong et al., 2008). SGK is known to response hyperosmotic stress (Bell et al., 2000) and also regulates proliferation and cell-cycle progression through phosphorylation of cell-cycle inhibitor p27(kip1) (Toker, 2008). Moreover like AKT, SGK can also phosphorylate FOXO and inhibit FOXO dependent gene expression (Brunet et al., 2001).

Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) can dephosphorylate PI_3K and inhibit further progression of ILS (Maehama & Dixon, 1998). PP2A and its regulatory subunit B56β (PPTR-1 in *C. elegans*) dephosphorylate AKT and

inhibit ILS (Padmanabhan et al., 2009; Yan et al., 2008). Conversely heat shock protein 90 (HSP90) binds to AKT and can prevent inactivation of AKT by PP2A-mediated dephosphorylation (S. Sato et al., 2000). Sirtuin (Sir2 family histone deacetylase) can deacetylate FOXO in the cytoplasm to facilitate nuclear translocation (Daitoku et al., 2011). Deacetylation of FOXO in nucleus and mitochondria initiates binding affinity to the promoter region of several genes.

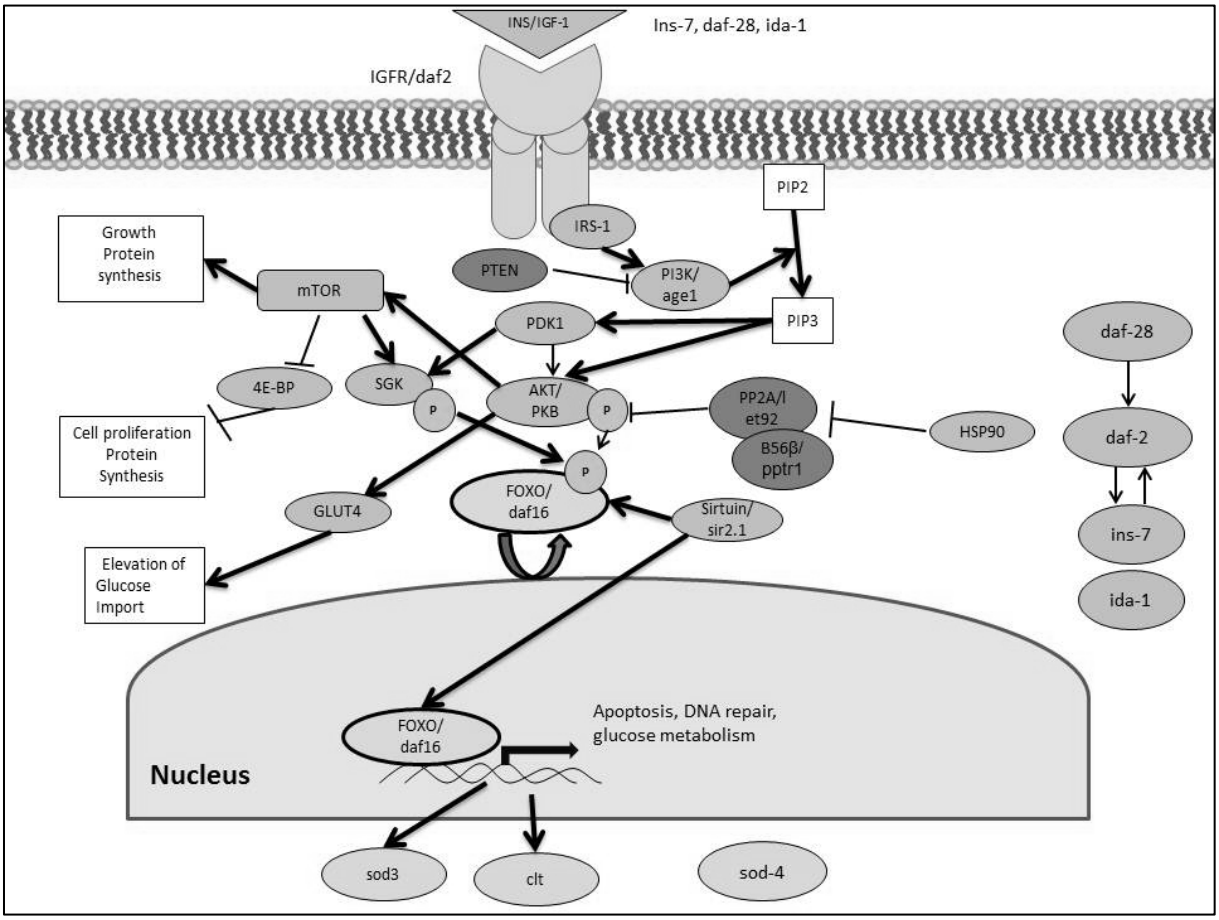

Figure 1. Model of insulin/insulin like growth factor-1 (INS/IGF-1) signaling pathway. INS /IGF-1 signaling modulates cell growth, metabolism, stress responses and apoptosis.

Dysregulation of INS/ILS and Diseases

ILS is sensitive to nutrient and metabolism changes and it is reported that type 2 diabetes is potentially caused due to dysregulation of ILS (X. Lin et al., 2004; Patil et al., 2011). Reduced IRS-1 and/or AKT expression, which is identified as insulin resistance, results in decreasing of glucose uptake due to the downregulation of GLUT4 (H. V. Lin et al., 2011). Also inhibition of PTEN is reported to decrease insulin resistance through upregulation of PI3K (Talbot et al., 2012). Dysregulation ILS can promote TGF-ß-mediated inflammation by insulin stimulated AKT phosphorylation (Remy et al., 2004). TGF-ß signaling is associated with growth inhibitor and apoptosis stimulator and ILS can regulate TGF-ß activity through phosphorylation of Smad3 by AKT, thus suppress inflammation (Remy et al., 2004). Also activation of ILS induces inflammation by phosphorylation of AKT its downstream target NF-κB (Michael & Delhase, 2000). ILS control neuronal oxidative stress through FOXO dependent gene expression and ILS potentially play key role in neurodegenerative disease, such as Huntington disease, Alzheimer disease (AD) and Parkinson's diseases (PD) (Craft & Watson, 2004). The mechanism of AD is not fully understood, but it is reported that downregulation of IRS-1 is occurred in AD patient brain (Talbot et al., 2012), and decreasing of Rho-activated protein kinase-1 (ROCK1) via inactivation of FOXO is shown to lower amyloid-β peptide, AD promoter (Qin et al., 2008). PD is described by massive loss of dopamine neurons in the substantia nigra (Damier, 1999) and it is reported that IGF-1 has protective effect against dopamine neurons by its survival and proliferation of neuronal cells (Ebert et al., 2008). Since ILS regulates apoptosis, cell growth, proliferation and cell cycle, the dysregulation of ILS can potentially be the causes of cancer development. It is known that one of causes of human breast cancer is over-expression of IGF-1 (Fukuda et al.,

2002). AKT activated mTOR leads to Hypoxia-inducible factors 1α (HIF-1α) translation and normally regulates oxygen homeostasis, but it is reported that over expression of HIF-1α causes tumor-associated angiogenesis and progress colon cancer (Fukuda et al., 2002). Also PTEN is known as human tumor suppressor by inhibition of PI_3K, and PTEN mutations are found in Brain, Breast, and Prostate Cancer (J. Li et al., 1997).

Cinnamon and INS/ILS

Cinnamon belongs to the family Lauraceae, and the genus *Cinnamomum* contains approximately 250 different species of which are widely distributed throughout many regions. Species of *Cinnamomum is* known to the traditional medicines of India and China include *C. zeylanicum* (Sri Lanka and India), *C. tamala* (south slopes of Himalayas), *C. burmannii* (West Sumatra), and *C. pauciflorum* (China, India, Assam, Khassia hills). The *Cinnamomum* species that commonly sold in the market as cinnamon today include *C. zeylanicum, C. loureirii, C. curmanni, and C. cassia* (Chinese Cinnamon) (Dugoua et al., 2007; R. Wang et al., 2009). Cinnamon has always been popular for its unique flavor components. More recently, research is advocating the use of cinnamon for various disease states that are prevalent in today's society. Cinnamon has been used since ancient age to treat or relieve symptoms of dyspepsia, gastritis, diabetes, poor blood circulation, and inflammatory diseases (R. Wang et al., 2009). Cinnamon is noted to be one of the earliest known spices used by humans, with Chinese texts dating back to 4,000 years ago. Cinnamon is mentioned in the Bible (Exodus and Proverbs), and there are reports of cinnamon importation from Egypt to China as early as 2000 BC (Dugoua et al., 2007). The part of the plant which is most commonly used for the production of cinnamon includes the thin inner bark of the evergreen tree. Cinnamon oil

and powder are commonly acquired from the dried bark of the plant (Kirkham et al., 2009; Peterson et al., 2009). Depending on the species of cinnamon, the bioactive compounds and concentrations at which they are found can vary. The preparation process of the cinnamon may also cause variations in the levels of bioactive compounds. Common and cassia cinnamon are known to contain volatile oils such as cinnamaldehyde (60-80%), eugenol (10%), and trans-cinnamic acid (5-10%). Phenolic compounds which are identified as approximately 4-10% of cinnamon by volume include condensed tannins, catechins, and proanthocyanidins. Mono and sesquiterpenes present include calcium-monoterpene oxalate, gum, mucilage, resin, starch, sugars, and traces of coumarin (Dugoua et al., 2007). In general, the three major compounds present in cinnamon include cinnamaldehyde, cinnamyl acetate, and cinnamyl alcohol, which become converted into cinnamic acid by oxidation and hydrolysis reactions after ingestion. Cinnamic acid is then converted to benzoate in the liver where it exists as a sodium salt in the body (Brahmachari et al., 2009). The analysis of cinnamon bark oil shows 13 active components, with the major compound present as cinnemaldehyde (97% by volume). Other compounds extracted from cinnamon bark include terpenes such as δ-cadinene (0.9%), α-copaene (4.6%), and α-amorphene (0.5%) (Gopu et al., 2008; Singh et al., 2007).

Overall, cinnamon can increase total serum antioxidant status, increase thiols, and decrease lipid. Cinnamaldehyde is an α,β-unsaturated carbonyl derivative which has been shown to exhibit antioxidant capacity through suppressing Toll-like receptors (TLR)- 4 activation (Youn et al., 2008). TLR4 is the known receptor for LPS, which upon engagement oligomerizes the receptor and induces the transcriptions factors NF-κB and

IRF3. These two transcription factors code for pro-inflammatory gene products such as cytokines, COX-2, and inducible NOS. Cinnamaldehyde is able to inhibit LPS-induced oligomerization of TLR4, thereby downregulating NF-κB, and the inflammation response genes towards foreign molecules (Youn et al., 2008). Cinnamon is known to contain the highest amount of procyanidins (A-type oligomeric form) with potent antioxidant activity, but the underlying mechanism of their action has not been fully determined (J. Lu et al., 2010). Advanced glycation end products, or AGEs, are known as the final products of reactions between sugars and amino groups (Peng et al., 2010). Upon accumulation in *vivo,* AGEs can be pro-inflammatory agents which have the ability to progress the pathogenic disease process, develop complications associated with disease, or facilitate the aging process. Aqueous cinnamon extract (ACE) is able to act as a natural antiglycation product through directly trapping reactive carbonyl species (Peng et al., 2010). The components found in ACE which inhibit the formation of AGEs include phenolics such as catechins, epicatechins, and procyanidin B2 (Peng et al., 2008a).

Cancer: *In vitro* as well as *in vivo* studies show great potential for aqueous cinnamon extract to induce cellular apoptosis, and inhibit tumor growth and progression. Cinnamaldehyde a component of cinnamon extract (CE) has been shown to have antitumor activity towards human solid tumor cells in vitro (Dugoua et al., 2007). This bioactive component is noted for its ability to block cellular proliferation at the M phase of the cell cycle (Dugoua et al., 2007) of specific cancer lines such as breast, leukemia, ovarian, and lung tumor cells both in vitro and in vivo (Koppikar et al., 2010). It should be noted that cinnamaldehyde is required at higher concentrations than CE to inhibit the same amount of proliferation on various cancer cells (Singh et al., 2007). While

cinnamaldehyde is noted to exhibit effects at dosages above 20 μM, cinnamon extract is able to induce the same effect as cinnamaldehyde at concentrations just above 1.28 μM (Singh et al., 2007). Other studies indicate that cinnamon extract can effectively inhibit growth factors and other receptors associated with angiogenesis and tumorigenesis. Binding of vascular endothelial growth factor (VEGF) secreted by cancer cells to two (VEGFR1 and VEGFR2) activates a signal transduction cascade resulting in endothelial cell morphogenesis and angiogenesis which eventually leads to tumor growth and metastasis. Treatment with CE considerably reduced the levels of pro-angiogenic factors, as well as metastasis of tumor tissues through inhibition of associated growth factors (EGF, VEGF, TGF α and β, Cox-2, HIF-1) (H. K. Kwon et al., 2009). Other *in vivo* studies demonstrated that CE treatment suppressed melanoma progression through down-regulation of NF-κB and AP1, though exact mechanisms of tumorigenesis suppression are not very well understood (H. K. Kwon et al., 2009; Singh et al., 2007).

Cardiovascular Disease**:** Recent studies have suggested that intake of cinnamon may decrease the risk of heart disease through reducing cholesterol and triglyceride levels (Khan et al., 2003). In mice studies, this reduction is attributed to the formation of sodium benzoate a metabolic byproduct of cinnamon that can suppress the mevalonate pathway and reduce cholesterol biosynthesis in vivo in mice (Brahmachari et al., 2009). In rats that were fed on a high fructose and high fat diet had reduced fat accumulation as a result of cinnamon treatment (K Couturier et al., 2010). Furthermore, cinnamon supplementation improved fasting blood sugar, systolic blood pressure, and body composition in rats that were genetically altered to develop metabolic syndrome (Ziegenfuss et al., 2006). Research on spontaneously hypertensive rats showed that

cinnamon supplementation had significant effects on lowering blood pressure in sucrose fed rats (18% sucrose in diet) and starch fed rats, even though the mechanisms of this reduction are not clearly understood (Preuss et al., 2006).

Diabetes**:** Cinnamon and its functional effect against diabetes is perhaps the longest studied property of cinnamon with the most amounts of resources The most commonly studied aspects of cinnamon and diabetes include blood glucose control, Hemoglobin A1C levels, satiety, gastric emptying rate, and gherlin values. Cinnamon has been shown to increase insulin sensitivity in healthy individuals (O'Keefe et al., 2008; Solomon & Blannin, 2007). *In vivo* studies indicate that cinnamon's effects on insulin sensitivity may be directly related to its ability to interfere with the insulin-signaling cascade associated with insulin resistance in type II diabetics. This interaction results in reduced glucose and lipid biosynthesis, increased GLUT4 translocation and reduced systolic blood pressure following ingestion of cinnamon (Solomon & Blannin, 2007). The bioactive components noted to increase insulin sensitivity include trimeric and tetrameric type A polyphenols (K Couturier et al., 2010). In vivo evidence suggests that addition of cinnamon to the diet counteracts insulin resistance brought about through a high fat/high fructose diet. Rats with elevated glucose rates administered cinnamon experienced a return of glucose levels to those of the control animals (K Couturier et al., 2010). This same data also suggests that cinnamon reduces fat accumulation in animal trials. A study conducted by Dugoa *et al.,* demonstrated that intake of 1, 3 and 6 grams of cassia cinnamon reduced fasting serum glucose by 18-29%, followed by improvements in triglyceride levels by 23-30%, LDL by 7-27%, and total cholesterol by 12-26% (2007). Cinnamon extract given to animals consuming a high fructose diet also helps with the

prevention of metabolic syndrome (Anderson, 2008; Solomon & Blannin, 2009). The ingestion of cinnamon at higher doses may potentially influence gastric emptying rate and postprandial glucose concentrations, as evidenced by reductions in gastric emptying rates with the ingestion of 6 g of cinnamon (Hlebowicz et al., 2007). The effect of gastric emptying is believed to improve blood glucose homeostasis through controlling the amount of carbohydrate which enters the small intestine. However, with every study the effect on blood glucose concentrations seem to be more noticeable than the lowering of gastric emptying rate, so it should be noted that gastric emptying is only a partial mechanism for aiding in the control of blood glucose levels (Hlebowicz et al., 2007). Advanced glycation end products (AGEs) accumulation in vivo has been associated with diabetes complications, including neuropathy, nephropathy, retinopathy, and cataracts (Peng et al., 2008a). As mentioned previously, cinnamon extract exhibits inhibitory activity towards AGEs by trapping reactive carbonyl species to avoid the accumulation of AGEs, and ultimately prevent diabetic complications (Peng et al., 2008a).

Neurodegeneration***:*** Neurodegenerative conditions such as AD or neural injury are characterized by the formation of extracellular plaques and intracellular neurofibillary tangles (tau), which result in a progressive loss in cognitive function (Peterson et al., 2009). Promising *in vitro* research suggests that cinnamon extract is able to inhibit aggregation of human tau due to the functional properties of proanthocyanidin trimer and cinnamaldehyde (Peterson et al., 2009). It is speculated that cinnamon extract can delay induction of neurodegeneration by preventing accumulation of AGEs (Brahmachari et al., 2009; Peterson et al., 2009).

It has been suggested that polyphenols such as proanthocyanids found in cinnamon possess properties that can inhibit the formation of amyloid fibrils in the brain that are developed during the progression of AD (Peterson et al., 2009). It is believed that neurofibrillary tangles typical in AD are formed in a downstream path leading to amyloid formation. Tangles occur when tau proteins accumulate in microtubules and detrimentally alter normal neuron pathways. The aqueous form of *C. zeylanicum* has recently been found to inhibit tau aggregation and filament formation, which are classic causes of AD (Peterson et al., 2009). Interestingly, the cinnamon extract did not appear to affect the normal cellular activity of tau proteins, such as microtubule support. Proanthocyanid trimer molecules but not epicatechin, (which is the main repeating unit of proanthocyanid trimer) are thought to be the key to the inhibitory activity in cinnamon extract, as well as cinnamaldehyde. Compared to whole cinnamon extract pure cinnamaldehyde fraction still exhibited 20% of the inhibitory action that the whole extract did, showing that there may be multiple beneficial factors from cinnamon involved in the inhibition of tau formation (Peterson et al., 2009). In genetically engineered flies overexpressing Amyloid-β_{42} peptide dietary intake of 75 mg/ml cinnamon extract showed no statistically significant difference in lifespan or climbing ability. However, in AD prone, 5XFAD knock-out mice intake of led to a 60% decrease in the accumulation of toxic Aβ oligomer (Frydman-Marom et al., 2011). Permeability of cinnamon bioactive compounds to the brain across the blood brain barrier is not known. The mechanisms for inhibition of Aβ aggregation is not clearly understood at this time and required further investigation.

Recent research has suggested potential benefits of cinnamon in a variety of health conditions including cancer, diabetes, heart disease and neurodegeneration; however, the biochemical and molecular mechanisms behind the bioactive effects of cinnamon have not been studies comprehensively. Moreover, dose-dependent effects of cinnamon at dietary relevant levels on critical regulatory pathways (eg. ILS) important in disease pathology, especially *in vivo* are sparse. Understanding mechanisms behind biological functionality cinnamon in various disease models is imperative to accurately assess the chemotherapeutic potential of cinnamon and cinnamon extracts as functional foods and pharmaceutical agents.

Methods

Nematode Propagation of INS and Cinnamon Treatment

Transgenic *C. elegans* strains carrying GFP-promoter fusions of genes relevant to the *daf-2/daf-16* signaling pathway were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN) (Table 1). Worms were grown on 35 mm or 60 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1mM CaCl, 1mM $MgSO_4$, 5mg/L Cholesterol, 2.5 mM KPO_4) at 18-20^oC (Brenner, 1974). Media was poured aseptically into culture plates (4.5 ml for 35mm, 10 ml for 60 mm) using a peristaltic pump and allowed to solidify for 36 hours. NGM culture plates were then inoculated with 50 µl of *Escherichia coli* OP50 overnight cultures and incubated for 8 hours at 37^oC. Strains of *C. elegans* were maintained by picking 2-3 young adult worms onto freshly inoculated NGM plates every 4-7 days.

As a control, NGM culture plates were prepared and inoculated as described previously. Treatment plates were made by supplementing NGM with aqueous extracts of cinnamon to final concentrations of 1 mg/ml, 5 mg/ml and 10 mg/ml. Aqueous extractions of cinnamon was prepared by heating 1.5g of the spice in 30 mL of distilled water, while stirring with a magnetic stir bar at 200 rpm for 30 minutes at 60^{o}C. The mixture was then vacuum filtered and centrifuged for 10 minutes at 4000 rpm. The supernatant was filter sterilized by passing through a 0.2µm syringe filter and added to the treatment medium just prior to pouring (Caldicott et al., 1994). After 36 hours, treatment plates were inoculated with 50 µL of *E. coli* OP50 supplemented with the appropriate concentration of cinnamon extract (1 mg/ml, 5 mg/ml or 10 mg/ml) and incubated for 8 hours at 37^{o}C. Two young adult worms were transferred to each plate, and allowed to lay hundreds of egg, hatch and grow to the young adult stage. This procedure was repeated with each strain. A minimum of 5 plates was used for each concentration.

Table 1. List of *C. elegans* strains used to evaluate dose-dependent effects of aqueous spice extracts on the *daf-2/daf-16* signaling pathway. Included are the *C. elegans* gene names and their human equivalents. Information obtained from www.wormbase.org.

Strain	Gene	Human Homolog	Wormbase Gene ID
GR1352	***daf-16α***	Forkhead Box O (FOXO)	WBGene00000912
TJ356	***daf-16***	Forkhead Box O (FOXO)	WBGene00000912
BC14074	***daf-2***	Insulin/Insulin growth factor (IGF) receptor	WBGene00000898
BC10837	***age-1***	Phoshoinositide 3-kinase (PI_3K)	WBGene00000090
BC10950	***let-92***	Protein Phosphatase 2A (let-92)	WBGene00002363
BC14613	***pptr-1***	Protein Phosphatase 2A regulatory subunit B56β	WBGene00012348
CF1553	***sod-3***	Iron/Manganese superoxide dismutase	WBGene00004932
BC13632	***sod-4***	Copper/Zinc superoxide dismutase	WBGene00004933
GA800	***ctl***	Catalase (ctl-1, ctl-2 and ctl-3)	WBGene00000830, WBGene00000831, WBGene00013220 respectively
UL3294	***sir-2.1***	Sirtuin 2	WBGene00004800
UL3351	***sir-2.1***	Sirtuin 2	WBGene00004800
CF2266	***ins-7***	Insulin/Insulin groth factor (IGF)-1-like peptide	WBGene00002090
GR1455	***daf-28***	Insulin-4 (ins-4), Human insulin	WBGene00000920
BL5752	***ida-1***	Type I diabetes autoantigens IA-1/phogrin	WBGene00002048

Fluorescence Imaging and Quantification

Images of 10 total adult worms from 5 plates were captured using the Nikon SMZ1500 fluorescence microscope with Ri1 CCD camera. Prior to capturing the images, worms were temporarily immobilized by chilling the cultures on ice for 15 minutes. Relative fluorescence, with respect to control, was quantified using the National Institute of Health's ImageJ software (Iser & Wolkow, 2007).

Statistical Analysis

Fold change in relative fluorescence was calculated between controls and each treatment concentration. Statistical analysis of data was performed using a two-tailed

Student's *t* test. *p* values $\leq$ 0.05 were considered statistically significant relative to control.

Results

Effect of Cinnamon on INS/ILS

Treatment with aqueous cinnamon extract resulted in an overall upregulation of multiple genes in the *daf-2/daf-16* pathway. Expression of *daf-2* (BC14074), the *C. elegans* insulin/IGF receptor ortholog, was significantly upregulated in response to all cinnamon treatments. Changes in relative fluorescence (RF) values were dose-dependent at concentrations of 1 mg/ml (RF= 1.039, *p=0.004*), 5 mg/ml (RF=1.560, *p=0.000*) and 10 mg/ml (RF=2.208, *p=0.001*) (Table 2). A similar trend was observed in the expression of *age-1* (BC10837), the p110 catalytic subunit of phosphoinositide 3-kinase (PI_3K), an ortholog of the mammalian PI_3K subunit. Relative to control, expression of *age-1* increased by fold changes of 1.36 (*p=0.000*), 3.05, (*p=0.000*), and 4.18, (*p=0.000*), in response to treatments at 1 mg/ml, 5 mg/ml and 10 mg/ml, respectively (Table 2). Upregulation of *let-92* (BC10950), which encodes the catalytic subunit for protein phosphatase 2A (PP2A) in *C. elegans*, occurred at both 1 mg/ml (RF=1.019, *p=0.005*) and 5 mg/ml (RF=1.116, *p=0.002*); however, at a dosage of 10 mg/ml, no change was observed in the expression of *let-92*, relative to control (Table 2). Expression of *pptr-1* (BC14613), a PP2A regulatory subunit, did not change in response to cinnamon at 1 mg/ml; however, significant upregulation was noted at 5 mg/ml (RF=1.117, *p=0.004*) and 10 mg/ml (RF= 1.110, *p=0.002*) (Table 2). Expression of the *C. elegans* FOXO homolog, *daf-16*, was evaluated using two strains (TJ356, GR1352), Significant upregulation of *daf-16* (TJ356) was observed in response to treatments of 1 mg/ml

(RF=1.069, *p=0.010*) and 10 mg/ml (RF=1.043, *p=0.013*). Expression of *daf-16α* (GR1352), an isoform of *daf-16*, did not change in response to cinnamon treatments (Table 2). Expression of *sod-3* (CF1553), which is under positive regulation of *daf-16* transcription, decreased in response to cinnamon concentrations of 1 mg/ml (RF=0.425, *p=0.011*), 5 mg/ml (RF=0.354, *p=0.004*) and 10 mg/ml (RF=0.545, *p=0.023*) (Table 2). Relative to control, the expression of *ctl* (GA800), which encodes three *C. elegans* catalases, did not significantly differ at concentrations of 1 mg/ml or 5 mg/ml; a decrease in *ctl* expression was observed in response to treatment with 10 mg/ml (RF=0.909, *p=0.032*). Expression of *sod-4* (BC13632), an extracellular superoxide dismutase, decreased in response to 1 mg/ml (RF=0.655, *p=0.000*) however, when the dosage of cinnamon was increased to 5 mg/ml, the expression of *sod-4* increased by 1.23 fold (*p=0.000*). A further increase in the dosage of cinnamon to 10 mg/ml resulted in a higher fold change of 1.34 (*p=0.000*) (Table 2). Increased expression of *ins-7* (CF2266), which encodes one of insulin-like peptides works in intestinal cells, was dose dependent, with relative fluorescence values of 1.152 (*p=0.002*), 1.967 (*p=0.000*), and 2.386 (*p=0.000*) in response to concentrations of 1 mg/ml, 5 mg/ml and 10 mg/ml, respectively (Table 2). Expression of *daf-28* (GR1455), which encodes an ortholog of human insulin, was reduced at a dosage of 1 mg/ml (RF=0.892, *p=0.000*). However, upregulation of *daf-28* was noted at 5 mg/ml (RF=1.267, *p=0.000*) and 10 mg/ml (RF=1.656, *p=0.000*) (Table 2). Relative to control, expression of *ida-1* (BL5752), an ortholog of the gene that encodes the mammalian type-1 diabetes autoantigens, was unchanged at a cinnamon concentration of 1 mg/ml. An upregulation of *ida-1* expression was noted at concentrations of 5 mg/ml (RF=1.256, *p=0.000*) and 10 mg/ml (RF=1.628, *p=0.000*)

(Table 2). Expression of *sir2.1* (UL3295, UL3351), a gene which functions upstream of *daf-16* and is positively correlated with increased lifespan in *C. elegans,* was upregulated in strain UL3295 at a concentration of 1 mg/ml (RF=1.490, *p=0.022*); while the same dosage resulted in the downregulation of *sir2.1* in UL3351 (RF=0.878, *p=0.*000). Upregulation of *sir2.1* was noted in both strains, in response to cinnamon treatments of 5 mg/ml (UL3295: RF=2.740, *p=0.022*; UL3351: RF=1.555, *p=0.000*) and 10 mg/ml (UL3294: RF=4.167, *p=0.000;* UL3351: RF= 2.968, *p=0.000*) (Table 2).

Table 2. Dose-dependent effect of cinnamon treatments on fold-change in gene expression relative to control (RF=relative fluorescence).

GENE	**Cinnamon Concentration**								
	1 mg/ml			**5 mg/ml**			**10 mg/ml**		
	RF	**SE**	***p value***	RF	**SE**	***p value***	RF	**SE**	***p value***
daf-16α	0.846	0.016	*0.379*	0.854	0.042	*0.265*	0.875	0.022	*0.225*
daf-16	1.069	0.046	***0.010***	0.842	0.061	*0.222*	1.043	0.050	***0.013***
daf-2	1.039	0.096	***0.004***	1.560	0.060	***0.000***	2.208	0.400	***0.001***
age-1	1.363	0.080	***0.000***	3.055	0.196	***0.000***	4.176	0.183	***0.000***
let-92	1.019	0.115	***0.005***	1.116	0.118	***0.002***	1.015	0.139	*0.064*
pptr-1	0.981	0.089	*0.166*	1.117	0.123	***0.004***	1.110	0.060	***0.002***
sod-3	0.425	0.064	***0.011***	0.354	0.081	***0.004***	0.545	0.126	***0.023***
sod-4	0.655	0.026	***0.000***	1.226	0.046	***0.000***	1.341	0.106	***0.000***
ctl	1.008	0.054	*0.380*	1.022	0.021	*0.187*	0.909	0.069	***0.032***
sir2.1	1.490	0.264	***0.022***	2.740	0.526	***0.004***	4.167	0.601	***0.000***
sir2.1	0.878	0.022	***0.000***	1.555	0.045	***0.000***	2.968	0.149	***0.000***
ins-7	1.152	0.044	***0.002***	1.967	0.050	***0.000***	2.386	0.073	***0.000***
daf-28	0.892	0.046	***0.000***	1.267	0.043	***0.000***	1.656	0.140	***0.000***
ida-1	0.955	0.036	*0.093*	1.256	0.040	***0.000***	1.628	0.060	***0.000***

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8.

Discussion

Insulin Signaling in Response to Cinnamon

The *C. elegans* homolog of insulin receptor, *daf-2* binds to insulin like peptides resulting in the activation of a phosphorylation cascade with mitogenic and metabolic implications. An active *daf-2* mediated signaling has been linked to increased metabolism and growth and proliferation. Consequently it has also been associated with a down regulation of redox stress response mechanisms. In response to cinnamon treatment the expression of *daf-2* was significantly upregulated, thus increasing the number of available insulin binding membrane receptor sites.

A conformational change resulting from the binding of insulin to insulin receptor activates an intrinsic tyrosine kinase that phosphorylates the cytoplasmic domain of the insulin receptor and serves as a docking site for IRS, facilitate interaction and activation of PI_3K(*age-1*). In our results we noted that treatment with cinnamon resulted in a significant upregulation of *age-1* expression in *C. elegans* (Prahlad & Morimoto, 2009).

An active *age-1* phosphorylates the transmembrane lipid PIP_2 to release the secondary messenged PIP_3. Cytoplasmic PIP_3 diffuses to allosteric site of another kinase PDK-1 resulting in its activation. An active PDK-1 in turn phosphorylates and activates a broad spectrum cytoplasmic kinase PI_3K/AKT (Lant & Storey, 2010). Several cytoplasmic proteins including the transcription factors are phosphorylation substrates for active PI_3K/AKT, their activation or inactivation in response to PI_3K mediated phosphorylation regulates cellular processes. FOXO/*daf-16* is an important transcription factor that regulates the expression of several antioxidant stress response genes such as superoxide dismutase and catalase (Barbieri et al., 2003).

However, *daf-16* is also a substrate for PI_3K/AKT and its phosphorylation causes cytoplasmic retention of *daf-16* preventing its nuclear translocation and thus inhibiting the expression genes under transcriptional regulation of *daf-16* (Hoogewijs et al., 2008). In response to cinnamon, our results showed that the expression of *daf-16* only increased marginally, however, the expression of mitochondrial Cu/Mn *sod-3* was significantly downregulated, suggesting a possible inactivation of *daf-16* and its cytoplasmic retention. Several protein phosphatases have been identified that can dephosphorylate and inhibit this signaling cascade.

In *C. elegans let-92* (protein phosphatase 2A PP2A), and its regulatory subunit *pptr-1* are known to dephosphorylate and inactivate AKT thus preventing cytosolic retention and facilitating nuclear translocation FOXO/*daf-16* (Lant & Storey, 2010; Shaw & Dillin, 2009). In response to cinnamon treatment, we only noted a marginal increase in the expression of *let-92* and *pptr-1* confirming that decreased expression of *sod-3* was indeed due to *daf-2* mediated phosphorylation of *daf-16*.

To confirm if the *daf-2* signal was truly active, we also looked at the expression of some genes that are positively regulated by *daf-2*. In response to cinnamon treatments, we observed that expression of *ins-7* and *ida-1* were significantly upregulated.

Insulin-7 (*ins-7*) has been identified as a putative agonist for *daf-2* (Murphy et al., 2007) and is positively regulated by the *daf-2* cascade in a feed-forward manner negatively regulated by active *daf-16* (Murphy et al., 2007). Recently *ins-7* RNAi in *C. elegans* was shown to promote life extension by inhibiting *daf-2*, which resulted in increased *daf-16* nuclear entry and increased expression of *sod-3* (Murphy et al., 2007).

In *C. elegans ida-1*, while in mammals (IA-2) expression is extended to pancreatic islet, adrenal medullary and pituitary cells (Zahn et al., 2001). The human IA-2 homologue in *C. elegans*, *ida-1* is expressed in peptidergic neurons and plays an important role in the regulation of secretory pathways of neuroendocrine cells such as pancreatic beta cells (Cai et al., 2009). In *C. elegans ida-1* causes exocytosis of dense-core vesicles and in humans IA-2 has been shown to be induced by insulin (Torii, 2009). Mutations in and *ida-1* and its deletion has shown to impair neuroendocrine processes insulin secretion (Torii, 2009). Upregulation in the expression of both *ins-7* and *ida-1* in response to cinnamon treatment suggests an active *daf-2* signaling mediated active insulin signaling cascade.

Moreover, the expression of *daf-28* an ortholog of mammalian insulin (INS-4) was also significantly upregulated in response to cinnamon treatment, however, it must be noted that unlike *ins-7*, expression of *daf-28* is not regulated by *daf-2* and it is also is known to positively regulate the expression and nuclear translocation of *daf-16* (Li et al., 2003). An upregulation in expression of *daf-28* in response to cinnamon suggest a possible tissue specific positive effect of cinnamon on neuronal growth and also an elevation in the *daf-16* mediated stress response in the ASJ and ASN neurons in *C. elegans.*

The gene *ctl* codes for three isoforms of the antioxidant enzyme catalase (Henderson et al., 2006) in *C. elegans* and is under positive transcriptional regulation of *daf-16.* In our study, we noted that in response to cinnamon treatment, the expression of *ctl* did not change significantly. Unlike *sod-3* which is strictly regulated by *daf-16* alone, expression of *ctl* is also under regulation of reactive oxygen and other metabolic

processes that enhance redox stress and is therefore considered an important indicator of cellular redox stress (Henderson et al., 2006). Therefore, a relatively constant expression of catalase upon treatment with cinnamon may reflect an enhanced oxidative stress response and redox balance perhaps via other mechanisms.

There is emerging evidence that shown a delineation in the activity of *sod-3* and *ctl* during conditions of oxidative stress. Recently, in *age-1* mutants the expression of *sod-3* and *ctl-1/2* increased during oxidative stress, however, in *daf-16* mutants, under the same conditions of hyperoxia, only *sod-3* expression decreased (Yanase et al., 2002). In *C. elegans* treatment with standardized extracts of *Ginkgo biloba* have been shown to decrease expression of stress inducible *ctl* genes during oxidative stress and under physiological conditions (Kampkötter et al., 2007).

The expression of *sod-4,* which is an extracellular Cu/Zn SOD and is important in redox balance in the extracellular matrix, was increased significantly upon treatment with cinnamon. However, unlike *sod-3* it has not been identified as a direct downstream target of *daf-16* protein (Murphy et al., 2007). Clearly treatment with cinnamon resulted in activation of *daf-2* signaling, however, contrary to expected results, even though there was a downregulation in the expression of *daf-2,* enhanced oxidative stress and decreased longitivity were not noted. These results suggest that cinnamon treatment resulted physiological redox balance even in the presence of an active *daf-2* signaling cascade.

Sir2.1, which has a human homolog of sirtuin 2 (Sir2) is a small NAD+ dependent histone deacetylase that can independently activate FOXO/*daf-16* by increasing its interaction with 14-3-3 protein (Lamming et al., 2004; Rizki et al., 2011; Y.

Wang et al., 2006). However, as described previously, in our studies, treatment with cinnamon did not result in any increase in the expression of genes positively regulated by *daf-16*, suggesting an alternate mechanism of stress reduction. In a separate pathway, *sir-2.1* acts to repress the expression of *abu-11* and a number of other pqn family genes believed to be involved in regulating protein folding within the endoplasmic reticulum (Viswanathan et al., 2005). Cinnamon via *sir-2.1*, may upregulate the expression of *abu-11* and other *abu/pqn* genes, potentially functioning to decrease ER stress ultimately resulting in enhanced redox balance and increase in lifespan (Viswanathan et al., 2005). Weather cinnamon treatment actually results in repression of ER stress and increased lifespan in *C. elegans* will be explored in future experiments.

Conclusion

Based on our preliminary results and study of literature survey we hypothesize that dietary cinnamon can modulate other important pathways in eukaryotic systems that regulate stress response signaling, such as Nrf2/ARE, HIF, HSP/UPR, MAPK and TGF-β. Positive modulation of stress response signaling by cinnamon may prove to be beneficial in management of neurodegenerative diseases that can result from their dysregulation.

CHAPTER II

CINNAMOMUM VERUM MODULATES NRF2/ARE (*SKN-1*/ARE) GENE EXPRESSION IN *C. ELEGANS*

Introduction

Nrf2/skn-1 Signaling Pathway

Expression of basic leucine zipper transcription factor, NF-E2–related factor (Nrf-2) is controlled by oxidative stress and PI_3K/Akt activities (Furukawa et al., 2010). It is reported to regulate antioxidative species against mitochondrial production of reactive oxygen species (ROS), phase II detoxification proteins and mitochondrial biogenesis (H. C. Huang et al., 2000; Suliman et al., 2007). In absence of oxidative stress Nrf2 exist in the cytoplasm bound with Kelch-like ECH-associated protein-1 (Keap-1) and GSK-3β which inhibit Nrf-2 nuclear translocation. In conditions of oxidative stress or caloric restriction, Nrf-2 translocates to the nucleus and binds to the many Nrf-2 dependent transcriptional factors, including antioxidant response element (ARE) (J. A. Johnson et al., 2009). ARE is present at the promoter region of genes for regulation of antioxidation and phase II detoxification. These genes include mitochondrial superoxide dismutase (*SOD2*), glutathione synthetase (*GSS*), glutathione reductase (*GSR*), glutathione S-transferase (*GST*), glutathione peroxidase (*GPx*), thioredoxin (*TRX*), thioredoxin reductase (*TRXR*), hemeoxygenase-1 (*HO-1*) and NAD(P)H quinone oxidoreductase 1

(*NQO1*) (Bardag-Gorce et al., 2011; Konstantinopoulos et al., 2011; Piantadosi et al., 2008; Tanito et al., 2007). Thus, increasing these antioxidative and detoxifying gene transcription via activation of Nrf-2 signaling is considered to play key role in neuroprotection, anticarcinogenicity, anti-inflammatory, and the aging processes. A number of studies have been done on the effect of phytochemicals from dietary spices and herbs as new potential prevention or treatment options against aging and age-related diseases, such as neurodegeneration and carcinogenesis. Some phytochemicals are known to modulate antioxidative gene expression and have potential benefit against aging and age-related diseases; for example, green tea catechins are shown to have antiangiogenic effect against colorectal cancer in animal models (Sukhthankar et al., 2008). Other studies reported that tea polyphenols modulate gene expression of Nrf2/ARE signaling and have protective effect against age related disease by inducing antioxidant and detoxifying enzymes (Na & Surh, 2008). *Cinnamomun* (cinnamon) has been known to the traditional spices and medicines since ancient age and in recent studies it is shown to have antioxidant, antidiabetic, antitumorigenesis and anti-inflammatory properties (Dugoua et al., 2007; X. Lin et al., 2004; R. Wang et al., 2009). For example, cinnamon polyphenol proanthocyanidins are shown to have antioxidative activity and prevent advance glycation endproducts as reactive carbonyl scavenger and prevent inflammation (Peng et al., 2008a). Trimeric and tetrameric type A polyphenols of cinnamon is noted to increase insulin sensitivity and have anti-diabetic properties (K Couturier et al., 2010). Cinnamon bioactive components are reported to suppress tumorigenesis by inhibiting angiogenesis and increase apoptosis of tumor cells (H. K. Kwon et al., 2010). In addition to these protective effects of cinnamon components, cinnamaldehyde (CA) was reported

to react with thiol groups of Keap1/Nrf2 and activate ARE mediated gene expression (T.-C. Huang et al., 2011). Activation of Nrf2-dependent gene is considered to have potent chemotherapeutic activities against cancer. It was reported that ethanol extract of cinnamon and aqueous cinnamon extract upregulated Nrf2 and its Nrf-2 dependent gene transcription and in cultured human epithelial colon cells *in vitro* (Georg Thomas Wondrak et al., 2010). Also potent therapeutic properties of cinnamon mediated by Nrf2 were reported against diabetic nephropathy in mice study, atherosclerosis *in vitro* and skin cell photo-oxidative stress *in vitro* (Jiang et al., 2010; Georg T Wondrak et al., 2008). In this study, we examined the effect of dietary cinnamon extract on modulation of Nrf2/ARE related gene expression in *C. elegans* model.

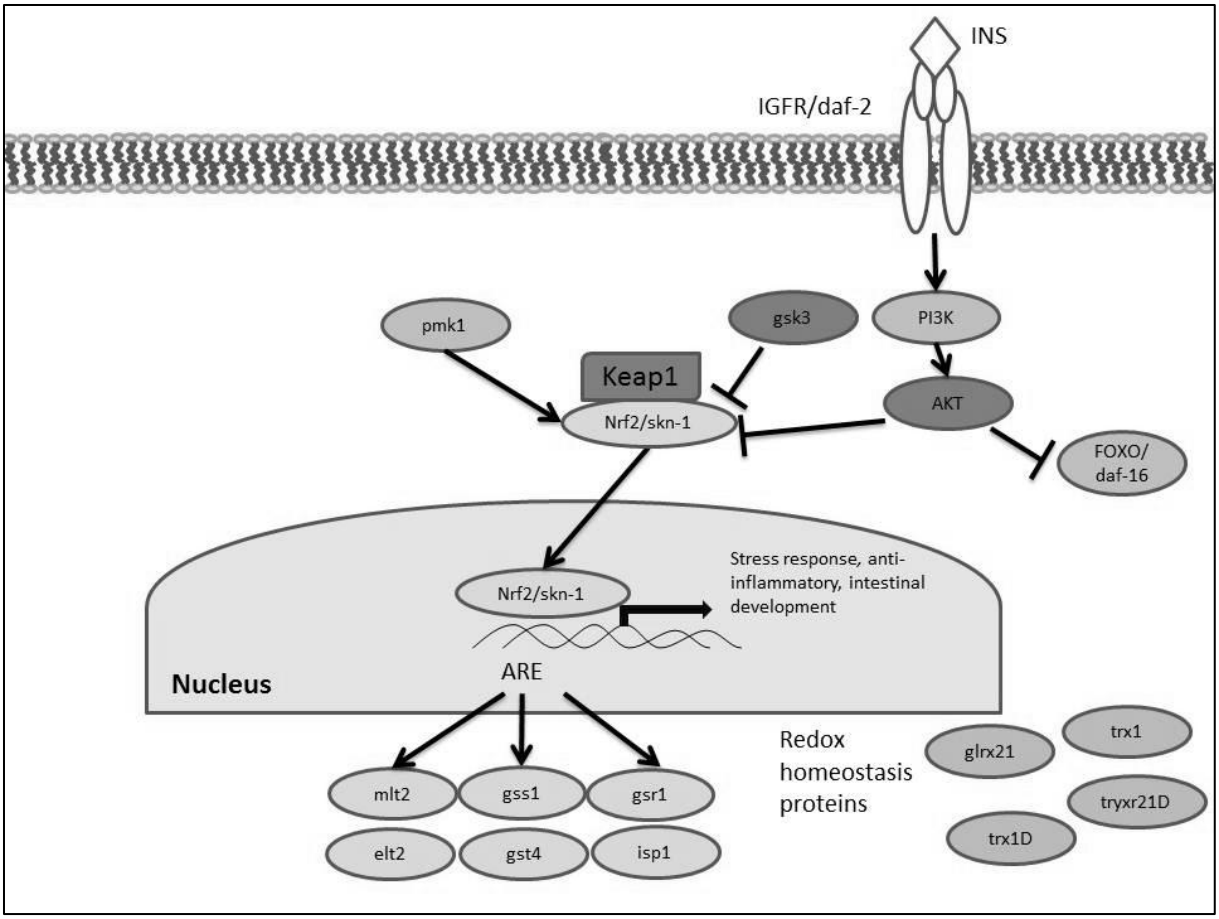

Figure 2. Nuclear factor-erythroid-2 related factor-2 (Nrf-2) signaling model. Nrf-2 is dissociated from Keap1 and translocates to the nucleus, then binds to the prompter region of the antioxidant response element (ARE). While expression of genes that function as redox sensors were also evaluated.

Methods

Nematode Propagation of Nrf-2/skn-1 Signaling Pathway and Cinnamon Treatment

C. elegans with GFP promoter constructs of genes relevant to *skn-1*/ARE (Human Nrf2/ARE) pathway (Table 3) obtained from the Caenorhabditis Genetics Center were used to study the effect of cinnamon on the expression and activity of genes regulated by

skn-1/Nrf2/ARE system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 3. List of C. elegans genes evaluated by strain, and their human equivalents in Skn-1 signaling. Information obtained from www.wormbase.org**.**

SKN-1			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
LG326	***skn-1***	Nuclear factor-erythroid-2 related factor-2 (Nrf2) transcription factor	WBGene00004804
MR142	***elt-2***	GATA4-6 transcription factors-primarily intestinal	WBGene00001250
MR164	***elt-2***	GATA4-6 transcription factors-primarily intestinal	WBGene00001250
OH7631	***elt-2***	GATA4-6 transcription factors-neuronal	WBGene00001250
BC12667	***gss-1***	Glutathione synthetase (GSS)	WBGene00010941
BC11072	***gsr-1***	Glutathione diSulfide Reductase (GSR)	WBGene00008117
BC13348	***gst-4***	Glutathione S-Transferase (GST)	WBGene00001752
CL2166	***gst-4***	Glutathione S-Transferase (GST)	WBGene00001752
CL2122	***mtl-2***	Metallothioneins (MTL)	WBGene00003474
BC14279	***isp-1***	Iron sulfur protein (ISP)	WBGene00002162
OE3010	***trx-1***	Thioredoxin (TRX)	WBGene00015062
BC13081	***trx-1 D***	Thioredoxin (TRX) driven (D)	WBGene00015062
BC12017	***trxr-2 D***	Thioredoxin reductase (TRXR) driven (D)	WBGene00014028
BC12039	***glrx-21 D***	Glutaredoxin (GLRX) driven (D)	WBGene00022663

Results

Effects of Cinnamon Nrf2/ARE Signaling

The effect of aqueous cinnamon extract on expression of *skn-1* (homolog of the mammalian *nrf-1* gene) and several genes downstream of *skn-1*, was evaluated at cinnamon concentrations of 1 mg/ml, 5 mg/ml and 10 mg/ml. In *C. elegans, skn-1*

functions within the p38 MAP-Kinase pathway to regulate the oxidative stress response. In response to treatment with cinnamon, dose-dependent upregulation of *skn-1* (LG326) was observed, relative to control. At a concentration of 1 mg/ml, relative fluorescence of *skn-1* was 1.130 (*p=0.001*), while increased dosages of cinnamon at 5 mg/ml and 10 mg/ml resulted in fold changes of 1.435 (*p=0.002*) and 1.680 (*p=0.000*), respectively (Table 4). Expression of *elt-2*, a gene downstream of *skn-1*, was evaluated in three strains (OH7631, MR142, MR164). In strain OH7631, *elt-2* expression increased, relative to control, in response to cinnamon concentrations of 1 mg/ml, 5 mg/ml and 10 mg/ml with relative fluorescence values of 1.121 (*p=0.020*), 1.449 (*p=0.000*) and 1.811 (*p=0.000*), respectively (Table 4). A similar dose-dependent trend was observed in *elt-2* expression when evaluated in strain MR142. At a concentration of 1 mg/ml, expression of *elt-2* (MR142) increased by a fold change of 1.419 (*p=0.000*), and when the dosage was increased to 5 mg/ml and 10 mg/ml, relative fluorescence values were 1.745 (*p=0.000*) and 2.542 (*p=0.000*), respectively. In strain MR164, expression of *elt-2* was unchanged at a concentration of 1 mg/ml; however, in response to 5 mg/ml and 10 mg/ml, relative fluorescence values were 1.218 (*p=0.005*) and 1.237 (*p=0.002*), respectively (Table 4). The *C. elegans* gene *gsy-1* (BC12667), an ortholog of the human glutathione synthase gene, was unchanged at concentrations of 1 mg/ml and 5 mg/ml. At a concentration of 10 mg/ml, expression of *gsy-1* increased by a fold-change of 1.912 (*p=0.000*), relative to control (Table 4). Upregulation of *gsr-1* (BC11072), an ortholog of the human mitochondrial glutathione reductase, was observed in response to cinnamon concentrations of 1 mg/ml (RF=1.132, *p=0.000*) and 5 mg/ml (RF=1.426, *p=0.000*); however, relative to control, at a dosage of 10 mg/ml, no change in *gsr-1* expression was

observed (Table 4). Expression of *gst-4*, a gene driven by glutathione s-transferase, was evaluated in strains BC13348 and CL2166. In both strains, significant dose-dependent upregulation of *gst-4* was observed (Table 4). Relative to control, fold changes of 1.423 (*p=0.009*), 2.352 (*p=0.000*) and 2.495 (*p=0.000*) were observed in strain BC13348 in response to treatments with 1 mg/ml, 5 mg/ml and 10 mg/ml, respectively. In strain CL2166, fold changes of 1.349 (*p=0.009*), 1.873 (*p=0.019*) and 2.558 (*p=0.032*), were observed in response to treatments at 1 mg/ml, 5 mg/ml and 10 mg/ml, respectively. Expression of *mtl-2* a gene which encodes for one of two metallothioneins, a protein which functions in metal detoxification and homeostasis, was unchanged at all concentrations of cinnamon. Expression of *isp-1* (BC14279), a gene involved in the expression of an iron-sulfur protein in *C. elegans*, was reduced, relative to control, when treated with cinnamon at 5 mg/ml (RF= 0.765, *p=0.000*) and 10 mg/ml (RF= 0.797, *p=0.007*); no change was observed in response to treatment with 1 mg/ml (Table 4). Thioredoxin-encoding, *trx-1* (OE3010), is required for normal adult lifespan in *C. elegans*. In response to cinnamon dosages of 1 mg/ml, 5 mg/ml, and 10 mg/ml, expression of *trx-1* increased dose-dependently, with fold changes of 1.138 (*p=0.000*), 1.729 (*p=0.000*) and 2.141 (*p=0.000*), respectively. A gene driven by *trx-1, trx-1-D* (BC13081) was observed to have a lower expression, relative to control, when treated with cinnamon at a concentration of 1 mg/ml (RF= 0.632, *p=0.000*). At 5 mg/ml, no change in *trx-1-D* expression was observed, however, in response to treatment at 10 mg/ml, a fold change of 1.465 (*p=0.022*) was noted. Expression of *trxr-2-D* (BC12017), a gene driven by thioredoxin reductase, increased dose dependently in response to cinnamon treatments. At a concentrations of 1 mg/ml, expression of *trxr-2-D* increased

by a fold change of 1.959 (*p=0.000*) and when the dosage was increased to 5 mg/ml and 10 mg/ml, relative fluorescence was 2.948 (*p=0.000*) and 3.405 (*p=0.000*) (Table 4). The expression of *glrx-2-D* (BC12039), a gene driven by glutaredoxin and related proteins, was also observed to increase, dose-dependently, in response to cinnamon treatment at 1 mg/ml, 5 mg/ml and 10 mg/ml, relative fluorescence, compared to control, was 1.396 (*p=0.000*), 2.290 (*p=0.001*) and 2.867 (*p=0.000*), respectively (Table 4).

Table 4. Dose-dependent effect of cinnamon treatments on fold-change in gene expression relative to control.

GENE	Cinnamon Concentration								
	1 mg/ml			5 mg/ml			10 mg/ml		
	RF	SE	*p value*	RF	SE	*p value*	RF	SE	*p value*
skn-1	1.130	0.035	***0.001***	1.435	0.127	***0.002***	1.680	0.074	***0.000***
elt-2	1.121	0.146	***0.020***	1.449	0.130	***0.000***	1.811	0.209	***0.000***
elt-2	1.419	0.147	***0.000***	1.745	0.120	***0.000***	2.542	0.452	***0.001***
elt-2	1.143	0.118	*0.061*	1.218	0.093	***0.005***	1.237	0.088	***0.002***
gss-1	0.844	0.063	***0.104***	0.827	0.060	***0.131***	1.912	0.266	***0.000***
gsr-1	1.132	0.044	***0.000***	1.426	0.077	***0.000***	1.168	0.141	*0.119*
gst-4	1.423	0.131	***0.009***	2.352	0.284	***0.000***	2.495	0.211	***0.000***
gst-4	1.349	0.177	***0.001***	1.873	0.211	***0.019***	2.558	0.125	***0.032***
mtl-2	0.389	0.129	*0.140*	0.481	0.057	*0.066*	0.469	0.055	*0.119*
isp-1	0.984	0.165	*0.150*	0.765	0.185	***0.009***	0.797	0.290	***0.007***
trx-1	1.138	0.044	***0.000***	1.729	0.147	***0.000***	2.141	0.169	***0.000***
trx-1-D	0.632	0.045	***0.000***	0.998	0.191	*0.339*	1.465	0.315	***0.022***
trxr-2-D	1.959	0.060	***0.000***	2.948	0.159	***0.000***	3.405	0.167	***0.000***
glrx-21-D	1.396	0.112	***0.000***	2.290	0.336	***0.001***	2.867	0.301	***0.000***

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8.

Discussion

Nrf2/ARE Signaling in Response to Cinnamon

The *C. elegans* homolog of basic leucine zipper transcription factor *Nrf2*, *skn-1* regulates level of antioxidants, phase II detoxification enzymes and glutathione synthesis (H. C. Huang et al., 2000; Suliman et al., 2007). *Skn-1* is involved in intestinal cell and ASI neuron development of *C. elegans* (Tullet et al., 2008). And similar to *daf-16*, decreasing of *daf-2* signaling increase *skn-1* nucleus translocation, and response to oxidative stress (Bishop & Guarente, 2007), thus modification of *skn-1* has potential benefit on neurodegenerative diseases and longevity (Dostal et al., 2010). In response to cinnamon treatment the expression of *skn-1* was significantly upregulated, suggesting a possible activation of *skn-1* dependent antioxidant proteins and phase II detoxification pathway.

C. elegans GATA-type transcription factor *elt-2* is required for differentiation of the post embryonic intestinal cell (McGhee et al., 2007). *Skn-1* signaling is also involved in intestinal cell differentiation, and it is reported that *elt-2* is indirectly or potentially directly regulated by *skn-1* (Maduro, 2009). To confirm if *elt-2* is truly active, three strains (M142, M164 and OH7631) were used to check their GFP level. In response to cinnamon treatment, we observed that in all three strains *elt-2* expression were increased (Table 4), suggesting active *skn-1* signaling and its dependent gene transcription.

Mtl-2 is *C. elegans* metallothionein, which expresses in intestinal cell have metal detoxification and heat shock response properties (Haqa et al., 2003). In mammal, metallothionein promoter region is overlapped with Nrf-2 dependent transcriptional

element ARE, and it is indicated *mtl-2* protects cell from free radical damage along with other detoxification enzymes (Haqa et al., 2003). In *C. elegans mtl*-2 is not directly upregulated by *skn-1* gene, but it is regulated via *elt-2* gene in the intestine and protect cells against cadmium exposure (Roh et al., 2009; Tvermoes et al., 2010). In response to cinnamon treatment, there was no significant difference of *mtl-2* gene expression (Table 4).

The gene *isp-1* codes the electron transport complex II subunit in mitochondria membrane and it is reported that the expression of *isp-1* related to extension of lifespan (Van Raamsdonk & Hekimi, 2009). *Isp-1* is involved in respiration of mitochondria, thus upregulation of *isp-1* expression indicates increasing of reactive oxygen spices (ROS). It is reported that loss-of-function mutation of *isp-1* results in low endogenous ROS production and increased life span of *C. elegans* (Feng et al., 2001). Also, it was observed that mild inhibition of respiration cause hypoxia condition and increased ROS level slightly, which activate *hif-1* and *hif-1* target genes expression and extend lifespan of *C. elegans* (S.-J. Lee et al., 2010). In response to cinnamon treatment *isp-1* expression was downregulated and *hif-1α* expression was significantly upregulated, suggesting activation of *skn-1* and possible lifespan extension via increasing *hif-1* target genes.

C. elegans gss-1 is the gene which codes human orthologous glutathione synthetase (GSS). Glutathione is synthesized by two enzymes γ-glutamylcysteine and GSS from glutamic acid, thus *gss-1* limit rate of glutathione synthesis rate. Glutathione is an important antioxidant protein to protect cell from ROS and it is involved in phase I and phase II detoxification by neutralizing free radicals. It is indicated that the transcription of g*ss-1* is induced by skn-1 (An & Blackwell, 2003). Upregulation in the

expression of *gss-1* in response to cinnamon treatment suggests an active *skn-1* signaling and increasing protection against ROS damage.

Gutathione disulfide reductase (GSR) is another protein that control redox balance. GSR is one of the enzymes of glutathione metabolism and catalyzes the reduction of oxidized glutathione (GSSG) along with cofactor NADPH and recycle GSH. The *C. elegans* gene *gsr-1* is an ortholog of mammalian GSR and it is predicted to be regulated by skn-1/Nrf-2 (An & Blackwell, 2003). The expression of *gsr-1* was also significantly upregulated in response to cinnamon treatment, suggesting increasing of GSH regeneration and keeping cellular redox balance.

Glutathione S-transferase (GST) is a type II detoxification enzyme which catalyzes binding of GSH to foreign compounds. The gene *gst-4* is one of *C. elegans* GSTs which is regulated by skn-1 (Park et al., 2009). One study reported that upregulation of *gst-4* increased protection against various stresses including oxidative stress, but did not affect lifespan (Leiers et al., 2003). Whereas, another study reported that diallyl trisulfide (DATS) of garlic upregulates skn-1 dependent gene transcriptions and increase mean lifespan but not maximal lifespan (Powolny et al., 2011). The mechanism of extension of lifespan and *gst-4* expression is still unclear, but it indicated that increasing of the expression of *gst-4* have protective effect against stress induced dysfunction. In response to cinnamon extract, the expression of *gst-4* was significantly increased compared with control, suggesting cinnamon treatment increase oxidative stress response via the glutathione system.

The gene *glrx-21* codes *C. elegans* glutaredoxin, which is an ortholog of the human glutaredoxin-2 protein (Sagemark et al., 2007). Reduced active form of *glrx-21* is maintained by GSH and protects cell from selenium-induced reductions in *C. elegans* (Morgan et al., 2010). The expression of *glrx-21* is not regulated by skn-1 signaling. An upregulation in expression of *glrx-21* in response to cinnamon suggests a possible positive effect of cinnamon on metal toxicity.

Thioredoxin (TRX) is a major antioxidant protein in cell universally presenting in organisms from archaea to humans. TRX contains SH group and promotes the reduction of disulfide bonds of proteins and recycled with NADPH and thioredoxin reductase (TRXR) (Arnér & Holmgren, 2000). In *C. elegans*, *trx-1* is one of ARE response genes expressed in ASJ sensory neurons (Oliveira et al., 2009) and it is reported that loss-of-function mutation of *trx-1* decreased lifespan of worm (Miranda-Vizuete et al., 2006). It is also indicated that upregulation of *trx-1* in neurons induced by dietary restriction increased lifespan of *C. elegans*. To confirm the *trx-1* expression change, two strains (OE3010 and BC13081) were used. In response to cinnamon treatment average of the expression of *trx-1* was upregulated, suggesting active skn-1 signaling and increasing of antioxidative activity in neurons. Also in response to cinnamon treatment mitochondrial form thioredoxin reductases *trxr-2* was upregulated, which suggesting whole thioredoxin system was activated. Recently it is reported that overexpression of *trxr-2* strongly decreased β-amyloid peptide and amyloid deposits in *C. elegans* model, thus cinnamon treatment has possible benefit to Alzheimer's disease.

Conclusion

The present study showed the effect of cinnamon extract on modification of Nrf-2/*skn-1* signaling *in vivo*, and many of these effects occur in a dose-dependent manner. The Nrf-2/*skn-1* signaling pathway regulate phase 2 detoxification genes, thus it is important pathway to protect cellular function from free radicals. Nrf-2/*skn-1* signaling has been indicated to influence inflammation, apoptosis, lifespan and neurodegenerative diseases. Therefore, cinnamon properties of activation of Nrf-2/*skn-1* may help as target molecules of neurodegenerative diseases by its stress responding activity.

CHAPTER III

THE *IN VIVO* EFFECT OF *CINNAMOMUM VERUM* ON HYPOXIA INDUCIBLE FACTOR, APOPTOSIS, AND CELL CYCLE REGULATION IN *C. ELEGANS*

Introduction

HIF-1 signaling pathway

Hypoxia-inducible factor 1 (HIF-1) protein is induced in hypoxia condition and functions as a transcription factor complex of hypoxia responsive element (HRE). HIF-1 plays a key role in managing energy metabolism (G. Semenza & Roth, 1994) , cell proliferation (Heiden & Cantley, 2009), cell death and survival (Mazure & Pouysségur, 2010), mitochondrial autophagy (H. Zhang et al., 2008), metastasis (Erler et al., 2006) and angiogenesis (Yamakuchi et al., 2010). HIF-1 is heterodimeric transcription factor consisting of HIF-1α and HIF-1β subunit. HIF-1α subunit is regulated by level of oxygen, but the expression of HIF-1β subunit is constitutive (G. L. Semenza, 2009). Expression of HIF-1α subunit is increased by several different pathways, such as PI_3K-AKT signaling pathway, TGF-β signaling pathway, NF-κB signaling pathway and MAPK pathway (BelAiba & Bonello, 2007; Frede et al., 2006; Mwaikambo et al., 2009). Thus, inhibition of these signaling causes modulation of various HRE dependent transcriptions. One of the HIF-1 inhibitor is PTEN. Since PTEN inhibit PI_3K-AKT signaling pathway, increasing expression of PTEN negatively regulate transcription of HIF-1α

(Emerling et al., 2008). Also under normoxia condition proline residue of HIF-1α/ HIF-1β is hydroxylated by enzymes, such as prolyl hydroxylases (PHD) and factor inhibiting HIF (FIH) (H. Zhang et al., 2007). Hydroxylated HIF-1α become oxygen-dependent protein which contains von Hippel Lindau E3 ubiquitin ligase (VHL) binding site, thus VHL can bind to hydroxylated HIF-1 α and targeted to the proteasome degradation (H. Zhang et al., 2007). Therefore, under the normoxia condition HIF-1α is inactivated and HRE dependent transcriptions will not be upregulated. On the contrary, under the hypoxia condition PHD and FIH hydroxylase activity is restricted, and stable HIF-1 translocates to the nuclear and binds with HRE and increase HIF-1 responsive genes (Berra et al., 2003).

HRE dependent transcription increases various genes, such as immune responsive genes, angiogenesis genes, cell proliferation genes, iron metabolism genes, energy metabolism genes (Glover et al., 2011; Haase, 2010). As a part of immune response, it is reported that HIF-1α regulate immune system by increasing antimicrobial peptides and macrophage activity (Zinkernagel et al., 2007). HIF-1α is also known to increase glucose metabolism and growth factor, such as glucose transporter 1, IGF2, and TGF-α (G. L. Semenza, 2012). Stimulation of HIF-1α increase vascular endothelial growth factor (VEGF) production and mediate angiogenesis (Ferrara et al., 2007). Also increasing epithelial levels of HIF-1α leads to induction of NF-κB and IL-8 and mediating angiogenesis is observed in cancer cell (Lurje et al., 2008; Végran et al., 2011). One of HIF-1 responsive protein adrenomedullin (ADM) is one of the regulatory factors to control angiogenesis and angiogenesis-dependent diseases (Fernandez-Sauze et al., 2004). ADM also promotes endothelial cell proliferation and has cardioprotective

properties by preventing apoptosis (Hamid & Baxter, 2006). HRE dependent transcription is also involved in the increase of iron homeostasis. In a mice study, it is reported that in condition of hypoxia, HIF-2 α bind with HRE and increase transferrin, ceruloplasmin, ferroportin, and erythropoietin in order to increase oxygen level (Mastrogiannaki & Matak, 2009). Thus, HIF-1 signaling responds to oxygen level and regulates cell survival and cell death via regulating IGF2, TGF-α and MAPK signalings.

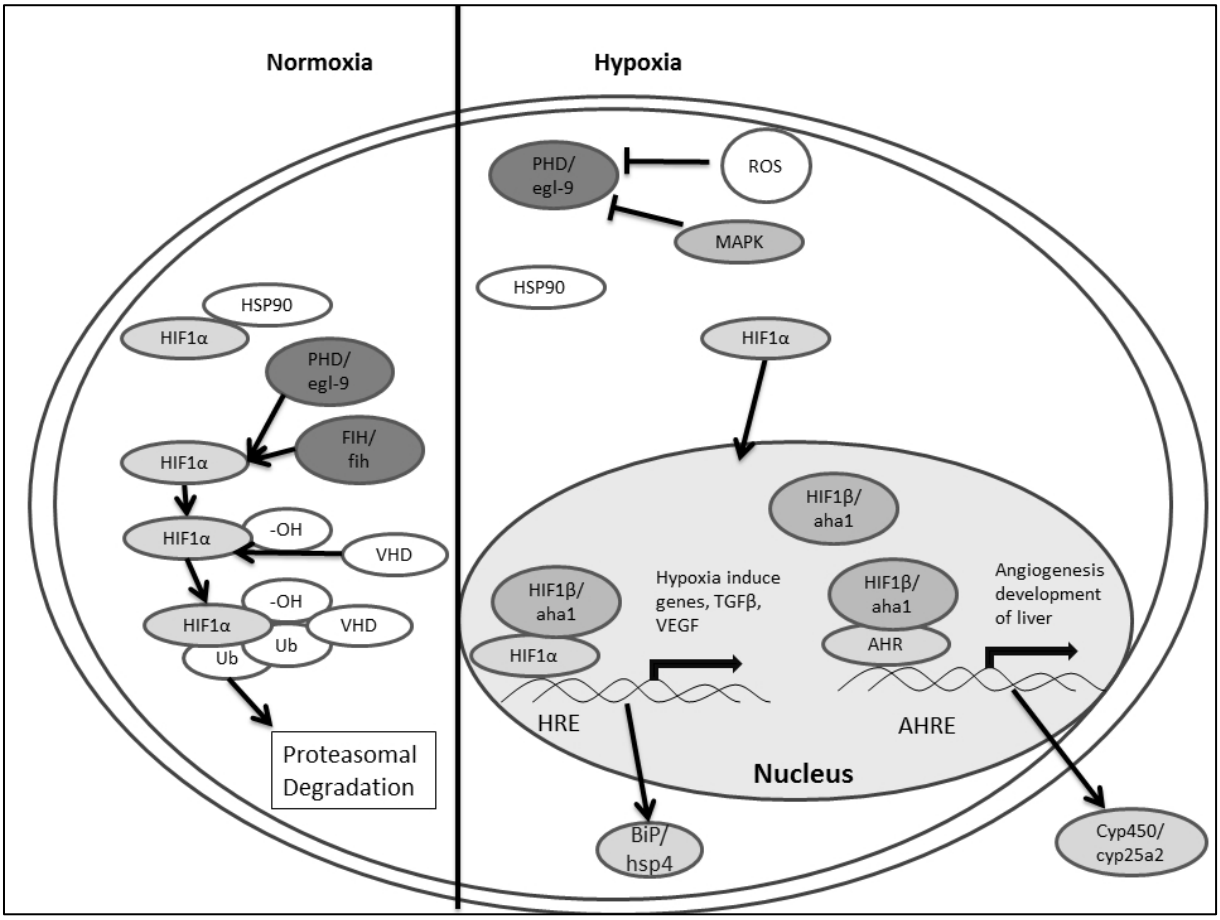

Figure 3. Hypoxia inducible factor-1 (HIF-1) signaling model. Under normal oxygen conditions (normoxia) HIF-1α is hydroxylated and ubiquitinated by HIF-1α inihibitor and targeted for proteasomal degradation. Under low oxygen conditions (hypoxia), HIF-1α and HIF-1β complex bind to hypoxia responsive element (HRE) and response to hypoxia condition.

Apoptotic Signaling

Apoptotic signaling is activated through extrinsic and intrinsic induction and also many pathways are involved in this apoptosis signaling in neuronal cell (Perera & Bardeesy, 2011). Extrinsic induction of apoptosis is activated when proapoptotic ligands, such as cytokines and death factors, bind to cytokines receptor or Fas receptor (Kong et al., 2001; C. Vincent et al., 2009). Oxidative stress and inflammation is involved in activating these receptors because various ligands of these receptors are induced or activated by oxidative stress and inflammation (Reuter et al., 2010). These signaling increase apoptosis signaling via activating of caspase-3 and caspase-8 and cause cell death of neurons (Sharifi et al., 2009). Intrinsic induction of apoptosis is activated through ER stress and mitochondrial stress (Martinez et al., 2010; Petit et al., 2003). ER stress increase enzymatic activities of caspase-12, then caspase-12 activate caspase-3 and caspase-9 which cause neuronal cell death (Martinez et al., 2010). Intrinsic induction of apoptosis is also mediated by mitochondrial stress (Petit et al., 2003). When mitochondria was damaged by U.V. or oxidative stress, cytochrome c is secreted from mitochondria due to increase of permeability of the mitochondrial membrane, cytochrome c then forms a complex with apoptotic protease activating factor-1 (Apaf-1) which activate caspase-9,-7 and -3 and cause apoptosis in neuronal cell (C. E. Johnson et al., 2007).

For activating pro-apoptosis signaling and anti-apoptosis signaling B-cell lymphoma 2 (Bcl-2) family proteins are reported to play important roles. Bcl-2 family proteins, Bcl-2 , Bcl-xL and Bcl-w are classified as anti-apoptotic proteins, which share four domains BH1, 2, 3 and 4 (Chipuk et al., 2010). Other Bcl-2 family proteins, Bax and

Bak are classified as pro-apoptotic proteins which share multi-domain of BH1, 2 and 3 (Chipuk et al., 2010). Also Bid, Bil, Bad, Bim and Bmf are classified as pro-apoptotic protein, but these protein share only BH3 domain, thus these are also called BH3-only proteins (Chipuk et al., 2010).

In normal condition, *ced-9*, homolog of human Bcl-2, suppresses apoptosis by binding with *ced-4*, homolog of apoptotic protease activating factor 1 (APAF1) or with *bec-1*, beclin-1 homolog in C. elegans neuronal cell (Y. Lu et al., 2011; Nehme et al., 2010). And an anti-apoptotic zinc-finger transcriptional repressor of the Snail/Slug family CES-1 (*ces-1* in *C. elegans*) is reported to bind and repress expression of the BH3-proapoptotic gene *egl-1* and prevent the death of motor neuronal cells (Hirose et al., 2010; Thellmann, 2003). The mechanism remain unclear but when Ces-1 was negatively regulated by Ces-2 or other environmental factors, when the mitochondrial *C. elegans* BH3-only protein *egl-1* will be activated (Hatzold & Conradt, 2008).

When *egl-1* binds to *ced-9* protein, the complex of *ced-9* with *ced-4* or *bec-1* will be broken and *ced-4* and *bec-1* will be released (Pinan-Lucarre et al., 2012). Free *ced-4* activate caspases, which results to cause apoptosis and regenerate neuronal cell (Pinan-Lucarre et al., 2012). Also free *bec-1* is suggested to be cleaved by caspase and its fragments does not increase autophagy but increase apoptosis signaling (Wirawan et al., 2010).

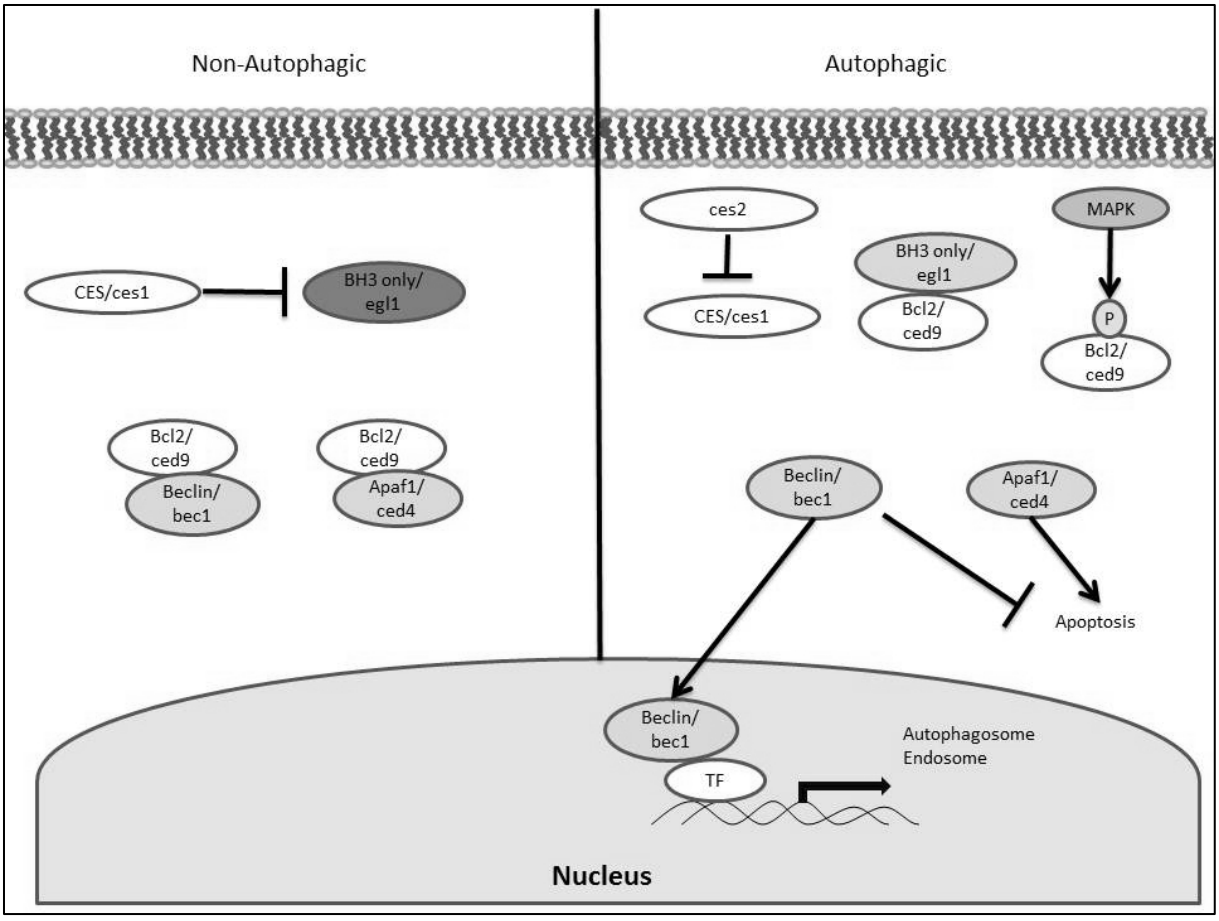

Figure 4. Autophagy and apoptosis signaling model. BH3 domain only protein egl-1 bind with Bcl-2/ced-9 protein and activate autophagy protein Beclin and pro-apoptotic protein Apaf-1.

Regulation of the Autophagy

After cell goes into apoptotic signaling, dead cell is engulfed by macroglial cell in neuron to inhibit increase of extracellular misfolded and unfolded which cause neuronal toxicity (Origasa et al., 2001). It is reported that BH3 only protein binds with Bc-2 and release Beclin-1 and free Beclin-1 make a complex with class the III PI3 kinase LET-512/Vps34 and trigger autophagy and endocytosis (Takacs-Vellai et al., 2005). Accumulation of extracellular apoptotic cells and misfolded or unfolded proteins cause extracellular toxicity, thus clearance of extracellular proteins and apoptotic cell is

important. It is reported that increase of Beclin-1 decreased extracellular synaptic protein α-synuclein and decreased development of Parkinson's disease (PD) *in vitro* study (Spencer et al., 2009). In *C. elegans ced-5*/Dock180, and *ced-12/*ELMO is reported to increase promotion of the basolateral recycling (Sun et al., 2012).

Ced-5 and *ced-12* are activated by RhoG and make a complex, and then this complex promotes endocytic trafficking of *ced-10,* which is homolog of Rac protein (Moreira & Barcinski, 2004). On the cell membrane, Rac protein recognizes misfolded proteins of apoptotic cells and uptake these to reduce extracellular toxicity from apoptotic cells (Moreira & Barcinski, 2004). Also, the transmembrane low density lipoprotein receptor molecule CD91 (*ced-1* in *C. elegans*) binds and recognizes the extracellular apoptotic cell proteins and promotes cell corpse engulfment of apoptosis cell (Moreira & Barcinski, 2004; Wang et al., 2011).

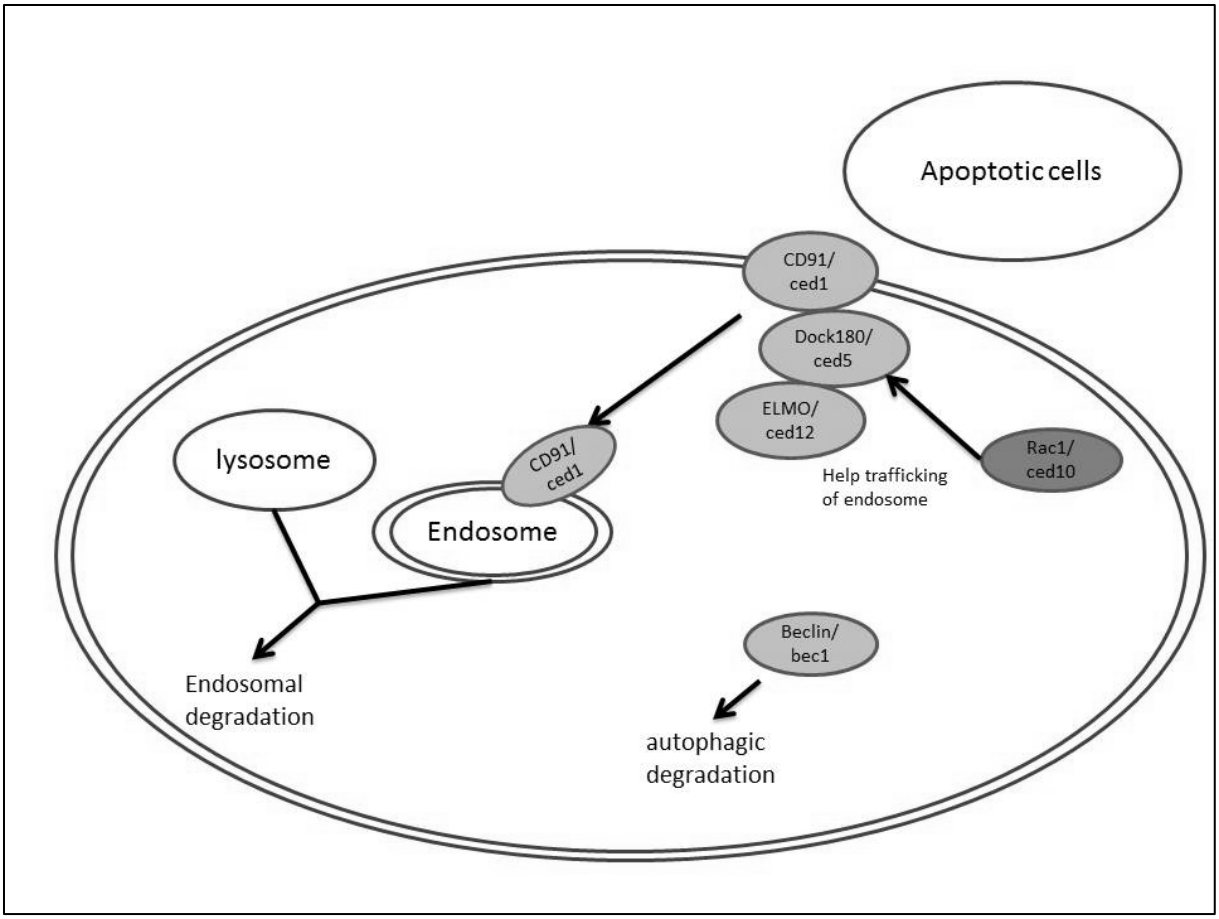

Figure 5. Phagocytic signaling involved in the uptake of apoptotic cells. CD91 receptor can recognize apoptotic cell and send misfolding proteins to endosomatic degradation.

Cinnamon Treatment for Modification of HIF, Apoptotic and Autophagy Signaling

There are several natural compounds containing potential compounds to modulate HIF-1 and apoptotic signaling and regulate cell death and cell survival (Nagle & Zhou, 2006; Xia et al.,2012). Cinnamon is reported to have potent therapeutic properties to decrease tumors because cinnamon is one of spices containing high level of bioactive compounds, such as polyphenols and procyanidins (Gu et al., 2004). Cinnamon extract is

suggested to reduce HIF-1α expression *in vitro* tumor cell culture study (H. K. Kwon et al., 2009). Also cinnamon is reported to inhibit vascular endothelial growth factor (VEGF) activity, thus considered to inhibit angiogenesis and potentially prevent cancer progression. In addition, *in vitro* study shown cinnamon extract to be a potent chemopreventive drug in cervical cancer because cinnamon extract reduced metalloproteinase-2 (MMP-2) activity and decrease mitochondrial membrane potential (J. Lu et al., 2010). However, cinnamon extract is also known to mimic insulin like properties and increase insulin signaling, which result in cell survival (Kirkham et al., 2009). There is little known about the affect cinnamon on hypoxia, apoptosis, phagocytosis and cell cycle regulation *in vivo* model and there are contradictory studies among cinnamon extract, we explored the interaction between cinnamon extract and key molecular.

Methods

Nematode Propagation of HIF-1/Apoptosis/Autophagy Signaling and Cinnamon Treatment

C. elegans with GFP promoter constructs of genes relevant to HIF-1/Apoptosis/Autophagy signaling (Table 5) were obtained from the Caenorhabditis Genetics Center. The effect of cinnamon aqueous treatment on the gene expression of HIF-1/Apoptosis/Autophagy signaling was evaluated using *in vivo C. elegans* model, as previously described in chapter 1.

Table 5. List of *C. elegans* genes in the HIF-1/Apoptosis/Autophagy pathways to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Hypoxia Induced Factor/Apoptosis/Cell Cycle			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
OH7688	***hif-1***	Hypoxia-induced factor 1	WBGene00001851
OH7692	***hif-1***	Hypoxia-induced factor 1	WBGene00001851
ZG494	***egl-9***	Prolyl hydroxylase (PHD)	WBGene00001178
BC16164	***fih***	Factor inhibiting HIF	WBGene00017304
UL1606	***aha-1***	Aryl-hydrocarbon receptor nuclear translocator (ARNT) HIF-1β	WBGene00000095
SD1444	***cyp-25a2***	Cytochrome P450 (CYP3A)	WBGene00007964
BC11941	***egl-1***	Bcl-2 homology region 3 (BH3) mammalian cell death activators	WBGene00001170
BC11395	***bec-1***	Autophagy regulating protein Beclin-1	WBGene00000247
XR-6	***ced-4***	Apoptotic protease activating factor 1	WBGene00000418
WS5770	***ced-4***	Apoptotic protease activating factor 1	WBGene00000418
MD701	***ced-1***	CD91-Low Density Lipoprotein Receptor	WBGene00002989
CU1546	***ced-1***	CD91-Low Density Lipoprotein Receptor	WBGene00000415
NK785	***ced-5***	Protein DOCK180	WBGene00000419
MT10865	***ced-10***	GTPase-Ras-Related C3 Botulinum Toxin substrate (RAC1)	WBGene00000424

Results

Effect of Cinnamon on the HIF-1/Apoptosis/Autophagy Signaling

Expression of first *hif-1* (OH7688), which encodes an ortholog of the mammalian hypoxia-induced factor HIF-1α was significantly higher at 5 mg/ml and 10 mg/ml of cinnamon extract compared to control (Table 6). RF value of *hif-1* at 5 mg/ml cinnamon treatment was 1.43 (*p=0.010*) and at 10 mg/ml it was 1.97 (*p=0.000*). At 1 mg/ml of cinnamon extract, RF value of *hif-1* was significantly lower and it was 0.94 (*p=0.040*). Expression of second *hif-1* strain (OH7692) was significantly upregulated at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment relative to control (Table 6). RF value of *hif-1* at 1 mg/ml cinnamon treatment was 1.23 (*p=0.000*),at 5 mg/ml it was 2.52 (*p=0.000*) and at 10 mg/ml it was 3.82 (*p=0.001*). Expression of *fkh-6* (DZ325), which encodes one of *C. elegans* forkhead transcription factors, was significantly lower than control at all three concentrations of cinnamon extract (Table 6). RF value of *fkh-6* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment was 1.29 (*p=0.001*), 2.64 (*p=0.001*) and 3.22 (*p=0.001*) respectively. *F09F7.7a* (BC16164) encodes hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) which belong to iron (II) and 2-oxoglutarate-dependent dioxygenase family and regulates HIF stability. Expression of *F09F7.7a* was evaluated and it was significantly upregulated at all three concentrations of cinnamon treatment relative to control (Table 6). RF value *F09F7.7a* at 1 mg/ml was 1.29 (*p=0.001*), at 5 mg/ml it was 2.64 (*p=0.000*) and at 10 mg/ml it was 3.22 (*p=0.004*). Expression of *aha-1* (UL1606), which encodes an ortholog of human aryl-hydrocarbon receptor nuclear translocator and form hetero-dimer with HIF-1 and AHR-1 and binds hypoxia response element, was evaluated. Expression of *aha-1* was significantly lower than control at 1 mg/ml and 5

mg/ml cinnamon treatment, but it was significantly higher at 10 mg/ml cinnamon extract (Table 6). At 1 mg/ml and 5 mg/ml cinnamon treatment RF value of *aha-1* was 0.62 (*p=0.010*) and 0.61 (*p=0.001*) respectively and at 10 mg/ml it was and 2.26(*p=0.008*) respectively. Expression of *cyp-25A2* (SD1444), which encodes cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies, was significantly upregulated relative to control at all three concentration of cinnamon treatment (Table 6). One mg/ml cinnamon treatment RF value of *cyp-25A2* was 1.07 (*p=0.044*). At 5 mg/ml and 10 mg/ml cinnamon treatment RF values *cyp-25A2* were 1.69 (*p=0.000*) and 1.92 (*p=0.000*) respectively. Expression of *egl-1* (BC11941), which encodes a protein containing a region similar to the BH3 domain of mammalian cell death activator, was evaluated. Expression of *egl-1* at all three concentrations of cinnamon extract was significantly higher than control (Table 6). RF values of *egl-1* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment were 1.00 (*p=0.042*), 1.62 (*p=0.000*) and 2.36 (*p=0.000*) respectively. Expression of *bec-1* (BC11395), which encodes a coiled-coil protein homolog to the Beclin-1 (human autophagy) protein, was significantly upregulated relative to control at 1 mg/ml and 5 mg/ml of cinnamon extract, but at 10 mg/ml it was not significant (Table 6). RF value of *bec-1* at 1 mg/ml, 5 mg/ml and 10 mg/ml was 1.09 (*p=0.000*), 1.29 (*p=0.000*) and 1.07 (*p=0.225*) respectively. Expression of *egl-9* (ZG494), which encodes dioxygenase and downregulate *hif-1*, was higher than control at all three concentrations of cinnamon extract, but only at 10 mg/ml cinnamon treatment there was significant different (Table 6). RF values of *egl-9* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment were 2.06 (*p=0.000*), 2.95 (*p=0.199*) and 2.76 (*p=0.002*) respectively. Expression of *ced-5* (NK785), which encodes human DOCK180-like

protein and is necessary for engulfing cell, was significantly higher than control at 1 mg/ml cinnamon treatment, but at 10 mg/ml cinnamon treatment it was significantly lower (Table 6). RF values of *ced-5* at 1 mg/ml cinnamon treatment was 1.07 (*p=0.033*) and at 10 mg/ml it was 0.95 (*p=0.044*). There was no significant effect of 5 mg/ml cinnamon treatment on *ced-5* expression. Expression of first *ced-4* strain (XR6), which encodes protein initiating programmed cell death pathway, was significantly higher than control at all three concentration of cinnamon treatment. RF values of *ced-4* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment was 1.37 (*p=0.000*) 2.02 (*p=0.000*) and 2.91 (*p=0.000*) respectively. Similarly, expression of second *ced-4* (WS5770) was higher than control at all three concentrations of cinnamon treatment (Table 6). RF values of *ced-4* at 1 mg/ml, 5 mg/ml and 10 mg/ml were 1.56 (*p=0.000*), 1.52 (*p=0.000*) and 1.71 (*p=0.000*) respectively. Expression of the *ced-10* (MT10865), which is encodes protein homologous to human GTPase RAC1 protein and is functions in the engulfing cell during apoptosis, was evaluated. Expression of the *ced-10* at all three concentrations of cinnamon extract was significantly lower than control (Table 6). RF value of *ced-10* at 1 mg/ml of cinnamon treatment was 0.68 (*p=0.000*), at 5 mg/ml it was 0.76 (*p=0.001*) and at 10 mg/ml it was 0.69 (*p=0.002*). Expression of the first *ced-1* strain (MD701), which encodes transmembrane receptor protein placed on phagocytic cell and is required for engulfment, was significantly upregulated relative to control at all three concentrations of cinnamon extract (Table 6). RF values of *ced-1* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment were 1.23 (*p=0.000*), 2.07 (*p=0.000*) and 1.68 (*p=0.000*) respectively. Similarly expression of the second *ced-1* strain (CU1546) at 5 mg/ml and 10 mg/ml cinnamon treatment was significantly higher than control; however, at 1 mg/ml

cinnamon treatment it was significantly lower than control (Table 6). RF values of *ced-1* at 5 mg/ml and 10 mg/ml cinnamon treatment were 1.24 (*p=0.000*) and 1.68 (*p=0.000*) respectively, and it was 0.86 (*p=0.000*) at 1 mg/ml cinnamon treatment.

Table 6. Dose dependent effect of cinnamon treatment on fold-change in gene expression relative to control. Bold and italic number indicates significantly lower than control. *p<0.05*

GENE	Cinnamon Concentration								
	1 mg/ml			5mg/ml			10mg/ml		
	RF	**SE**	***p value***	RF	**SE**	***p value***	RF	**SE**	***p value***
hif-1	0.936	0.043	***0.040***	1.429	0.158	***0.010***	1.972	0.150	***0.000***
hif-1	1.225	0.044	***0.000***	2.524	0.124	***0.000***	3.820	0.560	***0.001***
F09F7.7.a	1.286	0.079	***0.001***	2.639	0.126	***0.000***	3.215	0.498	***0.004***
aha-1	0.615	0.142	***0.010***	0.609	0.069	***0.000***	2.255	0.417	***0.008***
cyp-25A2	1.066	0.060	***0.044***	1.690	0.064	***0.000***	1.916	0.121	***0.000***
egl-1	1.004	0.037	***0.042***	1.615	0.063	***0.000***	2.364	0.124	***0.000***
bec-1	1.092	0.037	***0.000***	1.290	0.059	***0.000***	1.072	0.162	*0.225*
egl-9	2.063	0.087	*0.226*	2.951	0.278	*0.199*	2.764	0.479	***0.002***
ced-5	1.071	0.106	***0.033***	0.934	0.067	*0.183*	0.945	0.079	***0.044***
ced-4	1.372	0.084	***0.000***	2.019	0.142	***0.000***	2.913	0.244	***0.000***
ced-4	1.556	0.097	***0.000***	1.518	0.095	***0.000***	1.711	0.132	***0.000***
ced-10	0.683	0.088	***0.000***	0.756	0.093	***0.001***	0.688	0.084	***0.002***
ced-1	1.234	0.083	***0.000***	2.073	0.145	***0.000***	1.683	0.119	***0.000***
ced-1	0.855	0.043	***0.000***	1.236	0.049	***0.000***	1.684	0.138	***0.000***

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8.

Discussion

HIF-1/Apoptosis/Autophagy Signaling in Response to Cinnamon

Hypoxia condition is known to be regulated by HIF-1 signaling by activating many HRE dependent transcription and apoptosis and autophagy signaling (Greijer & van der Wall, 2004; Papandreou et al., 2008). Optimal management of oxygen levels at both cellular and organismal level is critical for overall health of the system. Conditions of hyperoxia are managed by Nrf2/ARE (Godman et al., 2010), whereas conditions of hypoxia are now understood to be managed by HIF-1 signaling (Chang & Bargmann, 2008). HIF-1 signaling regulate apoptotic signaling carefully controlling pro-apoptotic and anti-apoptotic proteins transcription, thus balance of activation of these factors are important since unregulated HIF-1 signaling is activated with concerned to contribute to develop tumors (Shen et al., 2005). In our studies we examined the effects of cinnamon on various critical factors involved in HIF, apoptotic and autophagy signaling. In hypoxia condition *C. elegans hif-1*, homolog of human HIF-1α interact with *C. elegans* HIF-1β or ARNT is *aha-1*, and bind to the HRE to increase HRE dependent gene transcription and directly and indirectly control energy metabolism (Zhang et al., 2009). We observed an increase in the expression of *hif-1*/HIF-1α in response to cinnamon treatment (Table 6). On the other hand, HIF-1β levels are not influenced by level of oxygen in the cell (Denison & Nagy, 2003). HIF-1β forms a complex with HIF-1α or AhR(aryl hydrocarbon receptor) to increase various genes expression via HRE and AHRE transcriptions (Denison & Nagy, 2003). The expression of *aha-1* (*C. elegans* HIF-1β) was found to be downregulated at lower concentration but it was significant upregulated at the highest concentration of cinnamon treatment (Table 6). These data suggests

upregulation of downstream gene of HIF-1 signaling. However, in normoxia condition HIF-1 is regulated several proteins. FIH (*fih-1* in *C. elegans*) is one of proteins to inhibit *hif-1*/HIF-1α activity normal oxygen levels by hydroxylated of asparagine residue of the C-terminal activation domain (CAD) of HIF-1α / HIF-1β complex (Lando et al., 2002). Also PHD-1 (*egl-9* in C. elegans) hydroxylate proline residue of HIF-1α (Shao et al., 2009). Hydroxylated HIF-1α bind with von Hippel Lindau E3 ubiquitin ligase (VHL) and targeted for proteasomal degradation, thus inhibiting HRE and AHRE transcription under normoxia condition (H. Zhang et al., 2007). In response to cinnamon extract we observed the expression of FIH was decreased and the expression of PHD-1 was increased (Table 6). The data indicated potential inhibition of HRE /AHRE transcription not by inhibition of HIF-1α / HIF-1β complex by FIH, but by degradation of HIF-1α targeted by PHD-1 proteins. However, we observed increasing of HIF-1α / HIF-1β, thus to confirm if HRE /AHRE transcription is modulated by cinnamon extract, we examined the expression of AHR/ARNT target genes cytochrome P450/CYP3A (*cyp-25a2* in *C. elegans*) (Nebert et al., 2004) in response to cinnamon extract. We observed several fold upregulation of CYP3A in response to cinnamon treatment (Table 6). This suggests that although the expression of HIF-1 degenerative protein is increased overall HIF-1α / HIF-1β expression was exceeded over PHD-1 protein and increased HRE/AHRE signaling in response to cinnamon extract.

Apoptotic pathway of higher animal and *C. elegans* share consisting genes, such as *egl-1*, *ced-9*/Bcl-2, and *ced-4*/Apaf-1 (Hirose et al., 2010). As we mentioned previously in non-autophagy condition, anti-apoptotic protein Bcl-2 bind with Apaf-1 and Beclin-1 and prevent activating apoptotic signaling (Y. Lu et al., 2011; Nehme et al.,

2010). But when BH-3 only protein binds to Bcl-2 or Bcl-2 is phosphorylated, complex of Bcl-2/Apaf-1 will be broken and Apaf-1 will be released (C. E. Johnson et al., 2007). Apaf-1 activate caspase-9, and caspase further activate caspase-3 and -7, thus apoptotic signaling will be increased and cause cell death (C. E. Johnson et al., 2007). We observed the increase of the expression of all three pro-apoptotic proteins *egl-1*/BH3-only protein, and *ced-4*/Apaf-1 in response to cinnamon extract (Table 6). These data may suggest that toxic compounds, in whole cinnamon extract increased apoptotic signaling.

We also wanted to examine the expression of gene related to phagocytosis in response to cinnamon treatment to evaluate clearance of apoptotic cells. Therefore, we looked at the expression of genes related to autophagy, such as *bec-1, ced-1, ced-5* and *ced-10* in *C. elegnas.*Bcl-2 homolog *ced-9* is also reported to binds with beclin-1 and inhibit autophagy (Spencer et al., 2009; Wirawan et al., 2010). It is reported that increase of Beclin-1 decreased extracellular synaptic protein α-synuclein and decreased development of PD *in vitro* (Spencer et al., 2009). Thus, our result may indicate possible beneficial effect of cinnamon extract on neurodegenerative disease. In addition, it is reported the transmembrane low density lipoprotein receptor molecule CD91 (*ced-1* in *C. elegans*) binds and recognizes the extracellular apoptotic cell proteins and engulf apoptotic cells (Moreira & Barcinski, 2004; Wang et al., 2011). *Ced-1*/CD91 expression in response to cinnamon treatment was upregulated (Table 6), suggesting possible clearance of apoptotic cell. Also in *C. elegans ced-5*/Dock180, and *ced-12*/ELMO is shown to increase increasing the efficiency of phagocytosis (Sun et al., 2012). RhoG protein activate c*ed-5* and *ced-12* and make complex then endocytic trafficking of *ced-10* (*C. elegans* Rac1) is promoted (Moreira & Barcinski, 2004). We observed that the expression of

both *ced-5*/Dock180 and *ced-10*/Rac-1 were decreased in response to cinnamon extract (Table 6). Our result of autophagy genes indicated increase in *bec-1*, which increase autophagy gene transcription and in *ced-1*, which utilize nutrient uptake and reduce oxidative LDL, but decreased in other apoptotic cell clearance gene *ced-5*/Dock180 and *ced-10*/Rac-1.

Conclusion

The overall effects of cinnamon on four important signaling pathways HIF-1, apoptosis, phagocytosis, and autophagy regulation were evaluated. Our result indicates that there was a marginal increase in HIF-1 signaling in response to cinnamon treatment. However, the result of increase in gene expression of apoptotic and decrease in gene expression of autophagy was inconsistent. Whole cinnamon extract containing various compounds, thus it is reported that cinnamon is involving in inhibition of tumor and cancer and cinnamon mimic insulin signaling and increase cell survival. This may indicate importance of fractionation of whole cinnamon extract. Clearance of apoptotic cell is important to reduced misfolded and unfolded protein in extracellular condition to reduce neurotoxicity. One of fractions may contain more anti-apoptotic and more autophagy protein and have beneficial effect on neurodegenerative diseases. Suggesting further investigation of cinnamon treatment on gene related to autophagy.

CHAPTER IV

TREATMENT WITH CINNAMON ON THE HSP AND UPR PATHWAYS

Introduction

Hypoxia Signaling Pathway and Unfolded Protein Response Signaling

Whenever translation takes place in ribosome, polypeptides, which are also called unfolded protein, are synthesis, then heat shock proteins (HSPs), such as HSP70 and HSP90 binds to unfolded proteins and facilitate unfolded proteins to proper folding in cytoplasm (Pratt & Morishima, 2010). However, not all proteins are folded properly, and high accumulations of unfolded or misfolded is reported to cause intracellular toxicity and contribute to cell death (Jäger et al., 2012; Loewen & Feany, 2010). These misfolded or unfolded proteins trapped in cytoplasm or endoplasmic reticulum (ER) and induce the expression of stress response protein, such as HSPs, IRE, PERK, and ATF-6 (Badiola et al., 2011; Gorman et al., 2012).

There are four main canonical response pathways to manage unfolded proteins via UPR signaling and HSP. PKR-related ER kinase (PERK) is one of receptors to detect ER stress at the ER lumen, which normally bind with BIP (GRP78) (Samali et al., 2010). When unfolded protein binds to BIP and dissociate BIP/PERK complex, PERK become active and phosphorylate eukaryotic initiation factor 2 initiation factor 2α (eIF2) (Samali et al., 2010). Phosphorylated eIF2α inhibit Cap-dependent translation and ATF4

transcriptional factor, thus ATF4 bind to promoter region site and increase gene associated to amino acid transport, chaperones (GRP78, GRP94), redox reaction, stress response proteins and CHOP (Harding et al., 2000; Yoshizawa & Hinoi, 2009). CHOP directly downregulates expression of Bcl-2 protein, thus increase beclin-1 dependent autophagy (Szegezdi et al., 2009). Also, PERK is known to activate Nrf2 and its target genes, such as GST, GSH and HO-1, thus activation of UPR signaling also involved in managing cellular redox condition (Szegezdi et al., 2009).

Activating transcription factor-6 (ATF-6) presents on ER membrane is also activated by dissociation of BIP by unfolded protein, and activated ATF-6 is reported to cause ATF-6 translocation to Golgi and become active transcriptional factor by cleavage (Y. Sato et al., 2011). Cleaved ATF-6 increase transcription of various genes, such as, CHOP, chaperones (GRP78, GRP94) and XBP-1u mRNA, thus reduce ER (Okada et al., 2002).

Inositol-requiring protein-1 (IRE-1) present on the membrane of ER, thus it can detect accumulation unfolded proteins in site of IRE-1 ER lumen region (Back et al., 2005). By binding of unfolded protein to BIP in the ER lumen, BIP become dissociated and RNase of IRE-1 at cytosolic region is activated then splice XBP1u mRNA to produce XBP1s mRNA (Deegan et al., 2012). XBP1s mRNA is translated and makes XBP1s protein, then XBP1s, as a transcriptional factor, translocates to the nucleus and bind to promoter region, thus activate numbers of gene transcripation such as BiP, PDI, HSP40/70, $p58^{IPK}$ protein disulfide isomerase (PDI), and 4-prorin hydrosyrase to manage for proper folding (Gupta et al., 2010; Yaohua Liu et al., 2012). Also, activation of IRE-1 recruits tumor necrosis factor-a (TNF-α) receptor-associated factor 2 (TRAF-2) and

makes a complex, which then recruits apoptosis-signal regulating kinase 1 (ASK-1) (Deegan et al., 2012). IRE-1/TRAF-2/ASK-1 complex phosphorylate Bcl-2 and p38 MAPK, and phosphorylated Bcl-2 is dissociated from Beclin, thus increase autophagy (Deegan et al., 2012). In addition, XBP-1s is reported to increase FoxO1 expression individually, thus involved in neuronal cell survival, autophagy and gluconeogenesis (Deegan et al., 2012; Sha et al., 2011).

As it is described, UPR signaling is essential to decrease misfolded and unfolded proteins. When UPR signaling is dysregulated, misfolded or unfolded protein accumulates and increase toxicity and activates apoptosis signaling (Fribley & Miller, 2011). *C. elegans* gene *abu-1* is reported to manage ER stress when regular UPR signaling is blocked, which is called noncanonical UPR pathway (Urano et al., 2002). Thus, we decided to evaluate the expression of UPR gene as well as *abu-1* as to check whether canonical UPR signaling is blocked or not.

As well as UPR signaling HSPs also manage misfolded or unfolded protein induced stress. HSP70 and HSP90 are normally bound to heat shock factor-1 (HSF-1) and stay inactive, but when ER stress increase HSP70/HSP90 are dissociated from HSF-1 and immediately bind to unfolded proteins to decrease ER stress (Wieten & Zee, 2010). Also dissociated HSF-1 become transcriptional factor and translocate to the nuclear (Hayashida et al., 2011). HSF-1 binds to promoter regions of various genes to manage unfolded and misfolded protein further facilitate HSP transcription (Hayashida et al., 2011). Also, HSF-1 can be regulated by several growth signaling, such as insulin like growth factor signaling (IGF), JNK/MAPK signaling (Prahlad & Morimoto, 2009). In *C. elegans* HSF-1 monomers were phosphorylated via stress induced hyperphosphorylation,

IGF or JNK/MAPK signaling and are trimerized (Prahlad & Morimoto, 2009). Trimerized phosphorylated HSF-1 become transcriptional, which bind to heat shock element site (HSE) factor and increase further HSP transcription (Prahlad & Morimoto, 2009).

One of the sources to increase unfolded protein comes from apoptosis cell. When scavenger receptor bind to apoptotic cell, it become transcription factor which get into nucleus and increase expression of UPR signaling (Devries-Seimon et al., 2005). In *C. elegans* it is reported that receptor mediated endocytosis 8 (*rme-8*) facilitate this process by trafficking early endosomes with *ced-1* (CD91 low density lipoprotein receptor) (Shi et al., 2009). When *rme-8* are increased on cell membrane, cell engulfs apoptotic cell and produce endosome and endosome eventually fuse to lysosome for degradation (Shi et al., 2009). Dynamin, which is GTPase scavenger receptor, helps engulfment activity of *rme-8* and produce endosome containing numbers of misfolded proteins (Shi et al., 2009). *Rme-8* is also required for activation HSP70 and hydrate ATP and remove inhibitory protein of HSP70 and decrease cytosol unfolded protein induced stress and ER stress (Girard & McPherson, 2008; Meimaridou et al., 2009).

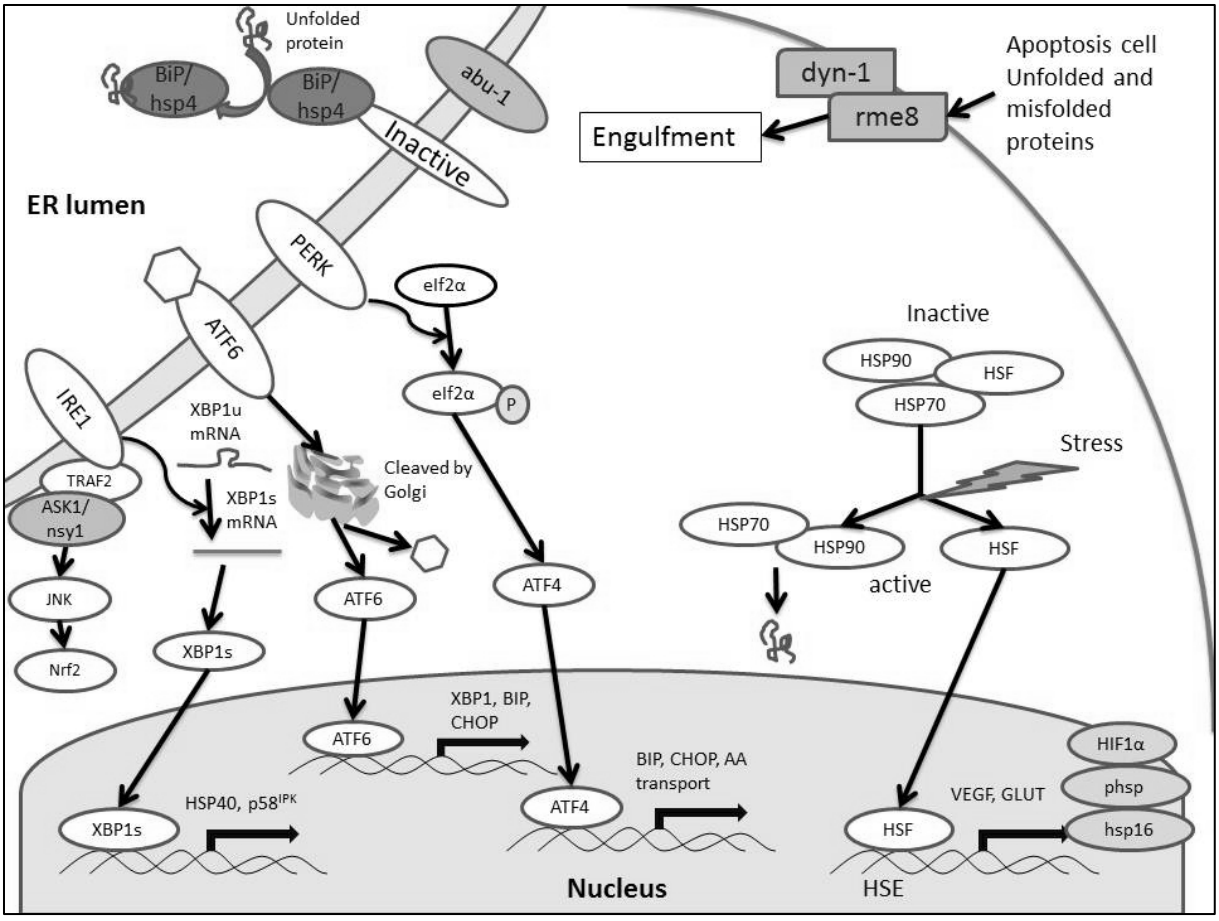

Figure 6. The heat shock protein response (Hsp) and the unfolded protein response (UPR) signaling model. Accumulation of unfolded protein upregulates HSP/UPR signaling and activation of HSP/UPR signaling lead to increase HSPs.

HSP/UPR Link to Neurodegeneration

UPR signaling is known to play important roles in neuronal protection from unfolded protein induced neurotoxicity, thus dysfunction of UPR is known to be involved in clinical neuronal diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), (H. Gao & Hong, 2008). Accumulation of Amyloid $\beta_{1\text{-}42}$ is known to be involved in progression of AD, and it has been reported that lowering of HSF-1 levels in AD brain (Magrané et al., 2005). Lowering *C. elegans* HSP *hsp16.2* have been shown to be associated with formation of $A\beta_{1\text{-}42}$ peptide aggregator plaques (Fonte et al., 2008). Thus,

the mechanism is not clear, but it is suggested that dysregulation of HSP is involved in progression of AD like symptom (Fonte et al., 2008; Kirbach & Golenhofen, 2011). Also, Hsp70 activity in PD patient neuronal cell is reported to involved in reducing α-synuclein misfolding and prevent the formation of neurotoxic neurofribrillary tangles known as Lewy bodies (Arawaka et al., 2010; Malkus et al., 2009). Thus, HSP/UPR regulation is interested in drug target for neurodegenerative disease therapy.

Cinnamon on Neurodegenerative Conditions/Protein Homeostasis

As previously described cinnamon contains rich bioactive compounds, such as polyphenols and procyanidins, thus it is interested in therapeutic research for neurodegenerative diseases (Gu et al., 2004). It is reported that cinnamaldehyde increases insulin signaling and suppress NF-κB mediated inflammation by activating oxidative stress response proteins via Nrf-2 activation (B.-C. Liao et al., 2008), thus it is indicated that cinnamon is involved in UPR signaling. Also, recent studies indicated cinnamon extract may decrease neurotoxicity by reducing misfolded α-synuclein by stabilizing the soluble oligomeric phase *in vivo* in fly model (Shaltiel-Karyo et al., 2012). Also, UPR signaling plays essential role to manage misfolded protein, but aggregation of misfolded α-synuclein is known to involve PD progression, and cinnamon extract is known to reduce aggregation (Shaltiel-Karyo et al., 2012).In addition, cinnamon extract is suggested to reduce HIF-1α expression and inhibit tumor growth *in vitro* tumor cell culture study (H. K. Kwon et al., 2009). Provided these evidences suggested the possible like between HSP/UPR signaling and cinnamon, but *in vivo* research of cinnamon extract on HSP/UPR signaling is not well known. Thus, we investigated the effect of cinnamon extract on HSP/UPR signaling gene using *in vivo C. elegans* model.

Methods

Nematode Propagation of Hsp/UPR pathway and Cinnamon Treatment

C. elegans with GFP promoter constructs of genes relevant to Hsp/UPR pathways (Table 7) obtained from the Caenorhabditis Genetics Center were used to study the effect of cinnamon on the expression and activity of genes regulated by Hsp/UPR pathways. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 7. List of *C. elegans* genes in the Hsp/UPR pathway to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Heat Shock Protein/ Unfolded Protein Response			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
CL2070	***hsp16.2 D***	16-kD heat shock protein; αB crystalline driven (D)	WBGene00002016
GS1826	***phsp***	Driven by heat shock protein-16.2,and heat shock protein-16.41; αB crystalline	WBGene00002016; WBGene00002018
SJ6	***hsp-4***	Immunoglobulin binding (Bip)	WBGene00002008
BC13292	***dyn-1***	Dynamin GTPase	WBGene00001130
SJ4063	***abu-1***	Activated in Blocked Unfolded Protein Response	WBGene00000024
DH1336	***rme-8***	Receptor Mediated Endocytosis	WBGene00004378

Results

Effect of Cinnamon on HSP/UPR Signaling

Effect of cinnamon extracts on the expression of *hsp16.2* (NL3401), which encodes HSP16 family heat shock protein and interact with beta amyloid peptide, was evaluated. Expression of *hsp16.2* was significantly lower than control at 1 mg/ml cinnamon extract, but at 5 mg/ml and 10 mg/ml it was significantly higher than control (Table 8). RE values of *hsp16.2* at 1 mg/ml cinnamon treatment was 0.43 (*p=0.000*) and at 5 mg/ml and 10 mg/ml cinnamon treatment it was 1.23 (*p=0.004*) and 1.22 (*p=0.003*) respectively. Effect of cinnamon extract on the expression of *hsp-4* (SJ6), which encodes heat shock protein homologs of the mammalian ER chaperone GRP78/BiP and ER stress reporter, was evaluated. Expression of *hsp-4* (SJ6) was significantly downregulated than control at all three concentration of cinnamon extract (Table 8). RF value of *hsp-4* at 1 mg/ml of cinnamon treatment was 0.76 (*p=0.000*) and it was 0.89 (*p=0.008*) at 5 mg/ml and it was 0.89 (*p=0.008*) at 10 mg/ml. Expression of *phsp* (GS1826), which encodes heat shock proteins driven by *hsp-16.2* and *hsp-16.41* and is secreted in body cavity and coelomocytes, was significantly higher than control at all three concentration (Table 8). RF values of *phsp* at 1 mg/ml, 5 mg/ml 10 mg/ml cinnamon treatment were 2.34 (*p=0.000*), 3.42 (*p=0.000*) and 4.05 (*p=0.000*) respectively. Expression of *dyn-1* (BC13292), which encodes GTPase orthologous to mammalian dynamin and is regulated CED-1 expression, was downregulated at all three concentration, but there was no significant difference to control (Table 8). Expression of *abu-1* (SJ4063), which encodes a transmembrane protein in endoplasmic reticulum and is expressed by blockage of the unfolded protein response, was significantly lower than control at all three concentrations

of cinnamon treatment (Table 8). RF values of *abu-1* at 1 mg/ml, 5 mg/ml and 10 mg/ml were 0.62 (*p=0.000*), 0.59 (*p=0.000*) and 0.53 (*p=0.000*) respectively. Expression of *rme-8* (DH1336), which encodes J-domain protein in coelomocytes and other cells and is required for endocytosis, was significantly upregulated relative to control at all three concentration (Table 8). At 1 mg/ml cinnamon treatment RF value of *rme-8* was 1.14 (*p=0.000*), at 5 mg/ml it was 1.50 (*p=0.000*) and at 10 mg/ml it was 2.01 (*p=0.000*).

Table 8. Dose dependent effect of cinnamon treatments on fold-change in gene expression relative to control.

GENE	Cinnamon Concentration								
	1 mg/ml			5mg/ml			10mg/ml		
	RF	**SE**	***p value***	RF	**SE**	***p value***	RF	**SE**	***p value***
hsp-16.2	0.426	0.034	***0.000***	1.230	0.042	***0.004***	1.216	0.081	***0.000***
phsp	2.339	0.047	***0.000***	3.417	0.111	***0.000***	4.048	0.171	***0.000***
hsp-4	0.763	0.049	***0.000***	0.891	0.076	***0.008***	0.920	0.071	***0.029***
dyn-1	0.863	0.141	*0.252*	0.828	0.141	*0.164*	0.893	0.146	*0.317*
abu-1	0.618	0.031	***0.000***	0.591	0.055	***0.000***	0.526	0.034	***0.000***
rme-8	1.143	0.041	***0.000***	1.495	0.052	***0.000***	2.006	0.082	***0.000***

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8.

Discussion

HSP/UPR Signaling in Response to Cinnamon

It is reported *C. elegans* heat shock protein *hsp16.2* may have protective effect against neurotoxicity induced by metal exposure on neuronal condition *in vivo C. elegans* study (D. Wang & Xing, 2009). Also, overexpression of *hsp16.2* is reported to suppress neurotoxicity induced by amyloid-β aggregation in a *C. elegans* AD model (Fonte, Kipp, Yerg, et al., 2008). In response to cinnamon extract, expression of *C. elegans* HSP family *hsp16.2* was reduced in low concentration, but at higher concentration the expression was

significantly increased (Table 8). We also evaluated *phsp*, which is a gene share promoter region with *hsp16.2* to confirm if *hsp16.2* is really changed in response to cinnamon extract. We observed several fold increase of *phsp* (Table 8), which also suggested increase of expression of *C. elegans* heat shock protein *hsp16.2* in response to cinnamon treatment.

Also, we evaluated the expression of *C. elegans hsp-4,* which is homolog of human UPR signaling related protein glucose-regulated protein 78 (GRP78, also called BIP). BIP is reported to be regulated by IRE1, PERK and ATF6 signaling via upregulation of transcriptional factors XBP1s, ATF6 and ATF4 when unfolded protein bind to BIP and dissociated from these receptors (Back et al., 2005; Badiola et al., 2011; Kishi et al., 2010). In response to cinnamon treatment we observed reduction of gene the expression of *hsp-4* (Table 8). At low concentration of cinnamon the gene expression of *hsp-4* was more suppressed than higher concentration of cinnamon, which suggesting BIP level are changing in response to cinnamon treatment dosage.

Another *C. elegans* gene *abu-1* is similar to mammalian scavenger receptor, which is present on ER membrane and response to ER stress to manage unfolded protein when regular UPR signaling is blocked (Urano et al., 2002). We observed reduced expression of *abu-1* in response to cinnamon extract (Table 8); suggesting UPR signaling was not blocked in response to cinnamon extract.

Also, to evaluate other protein to increase engulfment of apoptotic cell and response to ER stress we have evaluated the expression of *C. elegans* dynamin homolog *dyn-1* and *C. elegans* receptor mediated endocytosis (RME) gene *rme-8* in response to

cinnamon extract. RME protein is reported to facilitate engulfment of apoptotic cell and help trafficking early endosome process (Shi et al., 2009). When RME recognized apoptotic cell protein or unfolded protein, with help of GTPase protein dynamin REM can engulf extracellular apoptotic proteins or unfolded proteins and transport to ER. Also, RME can help HSP activity to reduce ER stress (Girard & McPherson, 2008; Meimaridou et al., 2009). We observed increased expression of *rme-8*, but there was no significant change in expression of *dyn-1* in response to cinnamon extract (Table 8). Our observation of increase of HSP and RME protein in response to cinnamon treatment may indicate activation of HSP via RME activity.

Conclusion

Heat shock protein and UPR signaling play important roles in managing of ER stress and unfolded protein induce intrinsic toxicity, thus it regulation of HSP and UPR signaling is suggested to involved in neurodegenerative diseases, such as AD and PD (Fonte, Kipp, III, et al., 2008; Wieten & Zee, 2010). Thus, we evaluated the expression of gene related to HSP and UPR signaling in *C. elegans* in response to cinnamon extract. We observed increase of *hsp16.2*, *phsp*, and *rme-8* gene expression, suggesting cinnamon treatment provides improved handling of intracellular unfolded protein via HSP and autophagy, but interestingly we observed decrease of UPR signaling gene *hsp-4* expression in response to cinnamon extract. Also, *abu-1* is known to upregulated when UPR signaling is blocked (Urano et al., 2002) and we also observed decrease of gene expression of *abu-1*, suggesting UPR signaling was not blocked and there was no ER stress increase in response to cinnamon extract.

It is considered that there are two ways to increase HSP expression. One is unfolded or misfolded protein induced stress and the other is phosphorylation of HSF-1 phosphorylation via IGF signaling and MAPK. In *C. elegans* it is reported that phosphorylated HSF-1 make trimerized complex which bind to heat shock element site (HSE) and increase transcription of HSP (Prahlad & Morimoto, 2009). Because we did not see increase of ER in expression of *hsp-4*, our upregulation of HSPs may indicated to be activated by these phosphorylation of HSF-1. Decrease in *hsp-4*, suggests that there is low level of ER stress, and downregulation of *abu-1* suggests that the canonical UPR signaling is properly functioning in *C. elegans*, because we know *abu-1* become active only when there is some type of trouble in UPR signaling mediated IRE1, ATF6 and PERK. Upregulation in *rme-8* along with previously reported increase in expressoin of *ced-1* gene suggests that upon treatment of cinnamon the number of receptors on the cell surface required for recognition of unfolded, misfolded proteins or apoptotic cell is increased. Thus, there is neurodegenerative condition these receptors actively bind to misfolded, unfolded or apoptotic cells and quickly remove it. Therefore, it could indicate potential preparedness for misfolded, unfolded protein induced toxicity.

In conclusion our data suggests cinnamon's insulin like function increases insulin signaling and activates HSP expression via phosphorylation of HSF-1. And treatment of cinnamon results in enhances ability of the cell to handle misfolded, unfolded or apoptotic cell, for removing aggregated protein toxicity and recycling. These two mechanisms could potentially indicate neuroprotective effect of cinnamon.

CHAPTER V
THE *IN VIVO* EFFECT OF CINNAMON EXTRACT ON MAPK SIGNALING

Introduction

Mitogen activated kinase (MAPK) is known to be involved in many cell functions, such as cell growth, differentiation, development, inflammation, immunity and apoptosis, thus MAPK signaling responses to oxidative stress, metal stress and microbial infection (Cargnello & Roux, 2011; Junttila et al., 2008; Kim & Choi, 2010). There are four different MAPK signaling (Erk, JNK, p38 and Erk4) and these pathways are activated by various factors, such as growth factors, cytokines, environmental stresses, osmotic stress, death signaling molecule, U.V. and oxidative stress (Cuadrado & Nebreda, 2010; Hsieh et al., 2010; G. Huang et al., 2009). Since MAPK signaling is involved in many cellular functions, dysregulation of this signaling is reported to be associated with cancer, immune disease and neurodegenerative diseases (Carracedo & Ma, 2008; Kim & Choi, 2010; Moyes et al., 2010). The complete mechanism of MAPK signaling remains unclear because of complex network with four signaling and other growth factor signaling (Yu et al., 2012); therefore, there are various researches of this signaling for therapeutic aims.

Regulation and Dysregulation of the MAPK Cascade

As described before many MAPK signaling has been identified in eukaryote and higher animal as well. Even though the diversity of this pathway is different among

different species, the fundamental function of this pathway is highly conserved. In *C. elegans* p38 MAPK signaling activation is initiated by toll-like receptor (TIR) containing protein *tir-1*. *Tir-1* is reported to regulate both innate immunity, neuronal differentiation and injury (Hayakawa et al., 2011; Jenkins & Mansell, 2010). *C. elegans tir-1* is homologous to human SARM, which is involved TLR signaling, but since TLR signaling and MAPK signaling shares their pathways, in *C. elegans* it is works at upstream activator of p38 MAPK signaling pathway (Silverman et al., 2009). *Tir-1* phosphorylates and activates *C. elegans* MAP3K (*nsy-1*), which is homolog of human ASK-1 and also reported to activate JNK and involved in apoptotic signaling (G. Huang et al., 2009). Then, *nsy-1* further phosphorylate and activates MAP2K (*sek-1*), which then phosphorylate and activate p38 MAPK (*pmk-1*) (Hayakawa et al., 2011). Phosphorylated *pmk-1* translocates to the nucleus and activate transcription factor, which then initiates many gene transcriptions, such as and neuropeptide-like peptide *nlp-29* and antimicrobial peptide *amp* (Ziegler et al., 2009). *Pmk-1* is also involved in inhibition of GSK-3 independently with PI_3K/AKT signaling (Thornton & Pedraza-Alva, 2008). Active GSK-3 in axon decreases their ability to stabilize microtubules and it is reported to cause apoptosis, thus it is considered p38 MAPK pathway plays important role in protection of axon (Thornton & Pedraza-Alva, 2008). *Pmk-1*can be dephosphorylated by phosphatase PP2A *let-92*, thus expression of *pmk-1* and *let-92* play important role in regulation of cell survival (Junttila et al., 2008).

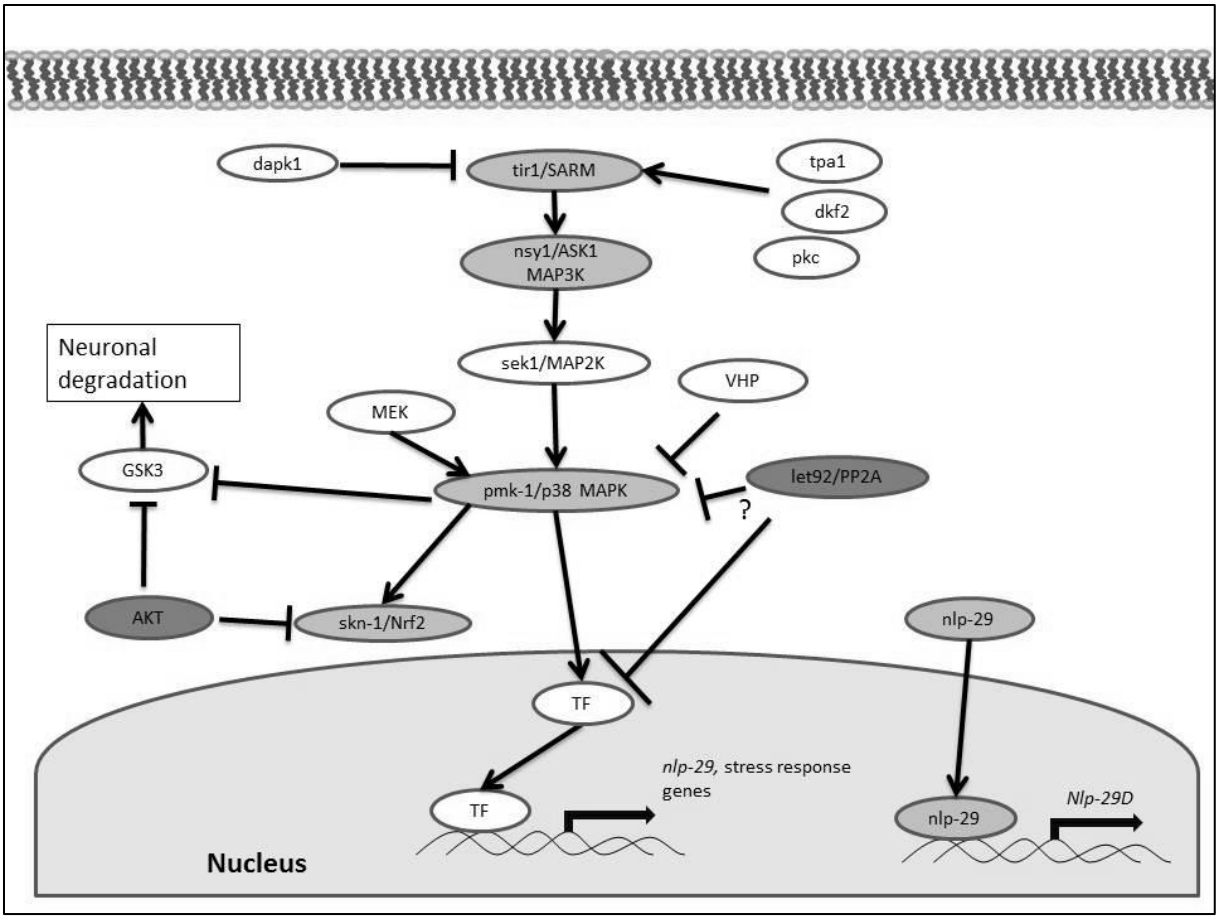

Figure 7. p38 mitogen activated kinase (MAPK) signaling model. p38 MAPK signaling response to cell stress and microbial infection.

Neurodegenerative Disease and p38 MAPK Signaling

Recent study reported that dysfunction of p38 or JNK of MAPK signaling is involved in development of Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) due to its effects on development and apoptosis (Kim & Choi, 2010). It is reported that overexpression of p38 in microglia, astrocyte and neuron may increase inflammation, excitotoxicity and synaptic plasticity via upregulation of TNF-α, IL-1β and nitric oxide (Munoz & Ammit, 2010). Also, accumulation of amyloid β plaques and overexpression of p38 were reported to involve tau

phosphorylation, thus it is suggested that p38 MAPK signaling dysfunction may contribute to PD (Munoz & Ammit, 2010). Also, activation of p38 MAPK signaling and inflammation via NF-κB was observed in MPTP treated mice brain, and it is reported upregulation of JNK mediated by ASK1 and MAPK increase neuronal cell death, thus p38 MAPK is considered to play important role in PD. Also, p38 can phosphorylate GSK-3 along with PI_3K/AKT, p38 MAPK is shown to involved in axonal regeneration (Thornton & Pedraza-Alva, 2008). As described activity of p38 MAPK regulate many cellular function, however during several pathology p38 MAPK is shown to be hyperactive, which have results in progression of neurodegenerative disease therefore proper regulation of MAPK signaling may become therapeutic target of neurodegenerative disease.

Cinnamon and p38 MAPK Signaling

P38 MAPK signaling is known to be involved in memory function because p38 indirectly increase expression of eEF2, which plays important role in developing synapses (Roux & Blenis, 2004). Recent study shown p38 MAPK signaling regulate inflammation in the accessory nerve, such as glial cell and astrocyte, which are important cell to stabilize proper neuron condition (Xie, Li, et al., 2010). Thus, modulating of p38 MAPK signaling of these cells plays important role in prevention and inhibition of progression of neurodegenerative disease (Xie, Li, et al., 2010). Cinnamon is reported to inhibit IFN-γ expression in activated T cells via inhibition of p38, JNK, ERK1/2, and STAT4 signaling and increases inflammation (Lee et al. 2011). Also, polyphenols of cinnamon is reported to be involved in proliferation of cell by regulating cell cycle via p38 MAPK signaling (Schoene et al., 2009). In addition cinnamon is reported to be

involved with inhibition of inflammation, thus immunomodulatory action of cinnamon bark of interests for neurodegenerative disease treatment. Because it is known that stress response signaling is involved in neurodegenerative disease, especially recent report of p38 MAPK signaling involvement in development of neurodegenerative disease (Munoz & Ammit, 2010), we have investigated the effect of cinnamon extract on modulation of p38 MAPK signaling using *in vivo C. elegans* model.

Methods

Nematode Propagation of MAPK pathway and Cinnamon Treatment

C. elegans with GFP promoter constructs of genes relevant to MAPK signaling pathway (Table 9) obtained from the *Caenorhabditis* Genetics Center were used to study the effect of cinnamon on the expression and activity of genes regulated by the MAPK system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 9. **List of C. elegans genes in the MAPK pathway to be evaluated by strain, and their human equivalents.** Information obtained from www.wormbase.org.

Mitogen Activated Protein Kinase Signaling Cascade			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
BC11466	***tir-1***	Toll-interleukin 1 receptor domain-containing protein SARM	WBGene00006575
BC10545	***nsy-1***	(MAP3K) Apoptosis signal-regulating kinase (ASK)	WBGene00003822
DA1750	***pmk-1***	(MAPK) Human p38 mitogen-activated protein kinase	WBGene00004055
IG692	***nlp-29***	Antimicrobial, neuropeptide-like protein	WBGene00003767
IG274	***nlp-29 D***	Antimicrobial, neuropeptide-like protein driven (D)	WBGene00003767

Results

Effect of Cinnamon on the p38 MAPK Signaling

Effect of cinnamon extracts on expression of *tir-1* (BC11466), which encodes the *C. elegans* ortholog of the human Toll/interleukin-1 receptor (TIR) domain-containing protein SARM and functions innate immunity and proper localization of NSY-1/MAPK to post-synaptic regions, was evaluated. Expression of *tir-1* was significantly lower than control at 1 mg/ml cinnamon treatment, but it was significantly higher at 10 mg/ml (Table 10). RF value of *tir-1* at 1 mg/ml cinnamon treatment was 0.60 (*p=0.000*) and at 10 mg/ml it was 1.11 (*p=0.015*). There was no significant effect of cinnamon extract on expression of *tir-1* at 5 mg/ml. Expression of *nsy-1* (BC10545), which encodes a homolog of the human MAP3K ASK1, was significantly downregulated at all three concentration relative to control (Table 10). RF values of *nsy-1* at 1 mg/ml, 5 mg/ml and 10 mg/ml cinnamon treatment were 0.50 (*p=0.015*), 0.66 (*p=0.049*), and 0.62 (*p=0.048*) respectively. Expression of *pmk-1* (DA1750), which encodes mitogen-activated protein kinase (MAPK) ortholog to human p38 MAPK and is required for program cell death was evaluated. Expression of *pmk-1* was significantly higher than control at 5 mg/ml and 10 mg/ml of cinnamon extract (Table 10). RF value of *pmk-1* at 5 mg/ml of cinnamon treatment was 1.58 (*p=0.001*) and it was 1.65 (*p=0.007*) at 10 mg/ml cinnamon treatment. At 1 mg/ml of cinnamon treatment expression of *pmk-1* was not significantly different from control. Effect of cinnamon extracts on expression of *Pnlp-29* (IG692), which encodes *nlp-29* promoter-*driven* antimicrobial proteins and is expressed by bacterial and fungal infection, was evaluated. Expression of *Pnlp-29* was significantly lower than control at 5 mg/ml and 10 mg/ml cinnamon treatment (Table 10). RF values of *Pnlp-29* at

5 mg/ml and 10 mg/ml were 0.40 (*p=0.002*) and 0.47 (*p=0.048*) respectively. There was no significant difference compared to control at 1 mg/ml cinnamon treatment. Expression of *Pnlp-29* (IG274) was significantly downregulated relative to control at all three concentrations of cinnamon treatment (Table 10). At 1 mg/ml cinnamon treatment RF value of *Pnlp-29* was 0.19 (*p=0.002*), at 5 mg/ml it was 0.30 (*p=0.004*) and at 10 mg/ml it was 0.42 (*p=0.009*).

Table 10. Dose dependent effect of cinnamon treatments on fold-change in gene expression relative to control. Bold and italic number indicates significantly lower than control. $p<0.05$

GENE	**Cinnamon Concentration**								
	1 mg/ml			**5mg/ml**			**10mg/ml**		
	RF	**SE**	***p value***	RF	**SE**	***p value***	RF	**SE**	***p value***
tir-1	0.603	0.053	***0.000***	0.906	0.134	*0.340*	1.112	0.197	***0.015***
nsy-1	0.499	0.047	***0.015***	0.656	0.113	***0.049***	0.632	0.095	***0.048***
pmk-1	1.146	0.134	*0.144*	1.579	0.126	***0.001***	1.646	0.208	***0.007***
nlp-29	0.411	0.244	*0.127*	0.401	0.115	***0.002***	0.470	0.169	***0.048***
nlp-29D	0.192	0.063	**0.002**	0.304	0.102	**0.004**	0.418	0.122	**0.009**

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8.

Discussion

MAPK Signaling in Response to Cinamon

The regulation of p38 MAPK signaling is considered to play important roles in neurodegenerative disease development since MAPK signaling responses to various stresses and regulates inflammation, apoptotic, cell growth, cell survival and microbial infection in neuronal tissue (Cargnello & Roux, 2011; Junttila et al., 2008; Kim & Choi, 2010). The mechanism remains unclear, but it is suggested in *C. elegans* p38 MAPK

signaling *tir-1* activation initiate the signaling cascade and control innate immunity and neuronal differentiation (Hayakawa et al., 2011; Jenkins & Mansell, 2010).

We observed decrease of expression of *tir-1* at low concentration, but observed increase of expression at high concentration of cinnamon extract (Table 10). It is reported that multiple proteins, such as tpa-1, dkf-2, pkc-3, and dapk-1, are involved in regulation of *tir-1* (Irazoqui et al., 2010). Our result shows change in expression of *tir-1* with dose dependent manner in response to cinnamon extract. Thus our data may suggest cinnamon extract had effect on expression of *tir-1*, but at low concentration and high concentration cinnamon extract may act on different protein involved in *tir-1* expression.

Downstream of *tir-1*, we observed downregulation of *nsy-1*/ASK-1; however, interestingly we observed upregulation of *pmk-1*/p38 in response to cinnamon extract (Table 10). In previous chapter, we observed ER stress was not increased in response to cinnamon extract, and it is known that *nsy-1*/ASK-1 is also activated via upregulation of IRE-1/TRAF-2 activity induced by ES stress. This data may indicate the expression of *nsy-1*/ASK-1 was not increased even in response to upregulation of *tir-1* because of low ER stress. ASK-1 can increase inflammation and autophagy via activating JNK and Bcl-2 proteins, it is also suggested cinnamon treatment may not increase inflammation via *nsy-1*/ASK-1 activity.

Even though we did not see increase of *nsy-1*/ASK-1, the expression of *pmk-1* p38 MAPK homolog was increased in response to cinnamon extract (Table 10). Activation of *pmk-1* is regulated by *tir-1* and *sek-1*, but expression of *pmk-1* is regulated by ROS, DNA damage and osmotic stress (Thornton & Rincon, 2009). In previous

chapter we observed increase of insulin signaling, but interestingly observed increase of *skn-1* dependent gene transcription (Table 2 & 4). Thus our data may explain upregulation of these gene transcriptions came from overall increase of p38 MAPK signaling.

We observed increase of the expression of *pmk-1*/p38 MAPK in response to cinnamon extract, however, we did not see an increase in the in the expression *nlp-29* and *nlp-29 driven,* which is downstream of *pmk-1* (Table 10). The activity of *pmk-1*/p38 is suggested to be regulated by other protein. For example, PP2A and VHP are reported to reduce activity of p38 MAPK and *mek*-1 is reported to increase p38 MAPK activity (Junttila et al., 2008; Yu et al., 2012). The decrease of expression of *nlp-29* and its driven gene may indicate inhibition of *pmk-1*. Also it is reported that sage treatment also increased the gene expression of *pmk-1*, but it decreased *nlp-29* and *nlp-29D* gene expression in *C. elegans* model (Jamison, 2012). Since Jamison's (2012) and our worms were not given microbial stress and our data indicates increase of p38 MAPK signaling, over expression of unnecessary anti-microbial peptide *nlp-29* may trigger proteasome degeneration and decrease amount of peptides. Then, it is suggested that decrease amount of *nlp-29* may cause decrease of it driven gene *nlp-29D.*

Conclusion

We evaluated the effect of cinnamon on p38 MAPK signaling because it is reported that MAPK signaling is involved to response various stress and proper regulation of p38 MAPK is essential to inhibit neurodegenerative disorders. We did not seen overall increase of MAPK signaling, but observed increase of *pmk-1*/p38 MAPK gene expression in response to cinnamon extract. Previously we observed increase of *skn-1* dependent transcription even in increase of insulin signaling (Table 4). It is reported *pmk-1* phosphorylates *skn-1* and increase stress response genes, such as *gst-1*, *gss-1*, *gsr-1* and *trx-1*to response redox homeostatic, thus this data may suggest cinnamon treatment increase p38 MAPK expression and manage intracellular stress. Oxidative stress is considered to be involved in development of neurodegenerative disease, such as PD and AD. Thus, cinnamon extract may have beneficial effects on inhibition of these diseases. Also, *pmk-1* is important to inhibit GSK-3 activity along with insulin signaling (Thornton & Pedraza-Alva, 2008), thus our result may suggest cinnamon's potential bioactivity to increase cell survival and formation of axon. Cinnamon extract as well as sage extract did not increase *nlp-29* and its driven gene. Since worms were not infected by microbe, *nlp-29* gene might be degenerated and decrease its driven gene, which suggests changing target gene in future research to study p38 MAPK signaling in *C. elegans* study.

CHAPTER VI

THE *IN VIVO* MODULATION OF TGF-β SIGNALING IN RESPONSE TO CINNAMON TREATMENTS

Introduction

TGF-β Signaling Pathway

Transforming growth factor (TGF)-β signaling is often known to be activated by many ligands, such as activin which promotes spermatogenesis and synthesis of follicle-stimulating hormone (FSH), and bone morphogenetic protein (BMP) which is cytokine to control bone formation, tissue homeostasis and regeneration (Knight & Glister, 2006; Nistala et al., 2010). Genetic defect of this signaling is known to cause progression and development of cancer, pulmonary arterial hypertension and telangiectasia (Goumans et al., 2009; Tian & Schiemann, 2009). In neuronal cell, TGF-β signaling regulates cell developmental processes and cell proliferation, also in response to neuronal injury and stress (Hawryluk et al., 2012; Krieglstein et al., 2011). When TGF-βs superfamily proteins bind to TGF-β signaling receptor type I and II, these receptors forms a heterocomplex and activate Smad-2/3 or Smad-1/5/8 by phosphorylation (Kang et al., 2009). Phosphorylated Smad proteins make complex with Smad4 and translocates to the nucleus, then its complex binds with CBP and regulates various transcription

(Kang et al., 2009). Activation of TGF-β receptors can influence PI_3K/AKT signaling as well as MAKP signaling, including Erk, JNK and p38 MAPK signaling (Kang et al., 2009).

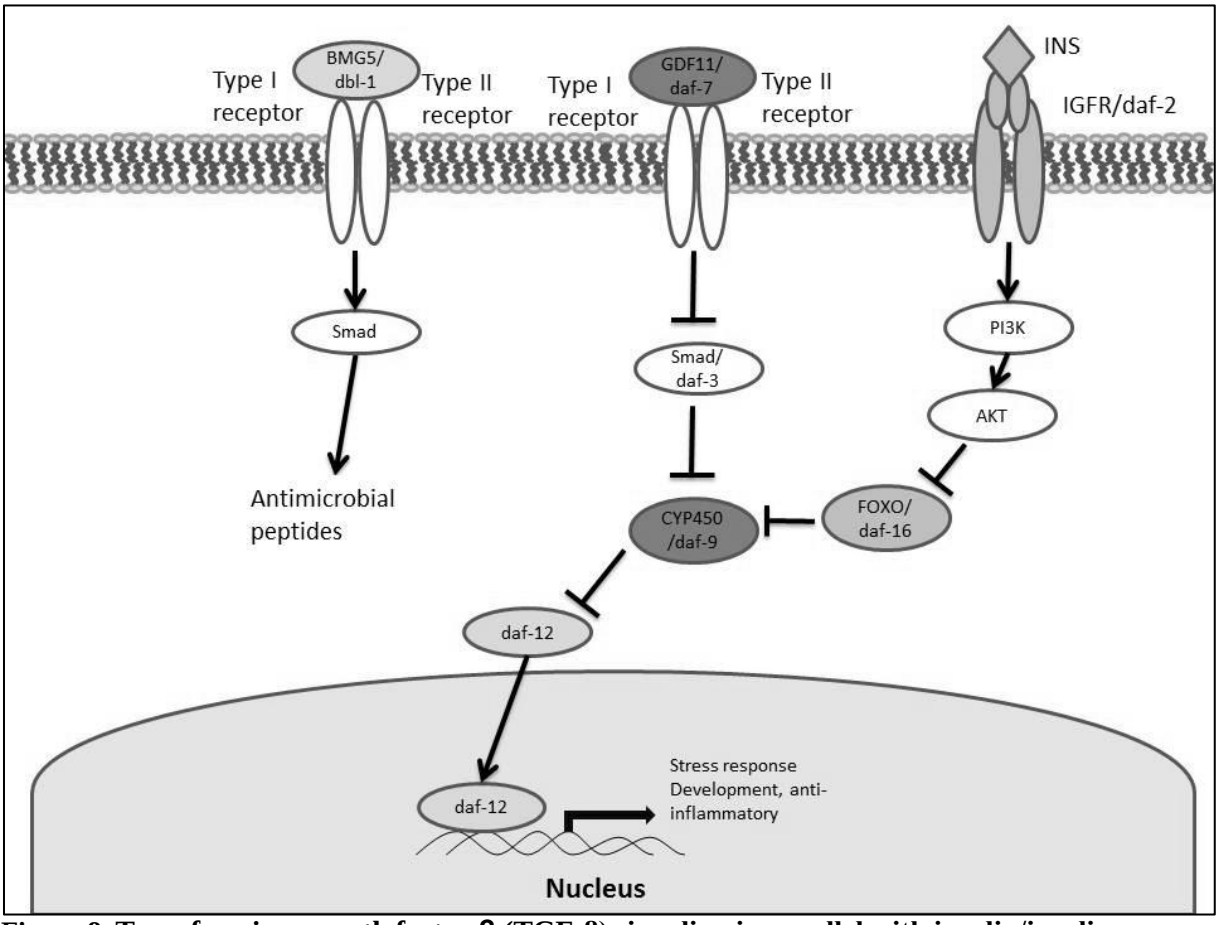

Figure 8. Transforming growth factor β (TGF-β) signaling in parallel with insulin/insulin growth factor (IGF) signaling.

Neurodegenerative Disease and TGF-β Signaling

In Alzheimer's disease (AD) patient brain, reduced TGF-β signaling are seen and suggested to progress formation of neurofibrillary tangles of aggregates of β-amyloid (Aβ), especially $Aβ_{1-42}$ (Caraci et al., 2011). It is reported reduction of TGF-β signaling reduce trophic support of neuron from glial cell and also reduce anti-inflammatory

proteins, thus it is considered to promote progression of AD (Caraci et al., 2010). However, overexpression of TGF-β signaling is reported to increase neuronal apoptosis and reduce phagocytosis, thus increases accumulation of aggregated Aβ which may contribute to AD (Lee et al., 2010).

Also, reduction of TGF-β is reported to trigger apoptosis and suggested to contribute to development of Parkinson's disease (PD) due to increase of ROS formation of microglia (L. Qian et al., 2008). Activation of TGF-β in macroglial cell attributes to inhibit PHOX induced ROS production via prevention of Erk activity, thus activation of TGF-β may play important role in preventing PD progression through reducing (L. Qian et al., 2008). Since there are controversial effects of TGF- β on neurodegenerative diseases, homeostatic regulation of TGF-β signaling may play essential roles on management of these diseases.

Modulation of TGF-β Signaling with Cinnamon extract

Cinnamon is one of spices that contains many bioactive compounds, thus recent study reported the effect of cinnamon extract on modulating many gene expressions (Gu et al., 2004). Related to the TGF- βsignaling, cinnamon extract is reported to increase expression levels TGF- β, thus cinnamon is suggested to have anti-inflammatory effect (H.-K. Kwon et al., 2011). Inflammation is known as one of risk factors of neurodegenerative disease, since inflammation increase peptide aggregation or production of ROS, which results in damage nerve tissues and may contribute to PD and AD (Lucas et al., 2006). However, in higher concentration of cinnamon extract is also reported as anti-angiogenic, pro-apoptotic compounds by reducing MAPK signaling including TGF- β pathway (H. K. Kwon et al., 2009), thus concentration of cinnamon

extracts and removing pro-apoptotic compounds of cinnamon may be important for therapeutic purpose. Thus, we evaluated the expression of genes relevant to TGF-β signaling in response to cinnamon treatments using a transgenic *C. elegans* model.

Methods

Nematode Propagation of TGF-β signaling pathway and Cinnamon Treatment

C. elegans with GFP promoter constructs of genes relevant to the TGF-β signaling pathway (Table 11) obtained from the Caenorhabditis Genetics Center were used to study the effect of cinnamon on the expression and activity of genes regulated by TGF-β system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 11. List of *C. elegans* genes in TGF-β pathway to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Transforming Growth Factor Beta			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
BW1940	***dbl-1***	Transforming growth factor β-bone morphogenetic protein 5 (BMP5)	WBGene00000936
FK181	***daf-7***	Transforming growth factor β-related proteins, Growth differentiation factor 11 (GDF11) (BMG-family)	WBGene00000903
AA278	***daf-9***	Cytochrome P450 (CYP2 subfamily) steroidogenic or fatty acid hydroxylase	WBGene00000905
AA120	***daf-12***	Nuclear receptor (Vitamin D Receptor)	WBGene00000908

Results

Effect of Cinnamon on TGF-β Signaling

The expression of *dbl-1* (BW1940), which encodes the *C. elegans* ortholog of the human Transforming growth factor β-bone morphogenetic protein 5 (BMP5), was downregulated at 1 mg/ml (RF=.0837, *p= 0.000*), and 10 mg/ml (RF=0.837, *p= 0.005*) of cinnamon but was no different from control at 5 mg/ml (Table 12). In response to cinnamon the expression of *daf-9* (AA278), which codes *C. elegans* cytochrome P450, was significantly decreased at 5 mg/ml concentration (Table 12). RF value of *daf-9* at 5 mg/ml cinnamon treatment was 0.65 (*p=0.042*), but there was no significant difference at concentration of 1 mg/ml and 10 mg/ml cinnamon treatment. Upregulations of expression of *daf-12* (AA120), which encode protein homologous to human vitamin D receptor were seen in response to cinnamon at 1 mg/ml (RF=1.28, *p=0.037*), 5 mg/ml (RF= 1.31, *p=0.027*), and 10 mg/ml (RF=1.29, *p=0.030*) (Table 12). Effect of cinnamon extracts on expression of *daf-7* (FK181), which codes transforming growth factor beta (TGF-β), was evaluated. Expression of *daf-7* at 5 mg/ml cinnamon treatment was significantly decreased (RF= 0.996, *p= 0.010*), but at 10 mg/ml cinnamon treatment it was significantly increased (RF= 2.171, *p= 0.000*) (Table 12). The expression of *daf-7* was not significant in response to 1 mg/ml cinnamon treatment.

Table 12. Dose dependent effect of cinnamon treatments on fold-change in gene expression relative to control.

GENE	Cinnamon Concentration								
	1 mg/ml			5 mg/ml			10 mg/ml		
	RF	SE	*p value*	RF	SE	*p value*	RF	SE	*p value*
dbl-1	0.837	0.098	***0.000***	1.017	0.076	*0.089*	0.837	0.131	***0.005***
daf-7	1.008	0.120	*0.180*	0.996	0.078	***0.010***	2.171	0.387	***0.000***
daf-9	0.589	0.028	*0.056*	0.650	0.101	***0.042***	1.016	0.102	*0.063*
daf-12	1.282	0.117	***0.037***	1.315	0.082	***0.027***	1.294	0.086	***0.030***

Cinnamon was tested at 1 mg/ml, 5 mg/ml, and 10 mg/ml concentrations, quantification of fluorescence expressed as RF (Relative Fluorescence) ± SE (Standard Error). N=8

Discussion

TGF-β Signaling in Response to Cinnamon

This TGF-β signaling is paracrine factor to regulate many hormone and cellular function, such as cell proliferation, cell differentiation and inflammation, and it is well conserved from nematodes to humans (Clarke et al., 2012). *C. elegans* TGF-β family members protein *daf-7* binds with TGF-β receptor I /II and activates downstream of TGF-β signaling *daf-9* and inactivate *daf-12* (Motola et al., 2006). In response to cinnamon extract, we observed several fold increase in highest concentration, but did not significant difference in low concentration (Table 12). However, downstream of *daf-7* we observed decrease of expression of *daf-9*, and observed increase of *daf-12* (Table 12). *C. elgans daf-9* and *daf-12* is also suggested be regulated by *daf-2* activation (Jia et al., 2002), our data also shown cinnamon extract increased expression of *daf-16* (Table 2). Since daf-16 inhibits daf-9 activity and results in activation of daf-12, our data may suggest overall activation of TGF-β signaling. It is also reported that increase of *daf-12* expression suppressed *daf-9* expression (Jeong et al., 2010), thus decrease of expression of *daf-9*

may confirm increase of *daf-12* expression in response to cinnamon extract. When *daf-7* levels are high, it typically indicates low stress, and it binds to TGF-β thereby activating the signaling cascade resulting in the overall activation of *daf-9* which encodes a cytochrome P450 (CYP450) enzyme that increases cholesterol delivery to cells and causes cells to grow and differentiate (Prahlad & Morimoto, 2008).

In our result we found an overall increase of *daf-7* and *daf-2* initiated TGF-β signaling. Even decrease and no significant difference of *daf-7* in lower concentration, *daf-12* was significantly upregulated in all concentration, suggesting perhaps upregulation of *daf-12* was mainly mediated by *daf-2* upregulation. In *C. elegans* it is reported that *daf-12* is essential for extension of longevity due to its stress response properties (Jeong et al., 2010). Also, recently TGF- β anti-inflammatory activity is interested in therapeutic target of neurodegenerative diseases (Appel et al., 2010). These anti-inflammatory properties may be beneficial for AD due to decreased ROS production and Aβ protein aggregation (Soler-López et al., 2012), thus upregulation of TGF-β signaling in response to cinnamon extract may indicate potential cinnamon therapeutic effects against neurodegenerative disease.

Conclusion

In response to cinnamon treatment we observed overall increase of TGF-β signaling. We did not observed increase of *daf-7* expression in lowest concentration of cinnamon extract, thus at lowest concentration *daf-12* expression is suggested to be increased by *daf-16* upregulation. Decrease of *daf-9* expression may due to *daf-12* negative feedback of the expression of *daf-9*. Overall activation of this signaling in response to cinnamon

extract may suggest beneficial effects of cinnamon extract. Over expression of TGF-β signaling cause negative effect on neurodegenerative disease pathway (H. K. Kwon et al., 2009), thus careful regulation of this signaling and proper concentration of cinnamon extract is important. Also fractionation and removing apoptotic compounds from whole cinnamon extract may be needed to get optimum benefit from cinnamon extract.

CHAPTER VII

CINNAMON DELAYS SYMPTOMS OF EXPERIMENTALLY INDUCED ALZHEIMER'S DISEASE IN *C. ELEGANS*

Introduction

Alzheimer's Disease Mechanism and Symptom

As average age rises in developed countries, cognitive disorders neurodegenerative diseases such as Alzheimer's diseases (AD) are increasing in elderly population. In the worldwide it is estimated that 26.6 million people, who were aged >65 years, living with AD in 2006 and by 2050 the prevalence of AD would grow to 106 million (Brookmeyer et al., 2007). Oxidative stress, tau protein or amyloid beta peptide are considered as possible etiological factor of AD, however the exact mechanism of AD remain unclear (Hung et al., 2008; Kitazawa et al., 2009; Murray et al., 2007) .

Oxidative stress is considered to involve to AD because of oxidative stress causes multiple damages to neuronal cell. It is reported that lipid peroxidation and its neurotoxic by-products 4-hydroxynonenal (HNE) and acrolein increased in AD brain (Williams et al., 2006). Mitochondrial membrane lipids vulnerable to oxidative stress, thus oxidative stress cause loss of function of mitochondria and cause further increase of ROS and kills neuronal cells (Harper & Bevilacqua, 2004). Also it is reported that reduction of nicotinic acetylcholine receptors (nAChRs) on neuron is disrupted by oxidative stress and

contribute to progression of AD (Picciotto & Zoli, 2008). nAChR agonists also increases the blood flow of the cerebral cortex as well as hippocampus and olfactory bulb, and it is known that number of nAChR on neuronal cell membrane significantly decrease with age (Shiba et al., 2006). Decreasing of nAchR decrease blood flow on neuronal cell and cause neuronal atrophy, thus drug to decrease oxidative stress in neuronal cell and inhibits degeneration of nAchR is interested for AD treatment.

Tau protein is also considered as one of major etiological factors of AD because it is reported that hyperphosphrylation of tau protein promotes neurofibrillary tangles and causes apoptosis activation (Cripps et al., 2006). Tau protein is one of the microtubule-associated protein, which stabilizes and promotes polymerization of microtubules and transport mitochondria in the axon (McVicker et al., 2011). However, in AD brain hyperphosphorylation of tau protein is observed, which is caused by increase of ROS or overexpression of CDK5 or GSK-3 via AKT gene (Hanger et al., 2009). When tau protein is hyperphosphorylated, neurofibrillary tangles are produced, thus inhibit mitochondria transportation in the axon and promote apoptosis (Shahpasand et al., 2012). The exact mechanism of neurotoxicity of tau protein is still unclear, but therapeutic efficacy of inhibitors of the phosphorylation of tau protein and anti-tau neurofibrillary tangles are investigated against AD.

Also accumulation of extracellular and intracellular misfolding and aggregation of amyloid β peptides, especially amyloid $\beta_{1\text{-}42}$ deposition is reported to progress AD (Flood et al., 2009; Knobloch et al., 2007). Amyloid precursor protein (APP) plays an essential role in growth repairing nerve cells, but it is reported to lead neuronal dysfunction and progression AD when one of cleaved APP $A\beta_{1\text{-}42}$ peptides increase in brain, (Kandimalla

et al., 2009) . In *C. elegans* it is revealed that increase in *daf-16* and *hsf-1* and reducing insulin signaling decrease neurotoxicity of $A\beta_{1-42}$ peptides (E Cohen & Dillin, 2008; Ehud Cohen et al., 2006). $A\beta_{1-42}$ peptides is reported to block postsynaptic neuron receptors, such as AMPA receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR) and acetylcholine receptors (AchR) and progress cognitive dysfunction by activating apoptosis signaling (Lipton, 2004; Rui et al., 2010; P. Wang et al., 2005).

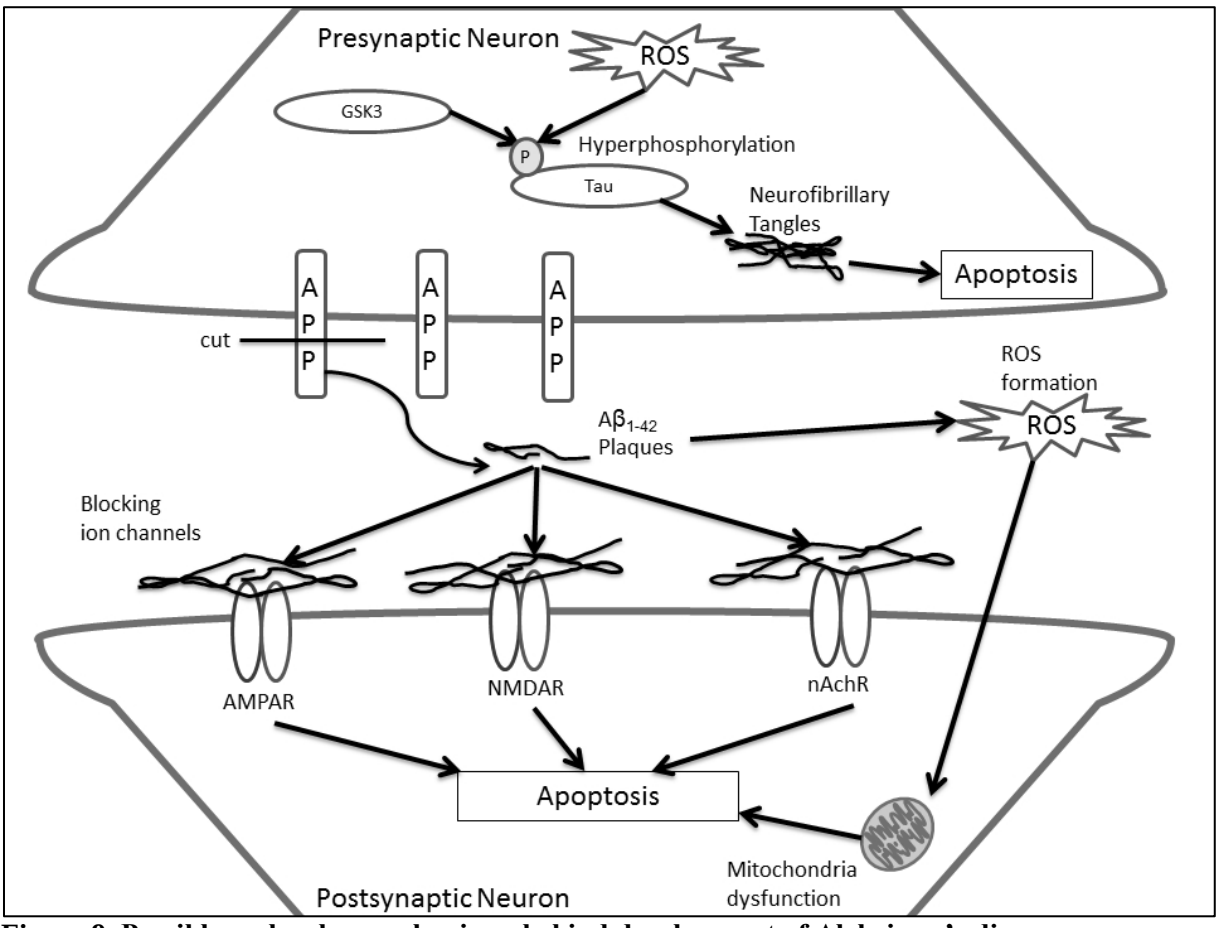

Figure 9. Possible molecular mechanisms behind development of Alzheimer's disease. Oxidative stress

Cinnamon for Management of Alzheimer's Disease

AD is a neurodegenerative basis of age-related cognitive decline disease which brain function is reduced by formation of extracellular Aβ peptides plaques and

intracellular neurofibillary tangles (tau) (Albert et al., 2011). Recent *in vitro* emerging research suggests that cinnamon extract has beneficial properties to reduce tau aggregation and filament formation associated with AD due to the functional properties of proanthocyanidin trimer and cinnamaldehyde (Peterson et al., 2009). The microtubule-associated protein tau stabilize translocation activity of microtubules in the axon, but hyperphosphrylated tau protein is known to become neurofibrillary tangles and promote apoptosis which may contribute AD (McVicker et al., 2011; Shahpasand et al., 2012). Recent *in vitro* studies shown that cinnamon polyphenols and a procyanidin type A trimer from cinnamon extract have inhibition effect on glial cell swelling and protective effect on mitochondrial function (K S Panickar et al., 2012; Kiran S Panickar et al., 2009), indicating a potential to inhibition effect against tau aggregation and filament formation. Furthermore, *in vivo* fly and mice and model studies suggested aqueous cinnamon extract has anti-amyloid aggregation properties which led to improvement in cognitive behavior (Frydman-Marom et al., 2011). The precise mechanism is not fully understood, but it is assumed that fibrillar Aβ, especially $A\beta_{1\text{-}42}$ is thought to cause neurotoxicity, which contribute to development of AD (Sakono & Zako, 2010). Cell culture *in vitro* studies and *in vivo* flies and mice models have shown that orally intake of cinnamon extract inhibit Aβ aggregation and reduced neurotoxicity of amyloid fibrils thereby increased cognitive performance (Frydman-Marom et al., 2011). The mechanisms of interaction of cinnamon extract and APP for inhibition of amyloid fibrils remain unclear therefore furthered investigation is required.

Also it is assumed that type 2 diabetes patients associated with insulin resistance frequently develop AD because of increase of neuritic plaques and oxidative stress,

decreasing glucose transporter and overactivation of GSK-3β (Deng et al., 2009; Matsuzaki et al., 2010). Cinnamon polyphenols has recently been found to improves insulin sensitivity via regulating insulin signaling in animal model (K Couturier et al., 2010; Karine Couturier et al., 2011), thus it is suggested that consumption of cinnamon extract intake has potential benefits on inhibition of AD progression.

Despite these emerging findings, the mechanism of cinnamon in effecting gene expression related various symptoms of AD and amyloid β peptide accumulation *in vivo* is not clearly understood at this time and required further investigation. Therefore in this research we have decided to evaluate the effectiveness of cinnamon extract at reducing amyloid β peptide induced paralysis in a novel *C. elegans* model.

Methods

Fractionation of Cinnamon extract

As described previously aqueous extracts (AE) of cinnamon was prepared by heating 1.5g of the herb in 30mL of distilled water at 60^{o}C for 30 minutes. The mixture was vacuum-filtered through Whatman filter paper, and filter sterilized. Using solvent fractionation aqueous extract of Cinnamon will be fractionated based on polarity as well as charge and mad polar acidic (PA), polar basic (PB) and polar neutral (PN) fractions from AE of cinnamon (Figure 10). The extract and its fractions of cinnamon extract was used to evaluate its effect on the neurodegeneration model.

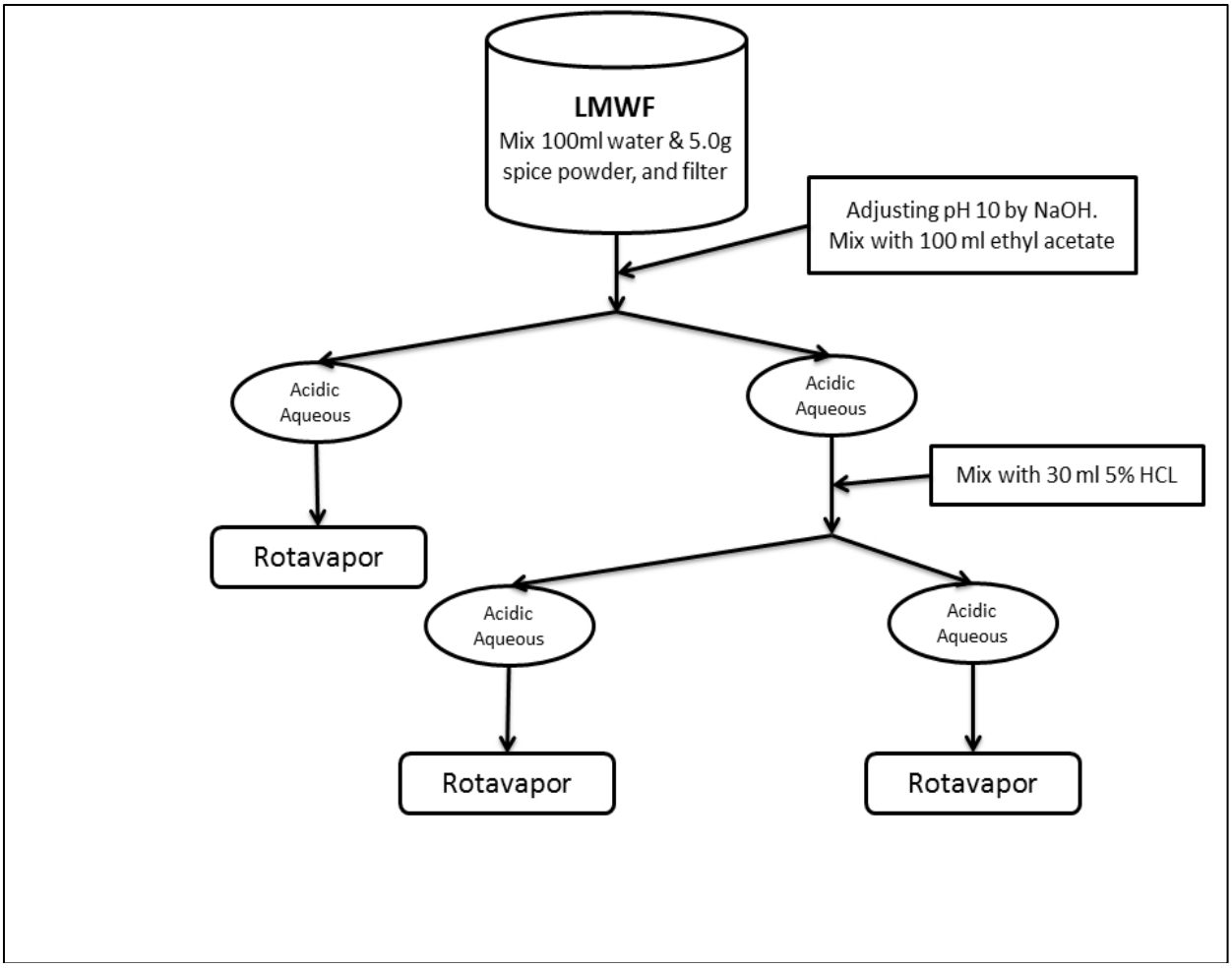

Figure 10. Method of solvent fractionation to collect samples of varying polarity and charge.

Nematode Propagation and Treatment

Transgenic *C. elegans* strain CL4176 was used for AD assay and the transgenic strain was obtained from the Caenorhabditis Genetics Center. Strain CL4176 has been commonly used for AD in *C. elegans* model and construction of CL4176 [smg-1 (cc546ts)] is previously described (Link et al., 2003). CL4176 contains a temperature sensitive mutation smg-1, thus at temperature 25°C CL4176 induce amyloid β protein to the body and muscle and will develop paralysis (Table 13). NGM and treatment plates were made as previously described in chapter 1 (Caldicott et al., 1994). 60 mm plates were used for NGM and cinnamon treatment plates and 100 µL of ten times concentrated

E. coli strain OP50 with or without cinnamon treatment were pipetted to plates. For treatment plate cinnamon AE, PA, PB or PN fractions were added to make 0.1% v/v. After overnight incubation at room temperature 25°C, plates were treated by UV light to inactivate *E. coli* OP50 using a Stratagene UV Stratalinker 2400. *C. elegans* model AD assays were performed as described previously (Dostal & Link, 2010). CL4176 strain worms were synchronized before the experiment to induce amyloid β protein at same life stage. Second days gravid adults were transferred to NGM or treatment plates and allowed to lay eggs for two to three hours. After removing gravid adults from plates, worms were grown to L3 stages in 16°C incubator. When worms were reached L3 stage, worms were moved in 25 °C incubator and amyloid β-induced paralysis was initiated as previously described (Dostal & Link, 2010). Twenty hours after the initiation, the mobility of worms was scored and scoring continued in 2 hour increments until worms are paralyzed. Worms that did not move even poke by a platinum wire or worms that were paralyzed whole body except head part were scored as paralyzed worms.

Table 13. ***C. elegans* transgenic gene to be evaluated in AD assay and the human equivalent. Information obtained from www.wormbase.org.**

Strain	Gene	Human Homolog	Construction
CL4176	***ABeta42***	Amyloid $\beta_{1\text{-}42}$	smg-1 (cc546ts); dvIs27

Results

Effect of Cinnamon in *C. elegans* Alzheimer's Neurodegeneration Model

In a transgenic *C. elegnas* model of AD (CL4176), the protective effect of cinnamon extracts on Amyloid $\beta_{1\text{-}42}$ induced neurotoxicity was examined. CL4176 worms were heat treated in 25°C incubator for 20 hours to induce Amyloid $\beta_{1\text{-}42}$ and number of non-

paralyzed worms and paralyzed worms were scored up to 38 hours. Figure 11 shown the result of the Amyloid β_{1-42} induced paralysis on no treatment plates (control). Time post induction of 50% paralysis of worms (PT_{50}) caused by Aβ on control plates was 30.0 hours and at 36 hours all worms were scored as paralysis (Fig. 11). Worms that treated with 0.1% (v/v) cinnamon AE the time to PT_{50} was 27.8 hours, which was 2.2 hours hastening paralysis compared with control (Fig. 11). Similarly, PT_{50} of 0.1% (v/v) cinnamon PA and PB treatments were 29.6 hours and 29.5 hours respectively (Fig. 12 and Fig. 13). Even hastening time is smaller than 0.1% (v/v) cinnamon AE, it suggested 0.1% (v/v) cinnamon PA and PB hastens paralysis compared with control. However, in contrast to 0.1% (v/v) cinnamon AE, PA and PB extract the time to PT_{50} of 0.1% (v/v) cinnamon PN treatment was 31.3 hours, which was 1.28 hours delaying paralysis compared to control with no treatment (Fig. 14). It is reported that Ginkgo biloba leaf extract delayed paralysis of transgenic strain *C. elegans* CL4176 by inhibiting Aβ oligomers (Wu et al., 2006), suggesting 0.1% cinnamon PN extract share similar mechanism of action.

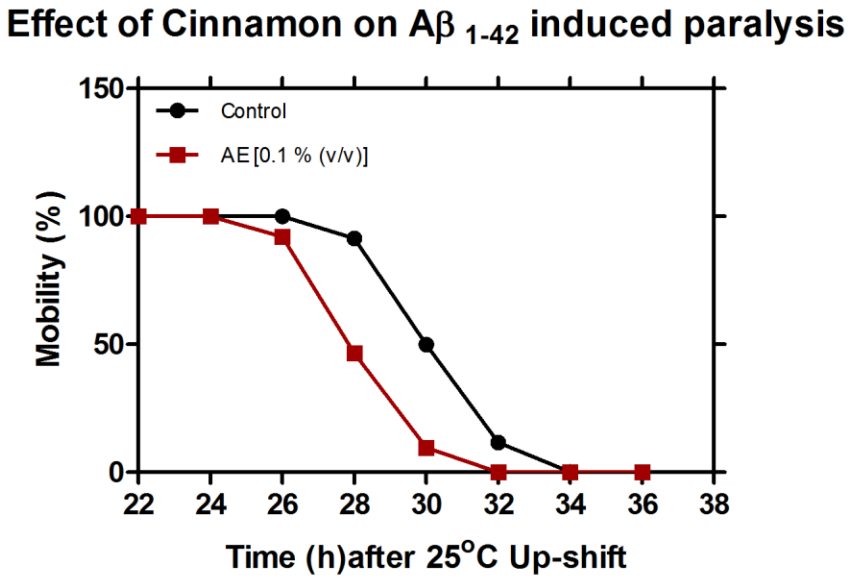

Figure 11. Mobility curve indicating percent of population mobile with AE treatment per time after 25°C temperature upshift.

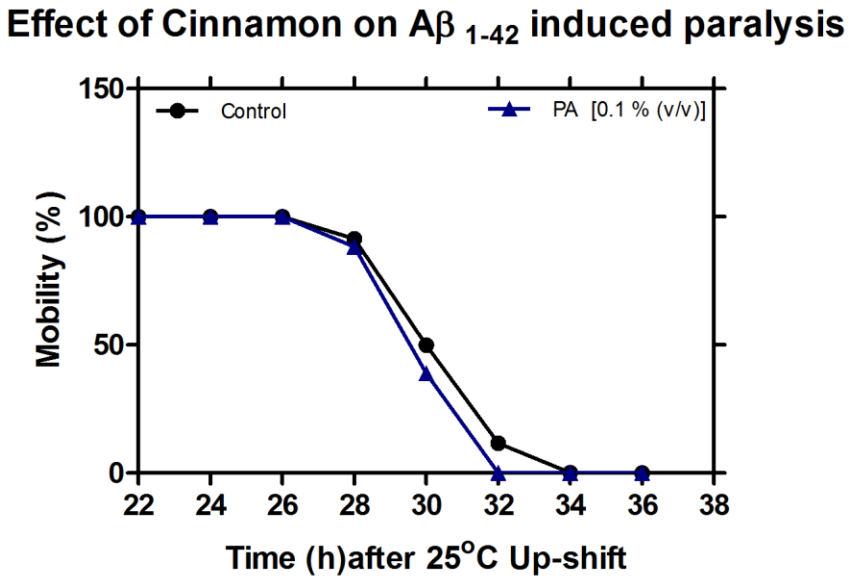

Figure 12. Mobility curve indicating percent of population mobile with PA treatment per time after 25°C temperature upshift.

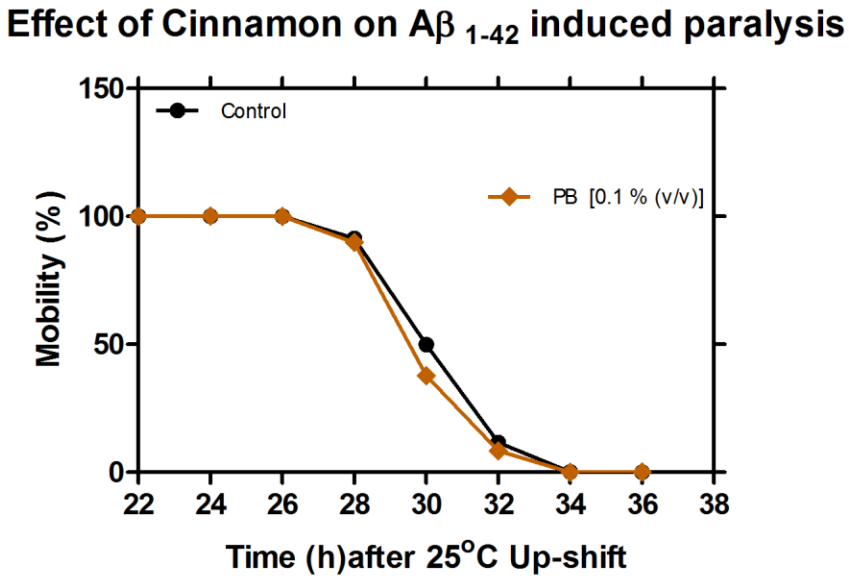

Figure 13. Mobility curve indicating percent of population mobile with PB treatment per time after 25°C temperature upshift.

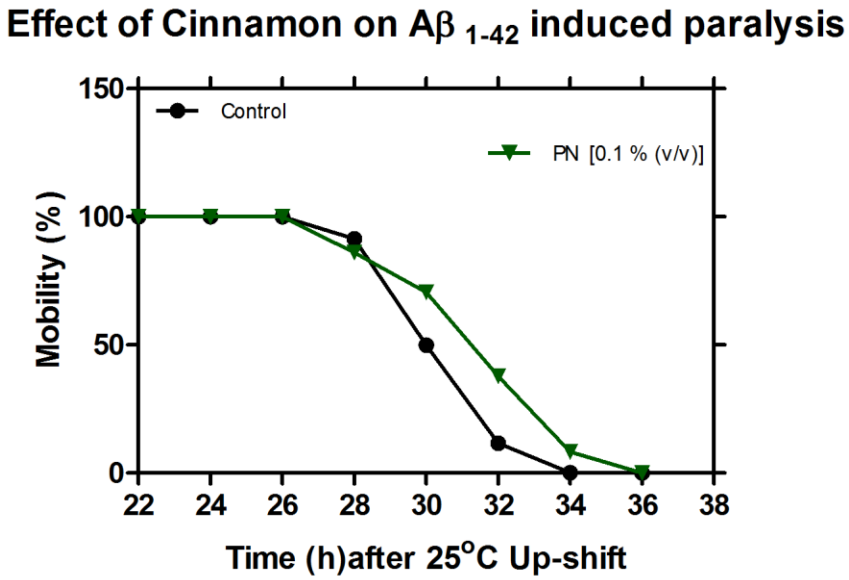

Figure 14. Mobility curve indicating percent of population moblie with PN treatment per time after 25°C temperature upshift.

Discussion

Effect of Cinnamon on Paralysis in Alzheimer's Disease Model

Alzheimer's disease is one of the most common cognitive or neurodegenerative disorder in the world population (Brookmeyer et al., 2007), but the mechanism of AD remain unclear because multiple factors are involved in the etiology of AD. Formation of neurofibrillary tangles of tau protein, accumulation of $A\beta_{1-42}$ peptides and dysregulation of stress response pathways have been implicated to development of neurodegenerative diseases (Caraci et al., 2012; De Felice et al., 2009; Di Domenico et al., 2010; Ying Liu et al., 2008; Munoz & Ammit, 2010; Riley et al., 2010).

We have evaluated the effect of aqueous cinnamon extract on different stress response pathways, such as those mediated by ILS, Nrf-2/ARE, MAPK, HIF-1, Hsp/UPR, TGF-β. The present study was designed to investigate the effect of cinnamon and its fractions on delaying $A\beta_{1-42}$ induced paralysis *in vivo* using *C. elegans* model.

As mentioned previously our result suggests that polar neutral (PN) cinnamon fraction was most effective for delaying $A\beta_{1-42}$ induced paralysis (Fig. 14). Similar research conducted by the other group have shown that oral intake of cinnamon aqueous extracts (AE) significantly reduced amyloid oligomerization and improved cognitive function associated with AD using *in vivo* fly as well as mice model (Frydman-Marom et al., 2011). However in our result delaying in paralysis was noted only with PN fraction of cinnamon, but not with AE, PA or PB fraction. Our result suggested that the possible presence of bioactive compound responsible for this delaying paralysis is PN in nature. The disparity in results observed with the fly and mice model compared to our model

suggests that there is a possible difference between the bioavailability and absorption of this polar fraction in *C. elegans* versus higher animals. *C. elegans* possess very rudimentary digestive system (Kormish et al., 2010), and perhaps the concentration of bioactive ingredients present in the whole cinnamon extract might not be adequately metabolized and activated to have the desired effects.

It has also been reported that cinnamon contributes to lowing blood glucose level by mimicking biochemical processes of insulin (Kirkham et al., 2009). In previous chapter we have reported increase in expression gene involved in insulin signaling, such as *daf-2, age-1, daf-16, let-92, daf-28, ins-7* and *ida-1* (Table 2). This is important because insulin resistance has previously been shown to be important in induction of neurodegeneration, possibly because of ether glycation of proteins, induction of oxidative stress which causes accumulation of improper processing toxic protein causing cell death; therefore, activation of insulin signaling may ameliorate many of the these effects associated with insulin resistance and improve cell survival (Talbot et al., 2012). Insulin signaling is also known to be mitogenic, thus activation of insulin signaling can down regulate many of genes involving in pro-apoptotic signaling causing and also concomitantly improve the expression of gene involved in promoting cell growth and cell division (Baur & Sinclair, 2006; Lant & Storey, 2010; Prahlad & Morimoto, 2009). Therefore, overall effects of cinnamon on reducing $A\beta_{1\text{-}42}$ peptide induce neurodegeneration could be due to these mechanism. However, under normal circumstance activation of insulin/*daf-2* signaling should result in inactivation and cytosolic localization of *daf-16* (FOXO) as well as of *skn-1* (Nrf-2) (Lant & Storey, 2010;

Prahlad & Morimoto, 2009). Interestingly we observed increase in expression of *daf-16* and *skn-1* in response to cinnamon extract (Table 2 & 4).

This may indicates increase of *sir2.1,* ortholog of human Sir2, and *let*-92, ortholog of human PP2A may deacetylate and dephosphorylate, which activate *daf-16* (Table 2). In *C. elegans* model it is revealed that increase of *daf-16* and *skn-1* expression reduced redox homeostasis and neurotoxicity of $A\beta_{1-42}$ peptides in the neuronal cell (E Cohen & A Dillin, 2008; Ehud Cohen et al., 2006). Also Protein phosphatase 2 (PP2A) is known to dephosphorylate GSK-3 and inhibit hyperphosphorylation of tau protein, which results in prevention of neurofibillary tangles formation (W. Qian et al., 2010). Although we did not specifically test neurofibrillary tangle formation, these finding may explain anti-neurodegnerative effects of cinnamon PN extract.

Also, we observed increase of *pmk-1,* ortholog to human p38 MAPK in response to cinnamon extract (Table 10).GSK-3 has been shown to hyperphosphorylates tau protein, which has been shown to be involved in oligomerization of $A\beta_{1-42}$ in neurodegenerative condition (Hanger et al., 2009). And active insulin signaling and MAPK signaling suggests that there is inhibition of GSK-3 activity, which may perhaps contributes to decrease in oligomerization of $A\beta_{1-42}$ upon its induction after thermal stress in *C. elegans* (Hanger et al., 2009) . Although the mechanism of the interaction between cinnamon PN fraction and GSK-3, it is assumed that increase of insulin signaling, *daf-16*, *skn-1* and MAPK signaling in response to cinnamon PN inhibited GSK-3 activity and reduced Aβ oligomer induced paralysis.

In addition, insulin signaling involves in trafficking of AMPA receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR), acetylcholine receptors (AchR) and glucose transporter (GLUT), which help the intracellular signal transduction system of neuron (M. de la Monte, 2012; Shonesy et al., 2012). Accumulation of $A\beta_{1-42}$ peptides blocks these postsynaptic neuron receptors and activate apoptosis signaling, which results in contribution of the neuropathology of AD (Lipton, 2004; Rui et al., 2010; P. Wang et al., 2005). Taken together, these data implicate upregulation of insulin signaling and stress response genes in response to cinnamon may suggest decreasing neurotoxicity of $A\beta_{1-42}$ peptides.

When intracellular or extracellular misfolded proteins are accumulated, it result in activation of UPR signaling (Salminen et al., 2009). UPR signaling function to reduce protein toxicity induced cell death by increase in the degradation of misfolded protein or uptaking and stabilizing misfolded proteins by heat shock proteins (Salminen et al., 2009). In a previous study we have reported several fold increase in gene expression of *hsp16.2* and *phsp*, which share the same promoter (Table. 8). Recent findings shows that constitutive overexpression of *hsp-16.2* expression reduce the formation of amyloid β plaques *in vivo C. elegans* (Fonte, Kipp, III, et al., 2008). The delaying in paralysis is perhaps due to the stabilization of the initially induced $A\beta_{1-42}$ peptides upon thermal shock in *C. elegans.* However, during late stage of induction $A\beta_{1-42}$ peptides formation of stress cellular oligomers of $A\beta_{1-42}$ eventually result in aggregation and plaques formation. One mechanism to reduce this plaques formation is to reuptake $A\beta_{1-42}$ peptides intracellularly either stabilized or degraded. HSP70 and its co-activator *rme-8* in *C. elegans* are known to increase receptor mediated endocytosis of misfolded protein

(Meimaridou et al., 2009). Treatment with the cinnamon results in increased expression of *rme-8*, perhaps suggesting that initial oligomers of $A\beta_{1-42}$ peptides are being endocytosis intracellularly and stabilized or degenerated which also be contributing factor to delaying paralysis.

Conclusion

We found that cinnamon PN treatment had positive effect on delaying the paralysis induced by Aβ neurotoxicity *in vivo C. elegans* model. Our previous study showed increase of insulin signaling and *daf-16, hsp-16.2, pmk-1* and *let-92* gene expression in response to cinnamon AE treatment. We found that increase of time post induction of 50% paralysis of worms (PT_{50}) in response to cinnamon PN treatment, but not to cinnamon AE, PA and PB treatment. This indicated cinnamon PN fraction may have potential therapeutic effects on slowing the progression of AD induced by Aβ neurotoxicity. In our future experiments we will attempt to characterize the chemical nature of the PN fractions of cinnamon, and further evaluate the neurodegenerative properties.

CHAPTER VIII

CINNAMOMUM VERUM AMELIORATES MPP^+ INDUCED PARKINSON'S DISEASE IN *C. ELEGANS*

Introduction

Parkinson's Disease Signs and Symptoms

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease caused by degeneration of nigrostriatal motor neuron and causes trembling limbs, fatigue or localized muscle pain (Hirsch & Hunot, 2009). It is found that some mutation of genes are associated to molecular dysfunction of oxidative stress and inflammation leading in to familial PD (Schulz, 2008); however, in majority cases of PD the result of complex environmental and genetic factors produced endogenously neurotoxins and progress PD (Banerjee et al., 2009). The mechanism of action of causation and progression PD remain unclear, but oxidative stress, inflammation, dysfunction of microglia, and increase of misfolding of alpha-synuclein are considered as major factors to develop PD (Whitton, 2007).

Dopamine Signaling

In normal state, L-DOPA is biosynthesized from L-tyrosine, then it is converted by enzyme tyrosine hydroxylase (TH) to form dopamine in the dopaminergic neurons

(Nakashima et al., 2011). When dopamine is released from substantia nigra pars compacta (SNpc), it binds to D1 type receptor and D2 type receptor resulting in increase or decrease of PKA, then send signal to muscle through controlling N-Nitrosodimethylamine (NDMA) receptor, AMPA receptor and Na+/Ca2+ channels, Na +, K +-ATPase via phospohorylation or dephosphorylation of DARPP-32 (Yuste et al., 2012). Also, it is known that NDMA and AMPA receptor can be bound by glutamic acid, nicotine and acetylcholine and increase excitability (Akaike & Takada-Takatori, 2010). Through these excitability, corticobulbar tracts regulates substantia nigra pars reticulata and thalamus by controlling the balance of direct pathway through D1 receptors of excitatory and indirect pathway through D2 receptors of inhibitory (Nishi et al., 2011). Also for feedback inhibition, D2 receptors present on SNpc, and negatively regulate dopamine releasing via inhibition of cAMP when dopamine bind to D2 receptors (Nishi & Snyder, 2010).

PD is caused by depletion of dopamine in the striatum via developing of degenerated dopamine neurons in the substantia nigra pars compacta (Hara et al., 2010). When dopamine neurons is loss in PD, D1 type and D2 type dopamine receptor do not bind to dopamine and the pathway to the internal segment of the globus pallidus is overexcited, thus result in suppression of the activity of the cerebral cortex and the thalamus in the end (Hara et al., 2010). Depletion or degeneration of dopamine neuron is mainly caused by over-activation of microglia, increasing ROS and misfolding of alpha-synuclein (Whitton, 2007).

It is reported that over-activation of microglia increase neurotoxicity because it increase production of ROS and leads to cell death by increasing apoptosis signaling

(Xie, Li, et al., 2010). Microglia is a type of glial cells present in the cerebrospinal and has macrophage-like phagocytosis (Sierra et al., 2010). Heme oxygenase-1 (HO-1) ,Lipopolysaccharide (LPS), and opioids are reported to induce microglia activation (Chinta et al., 2012; Jack et al., 2005; Xie, Li, et al., 2010) , and these compounds commonly activate p38 MAPK and JNK signaling in resulting apoptosis in dopaminergic neurons (Xie, Wang, et al., 2010). Also, apoptosis signal-regulating kinase-1 (ASK-1) is one of MAP3K, and it is reported as one of causation of PD (W. Yang et al., 2009). Similar to 1-methyl-4-phenyl-pyridinium (MPP^+), paraquat is suggested to cause PD like symptom by inducing neuronal cell death (W. Yang et al., 2009). Paraquat is reported to activate ER stress receptors IRE-1 and produce ROS in substantia nigra pars compacta, then activate ASK1-dependent apoptosis resulting in progress Parkinson's like symptom (Karunakaran et al., 2007). Thus, molecules what inhibit pro-apoptosis pathway are targeted for therapeutic intervention in PD.

Since anti-inflammatory drugs, such as ibuprofen, slow the progression of PD, it is known that inflammation is one of causes of PD (X. Gao et al., 2011). Inflammation of PNpc is caused by neuronal injury, oxidative stress or infection, and increase cytokines, IL-1β and TNF-α (Dantzer et al., 2008; Whitton, 2007). These cytokines increase pro-apoptosis signaling via Nf-κB and enhance transcription levels of apoptosis genes, thus suggested to trigger neurodegeneration in PD (Whitton, 2007). It is considered that ibuprofen antagonize the action of pro-apoptosis transcription factors, such as NF-κB, Ap-1 and NFAT by reduction of PPARγ expression (X. Gao et al., 2011), thus it is interested as therapeutic potential of PD.

Additionally, it is reported that misfolding of α-synuclein is involved to develop PD (Yakunin et al., 2012). Lewy bodies are circular and eosinophilic cytoplasmic inclusions appearing in central and peripheral neurons, which are made of misfolding α-synuclein (Duda et al., 2000). Ubiquitin ligase parkin is normally bound with ubiquitin-conjugating enzyme along with HSP70 and CHIP , and ubiquitinate misfolded α-synuclein, thus misfolded α-synuclein will be targeted for proteosomal degradation (Witt, 2010). Thus, dysfunction of parkin, ubiquitin-conjugating enzyme, HSP70 or CHIP is reported to associate to PD (Witt, 2010). Also, α-synuclein is associated with Sept4 protein, and decreasing of Sept4 is reported for increasing of misfolded α-synuclein, thus Sept4 is considered for suppression of misfolding α-synuclein neurotoxicity (Shehadeh et al., 2009). In addition, misfolded α-synuclein is normally degenerated by lysosome, thus lysosomal activity plays important role in protein clearance to slow progression of PD (Chu et al., 2009). It is reported that *in vivo* mice research for degradation of misfolded α-synuclein chaperone-mediated autophagy (CMA) by lysosome played important role for anti-neurotoxicity associated with PD (Mak et al., 2010). Thus, both of decrease degradation of α-synuclein caused by dysfunction of parkin and lysosome can result in accumulation of high levels of misfolded α-synuclein and can lead to neuronal cell death (Mosharov et al., 2009).

Cinnamon and Parkinson's Disease

The exact mechanism of causing PD remain unclear, but it is reported that majority causes of PD is initiated by neurotoxins caused from aging and environmental factors (Banerjee et al., 2009). It is well known that 1-methyl-4-phenyl-1 ,2,3,6-tetrahydropyridine (MPTP) cause the injury of nerve cells in the substantia nigra resulting

in Parkinson like symptom (Chen & Vargas, 2009). When MPTP passes through the blood-brain barrier, it is converted to 1-methyl-4-phenylpyridinium ion (MPP^+) and taken into substantia nigra neurons, then MPP is incorporated into the mitochondria and suppress the NADH dehydrogenase (complex I) in the electron transport chain increase ROS, thus MPP causes cell death (Marella et al., 2009). MPTP and MPP do not exist in nature, but oxidative stress and inflammation is considered to be involved in causing PD (Whitton, 2007).

Insulin signaling is one of the pathways to regulate inflammation, and it is reported that cinnamon extract mimic biochemical properties of insulin and reduced inflammation caused neuronal injury *in vivo* mice model (Azab et al., 2011). Neuronal inflammation caused by increase of ROS is assumed to involved in progression of PD because it activate apoptosis signaling and degenerate dopaminergic neurons (Xie, C. Wang, et al., 2010). Active microglia and their pro-apoptotic protein in activating JNK signaling via inflammatory cytokines MAPK are known to involved PD, but it is reported that cinnamon compound cinnamaldehyde increase insulin signaling and suppress Nrf-2 mediated inflammation (B.-C. Liao et al., 2008). Also it is reported that sodium benzoate (NaB), a metabolite of cinnamon, reduced the activity of NF-κB and expression of iNOS, thus NaB has neuroprotective effect on PD via modulation of pro-inflammatory pathway and $p21^{ras}$ (Brahmachari et al., 2009).

Misfolded α-synuclein is reported be involved for development of PD because it contributes to α-synuclein aggregation in Lewy boddies (Yakunin et al., 2012), but recent studies shown that cinnamon extract may protect neurotoxicity of misfolded α-synuclein by stabilizing the soluble oligomeric phase *in vivo* fly model (Shaltiel-Karyo et

al., 2012). Also, it is reported that ROS produced by mitochondria increase misfolded α-synuclein which contribute to the development of PD, but recent studies reported that cinnamon polyphenols and a procyanidin type A trimer from cinnamon extract attenuate glial cell swelling and mitochondrial dysfunction (K S Panickar et al., 2012; Kiran S Panickar et al., 2009). Thus, cinnamon is suggested to have potential protective effects on misfolded α-synuclein induce neurotoxicity to contribute PD.

In addition, type 2 diabetes patients is known to be associated with insulin resistance and frequently develop PD mainly because of mitochondrial dysfunction (Aviles-Olmos et al., 2012). Recent studies shown polyphenols from cinnamon extracts reduced insulin resistance *in vivo* animal study (K Couturier et al., 2010; Karine Couturier et al., 2011), thus it is suggested that cinnamon extract has potential therapeutic effect on PD. The effect of cinnamon on PD related phenotypic changes are not very well understood.

The excitation toxicity mediated causes for PD have not been investigated, therefore in this thesis, we have looked at the effect of cinnamon extract and it fractions on reducing L-glutamate (LG) and NDMA induced excitation toxicity in chick primary neural retina culture. LG and NDMA are reported to cause PD like symptoms because these increase excitotoxicity in neuronal cell and cause cell death (Nishi et al., 2011). In this research we evaluated the effectiveness of cinnamon on delaying PD like paralysis induced by MPP^+ treatment using *in vivo* C. *elegans* model and delaying PD like neurotoxicity induced by LG and NDMA treatment using *in vitro* chick primary neural retina cell culture.

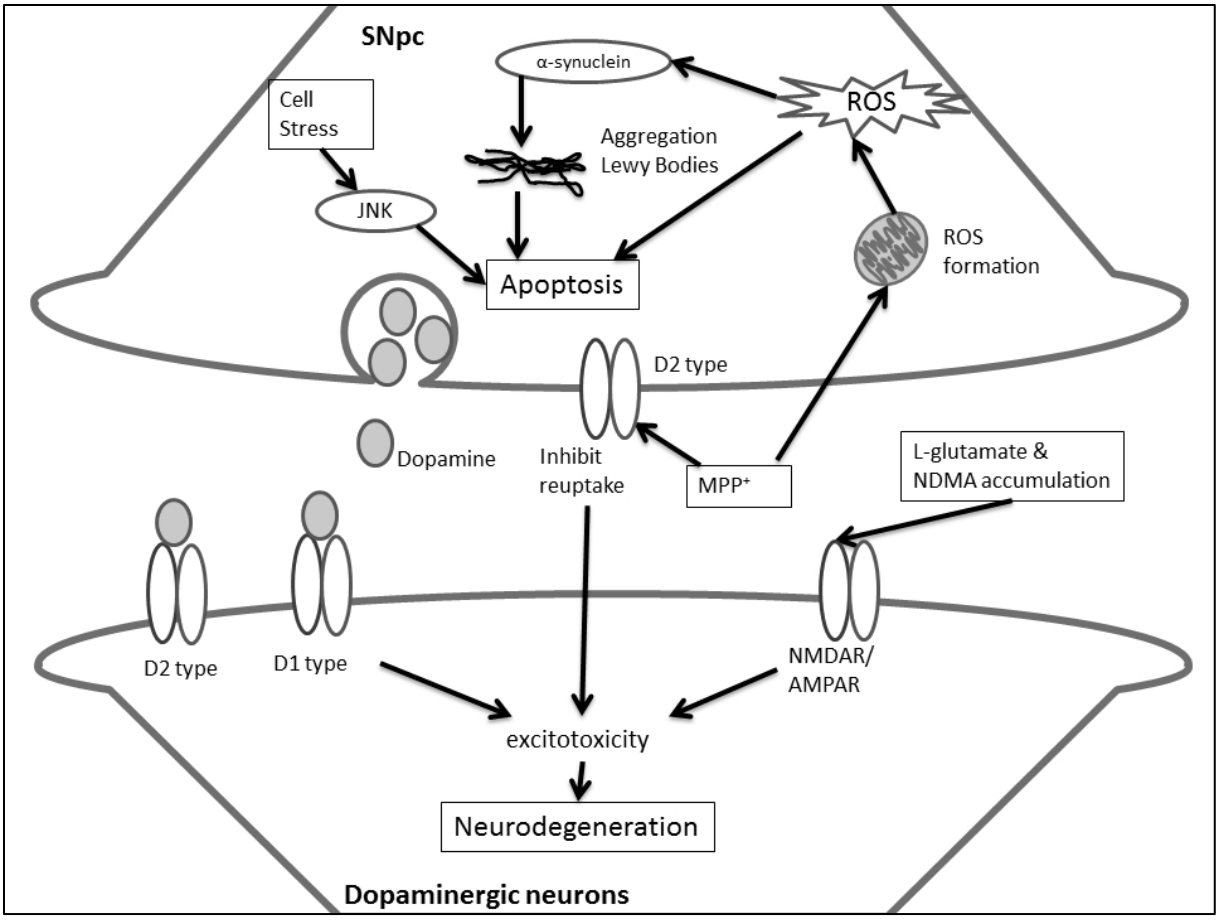

Figure 15. Parkinson's disease like symptoms model induced by MPP^+ and L-glutamate and NDMA.

Methods

Chick Retinal neurons viability assay

Retinal neurons were prepared from 8-day-old chick embryos by dissecting the eye and removing the pigmented epithelial tissue to expose the retina (Louzada et al., 2004). Retinas were dissected from several developing embryos under sterile conditions in a calcium/magnesium-free solution. Trypsinization (0.05% trypsin) was carried out for 10 min at 37°C in calcium/magnesium-free solution and the dissociated tissue was centrifuged for 2 min at 2000 rpm to remove all cell and tissue debris. The supernatant

was discarded, and the pellet was carefully resuspended in Basal Medium Eagle (BME) supplemented with 5% (v/v) fetal calf serum and mechanically dissociated by gently pipetting the tissue 10 to 20 times. Final concentration retinal cell was around 105 cells/ml. Five hundred µl the cell suspension was added to poly-L-lysine-coated 24 wells plates. 24 wells plates were incubated with 500 µl of 20 µg/ml poly-L-lysine hydrobromide per each well for 24 hours. The wells were then washed three times with 500 µl sterile distilled water to remove excess poly-L-lysine. Cultures were kept at 37°C in a humidified atmosphere of 92% air and 8% CO2 and the medium was changed 24 hours after plating. Excitation toxicity was induced by the addition of 400 µM of L-glutamate (LG) or N-Nitrosodimethylamine (NDMA). For neuroprotection assays, sterile aqueous extract of cinnamon (AE), polar acidic (PA), polar basic (PB), and polar neutral (PN) fractions were added at 0.1% (v/v) or 0.25% (v/v). Cinnamon aqueous extracts and fractions were prepared previously described in chapter 7. Cell survival and viability was evaluated 48 hours after addition of treatments to the cultures using the trypan blue exclusion method. Individual wells were also imaged under transmitted light using Nikon Ti Research microscope equipped with Qi CCD camera.

Nematode Propagation and Treatment for Parkinson's disease assay

The wild type *C. elegans* strain N2 was obtained from *Caenorhabditis* Genetics Center. The neurotoxin MPP^+ was used to induce Parkinson's like symptom by intracellular oxidative stress and neurodegeneration (Braungart & Gerlach, 2004). N2 strains were propagated as previously mentioned (Brenner, 1974).N2 worms were age synchronized and synchronized gravid adults were allowed to lay eggs on NGM plates.

After removing gravid adults from plates, eggs were incubated to hatch and synchronized L1 worms were pipetted out by with S-complete liquid media (0.59% NaCl, 5% 1M KPO4, 5 mg/ml Cholesterol in ethanol) (Fabian & Johnson, 1994) into a sterile 50 ml Falcon tube. Then, worms were washed by S-complete liquid media (97.7% S-basal, 1% potassium citrate, 1% trace metals, 0.3% $CaCl_2$, 0.3% $MgSO_4$) and *E. coli* OP50 yielding a 5 mg/ml was added food supply (Fabian & Johnson, 1994). 40 µl of S-Complete (around 17-23worms) and 10µl of IC_{90} MPP^+ (treatment group) or 10 µl (control group) were pipetted to a 96 well plate, and also cinnamon extracts and its fractions (AE, PA, PB, or PN), which were prepared as previously described, were added to reach 0.1% v/v concentration to treatment groups. The mobility of worms was scored as unparalyzed and paralyzed after 48 hours incubation.

Results

The Effect of Cinnamon extract and Cinnamon Fractions on LG and NDMA on Excitation Neurotoxicity in Chick Primary Neural retina Culture

Figure 16 represents effects of 400 µM of L-glutamate (LG) or 400 µM of N-Nitrosodimethylamine (NDMA) on of PD on chick primary neural retina cultures. Compared with control, neurotoxicity was seen in neural retina cultures treated with LG or NDMA. After 48 hours, cell viability in chick primary neural retina culture in response to LG treatment was 77 % ($p<0.05$) of control, and in response to NDMA treatment it was 86% ($p<0.05$) of control (Fig. 16). When chick primary neural retina culture was treated only with 0.1 % (v/v) and 0.25 % (v/v) cinnamon aqueous (AE) extracts and

cinnamon fractions, such as polar acidic (PA), polar basic (PB), and polar neutral (PN) for 48 hours, there was no significant effect on cell viability in primary neural retina relative to control, except 0.1% (v/v) PA and 0.1% (v/v) PB extracts (Fig. 17).

In response to 1.0% (v/v) and 0.25% (v/v) cinnamon AE/400 µM LG co-treatment, cell viability in the neural retina culture after 48 hours were121% ($p<0.05$) and 128% ($p<0.05$) of 400 µM LG treatment alone respectively (Fig. 18). In response to 1.0% (v/v) and 0.25% (v/v) cinnamon PA/400 µM LG co-treatment, cell viability in the neural retina culture after 48 hours were117% ($p<0.05$) and 115% ($p<0.05$) of 400 µM LG treatment alone respectively (Fig. 18). In response to 1.0% (v/v) and 0.25% (v/v) cinnamon PB/400 µM LG co-treatment, cell viability in the neural retina culture after 48 hours were107% ($p<0.05$) and 110% ($p<0.05$) of 400 µM LG treatment alone respectively (Fig. 18). In response to 1.0% (v/v) and 0.25% (v/v) cinnamon PN/400 µM LG co-treatment, cell viability in the neural retina culture after 48 hours were130% ($p<0.05$) and 123% ($p<0.05$) of 400 µM LG treatment alone respectively (Fig. 18).

In response to 1.0% (v/v) and 0.25% (v/v) cinnamon AE/400 µM NDMA co-treatment, cell viability in the neural retina culture after 48 hours were121% ($p<0.05$) and 130% ($p<0.05$) of 400 µM NDMA treatment alone respectively (Fig. 19). In response to 0.25% (v/v) cinnamon PA/400 µM NDMA co-treatment, cell viability in the neural retina culture after 48 hours was 110% ($p<0.05$) of 400 µM NDMA treatment alone, but in 1.0% (v/v) cinnamon PA/400 µM NDMA co-treatment there was no significant difference (Fig. 19). In response to 1.0% (v/v) and 0.25% (v/v) cinnamon PB/400 µM NDMA co-treatment, cell viability in the neural retina culture after 48 hours were123% ($p<0.05$) and 128% ($p<0.05$) of 400 µM NDMA treatment alone respectively (Fig. 19).

In response to 1.0% (v/v) and 0.25% (v/v) cinnamon PN/400 µM NDMA co-treatment, cell viability in the neural retina culture after 48 hours were115% ($p<0.05$) and 108% ($p<0.05$) of 400 µM NDMA treatment alone respectively (Fig. 19).

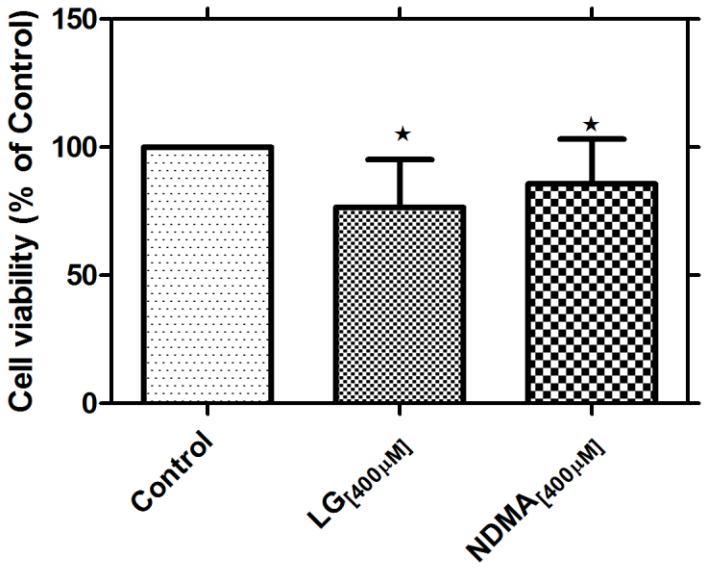

Figure 16: Neurotoxic effects of L-glutamate (400 µM) or NDMA (400 µM) on primary neural retina cultured in BEM after 48 hours of treatment. (*) $p < 0.05$

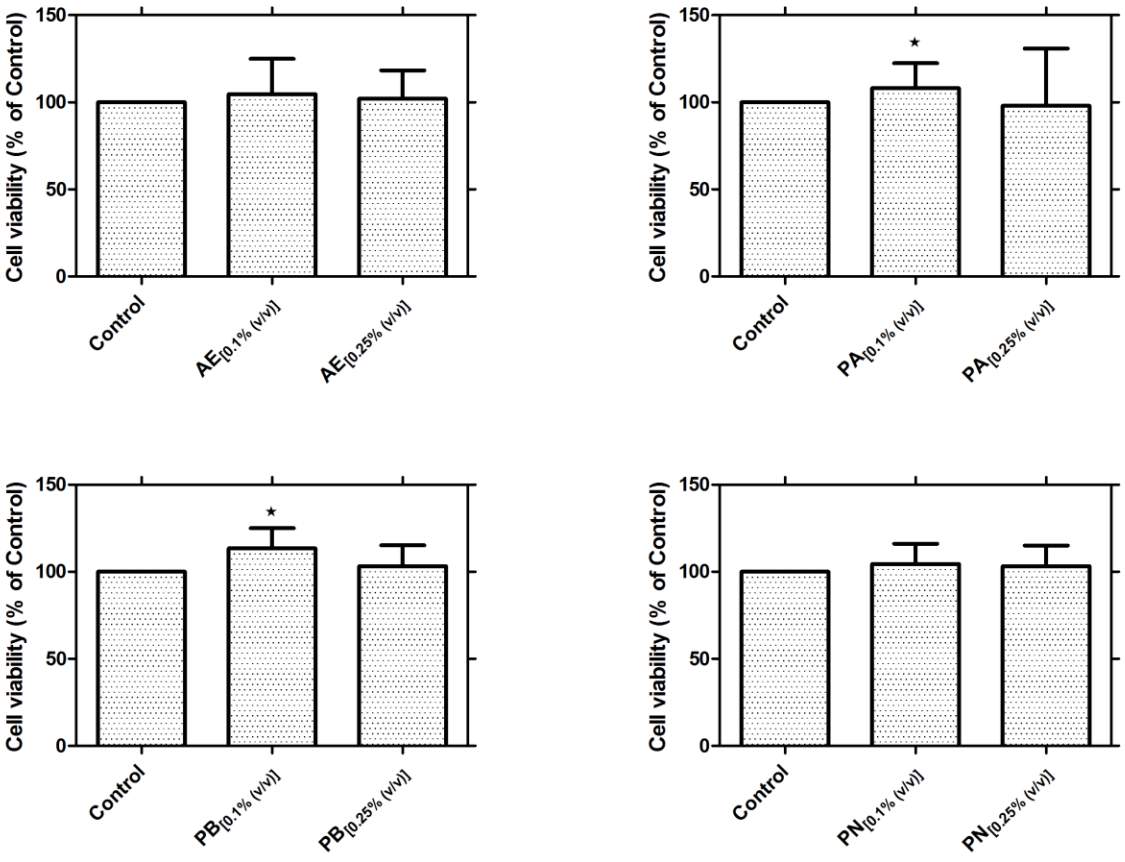

Figure 17. **Effects of aqueous extract (AE) of cinnamon or one of its fractions, polar acidic (PA), polar basic (PB), and polar neutral (PN) at 0.1-0.25 % (v/v) for 48 hours on primary neural retina cultures. (*) $p < 0.05$**

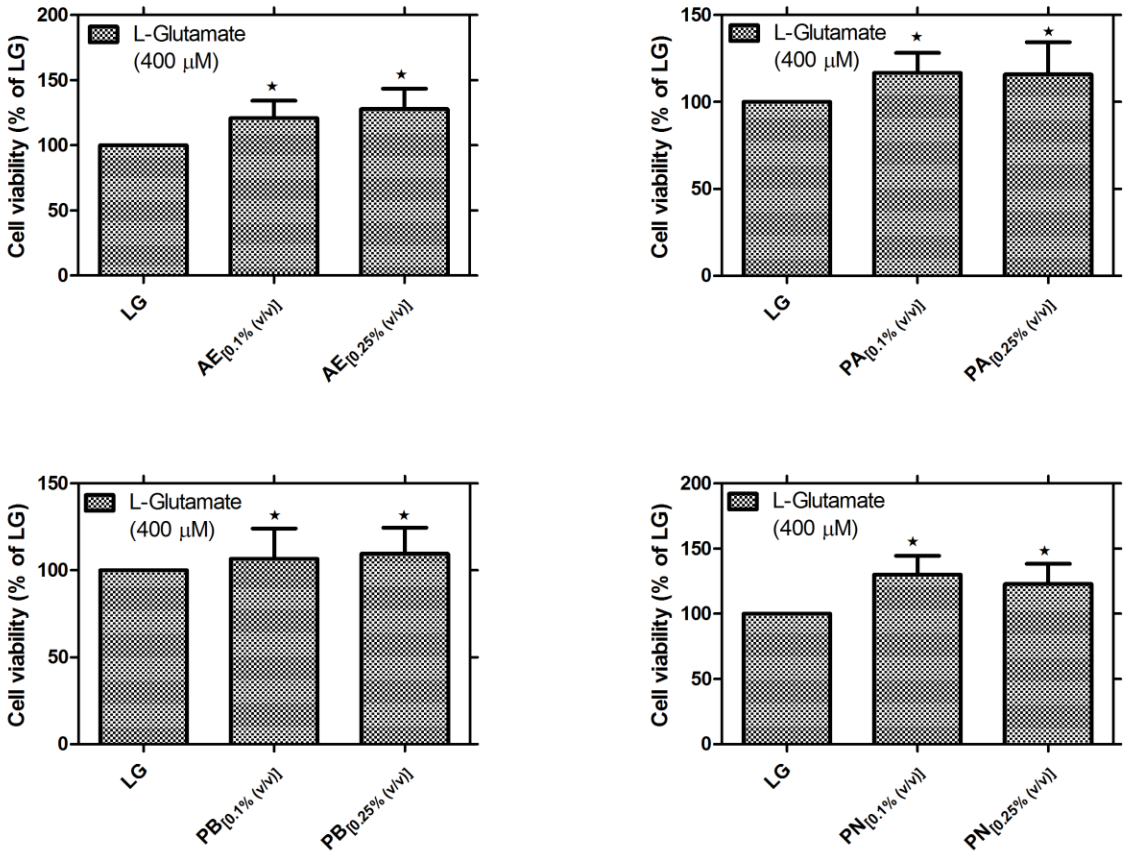

Figure 18. Effects of aqueous extract (AE) of cinnamon or one of its fractions, polar acidic (PA), polar basic (PB), and polar neutral (PN) at 0.1-0.25 % (v/v) for 48 hours on L-glutamate (400 µM) induced neurotoxicity in primary neural retina cultures. (*) *p < 0.05*

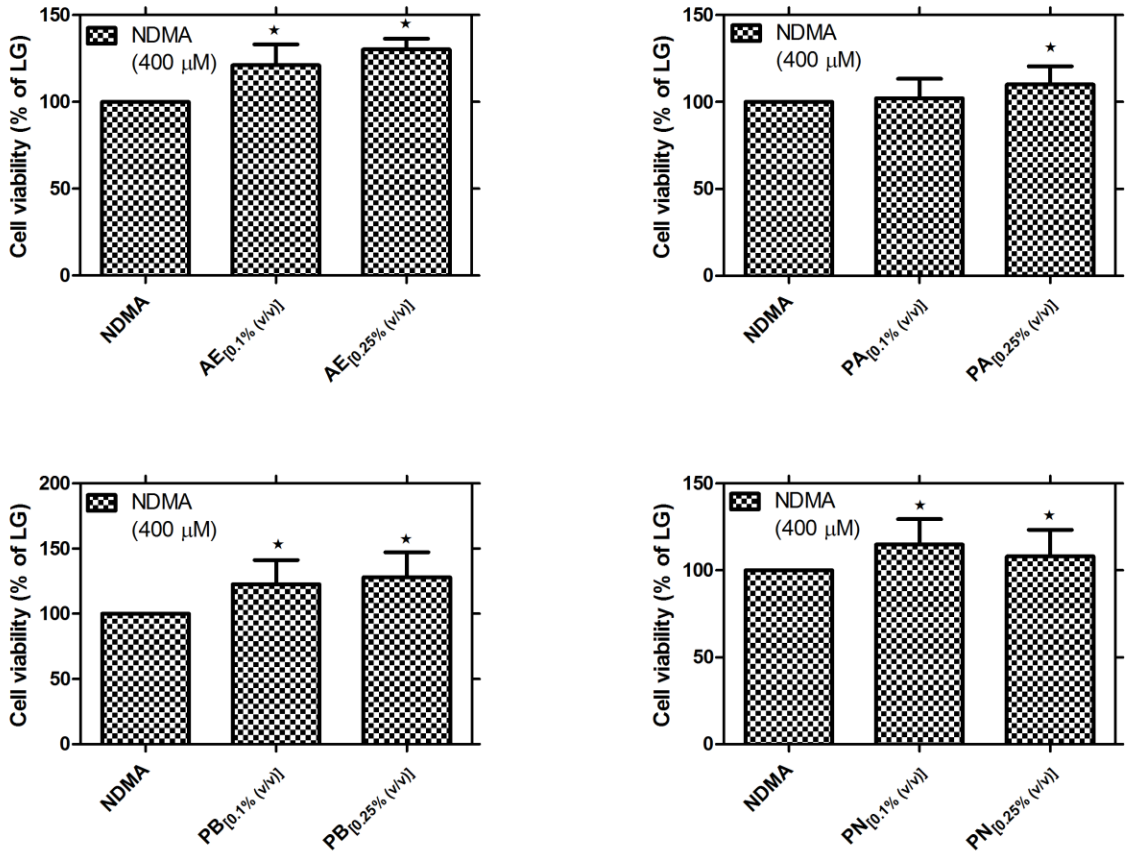

Figure 19. **Effects of aqueous extract (AE) of cinnamon or one of its fractions, polar acidic (PA), polar basic (PB), and polar neutral (PN) at 0.1-0.25 % (v/v) for 48 hours on NDMA (400 µM) induced neurotoxicity in primary neural retina cultures. (*)** ***p < 0.05***

Effect of Cinnamon on MPP^+ induced Paralysis in *C. elegans* after 48 hours

1-methyl-4-phenylpyridinium (MPP^+) kills dopaminergic neurons by inhibiting production of ATP and preventing mitochondrial DNA replication of NADPH dehydrogenase (complex I) (Cochemé & Murphy, 2008). When *C. elegans* worms are treated with MPP^+, worm's dopaminergic neurons are destroyed and cause paralysis. Wild type strain (N2) *C. elegans* was co-treated with MPP^+ and 0.1% cinnamon AE, PA, PB and PN extracts for 48 hours and the effect of cinnamon extracts on delaying paralysis of worms was evaluated.

Mobility of worms was recorded as either unparalyzed or paralyzed. In control (without MPP^+), all worms scored as unparalyzed after 48 hours; whereas, 45.3% worms which were exposed to IC_{90} dose of MPP^+ were scored as paralyzed (Fig. 20). Worms, which were co-treated with MPP^+/0.1% (v/v) cinnamon AE for 48 hours, 79.0 % paralyzed (Fig. 20), which was 33.7% more paralysis compared with MPP^+ treatment only. Worms, which were co-treated with MPP^+/0.1% (v/v) cinnamon PB for 48 hours, 65.0% paralyzed (Fig. 20), which was 19.7% more paralysis compared with MPP^+ treatment only. Worms, which were co-treated with MPP^+/0.1% (v/v) cinnamon PN for 48 hours, 46.5% paralyzed (Fig. 20), which was 1.3% more paralysis compared with MPP^+ treatment only. However, worms, which were co-treated with MPP^+/0.1% (v/v) cinnamon PA for 48 hours, 33.4% paralyzed (Fig. 20), which was 11.9% less paralysis compared with MPP^+ treatment only.

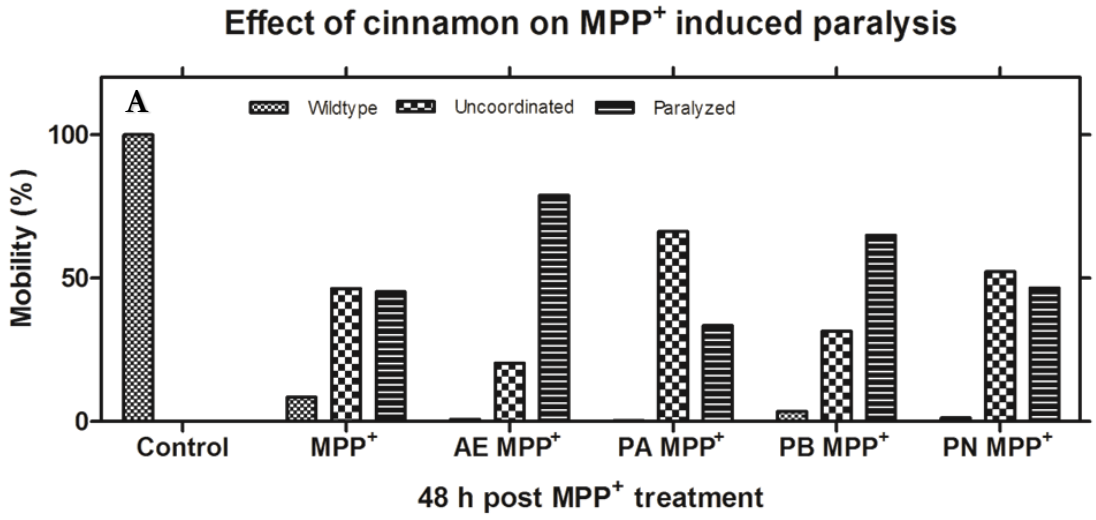

Figure 20. Mobility (%) of worm after 48 hours treatment without MPP^+ (control), with MPP^+ (MPP^+), MPP^+ with cinnamon aqueous extract (AE MPP^+), polar acidic fraction (PA MPP^+), polar basic fraction (PB MPP^+) and polar neutral fraction (PN MPP^+)

Discussion

Effect of Cinnamon on Chemically Induced Parkinson's Like Symptoms

The mechanisms of Parkinson's disease (PD) remain unclear, but it is reported that familial PD is rare and majority of PD is caused by neurotoxicity and environmental factors. It is well known that MPTP induce neurodegeneration because it is converted to MPP^+ in the dopaminergic neuron and increase ROS mediated neuronal cell death, resulting in PD like symptoms (Chen & Vargas, 2009). Since oxidative stress, inflammation, dysfunction of microglia, and increase of misfolding of alpha-synuclein are suggested to be involved in the progression of PD (Whitton, 2007). Previously we have evaluated the effects of aqueous cinnamon extract on stress response pathways, such as ILS, Nrf-2/ARE, MAPK, HIF-1, HSP/UPR, TIR-1 and TGF-β.

The present study was designed to investigate the neuroprotective effect of cinnamon on delaying PD like paralysis induced by MPP^+ treatment and its factions on L-glutamate (LG) and N-Nitrosodimethylamine (NDMA) induced neurotoxicity using chick primary neuronal neural retina culture, additionally we also investigated the effect of cinnamon and cinnamon fractions on PD like paralysis induced by MPP^+ neurotoxin using *in vivo C. elegans* model.

LG and NDMA both activate NDMA receptor and increase excitation of direct and indirect pathway and control substantia nigra pars reticulata and thalamus (Nishi et al., 2011). MPP^+ and 6-hydroxydopamine (6-OHDA) are known to produce neurotoxicity and progress PD like symptom because these inhibit glutamate reuptake into axon and cause accumulation of dopamine *in vivo* and *in vitro* study (Y. Yang et al., 2005), and excessive activation NDMA receptor also cause excitotoxicity and leads dopaminergic neural cell death and contribute to PD like symptoms (Sheldon & Robinson, 2007).

In chick primary neuronal neural retina cultures study, we treated cell culture by 400 μM LG and NDMA for 48 hours and induced neurotoxicity by over exciting NDMA receptor (Fig. 17) and examined if co-treatment with cinnamon extract and its fractions protect neuron from LG and NDMA induced excitotoxicity (Fig. 19 and Fig. 20). We have revealed that cinnamon aqueous extract and its fractions were effective for protect neural retina from LG and NDMA induced excitotoxicity, and especially cinnamon polar basic (PB) was most effective (Fig. 19 and Fig. 20).

Previous studies suggested that activating insulin signaling have neuroprotective effect on dopaminergic neurons SNpc (Quesada et al., 2008). However, treatment of neurons with NDMA is reported to cause decrease of Akt activity by dephosphorylation and suggested to induced NDMA mediated neuronal apoptosis (Yitao Liu et al., 2007). Cinnamon is described as mimic of biochemical properties of insulin and it is reported that cinnamon increased insulin signaling *in vivo* mice model (Azab et al., 2011), which may explain our observation of neuroprotective effect on LG and NDMA treated chick primary neural retina cluture. Also TGF-β signaling is known to inhibit apoptosis and enhance cell survival, and it is reported to be involved inhibition of NDMAR mediated excitotoxicity (Krieglstein et al., 2011). Our previous results shown that cinnamon extract significantly increased *C. elegnas daf-7* gene, which codes ortholog of TGF-β (Table 21).Thus, it is suggested that both increase of insulin signaling and TGF-β by cinnamon treatment may contribute to protect neural retina cell.

Also, out result shown co-treatment of cinnamon polar acidic fraction (PA) and MPP^+ decreased paralysis of worms compared with MPP^+ only treatment (Fig. 18). In cinnamon AE, PB and PN treatment, we did not observed positive neuroprotective effects, which suggest cinnamon PN fraction may contains neuroprotective compounds to MPP^+ induced PD symptoms.

In previous result, cinnamon AE increased the Nrf2/ARE signaling cascade and oxidative protection genes, such as *skn-1, elt-2, gss-1, gsr-1, gst-4, trx-1, trx-1-D, trxr-2-D* and *glrx-21-D in vivo C. elegans* model (Table 4). Researches indicated that dysregulation of Nrf-2 pathway is involved in neurodegeration because of dysregulation of redox condition in neuron , thus this pathway is associated to neuroprotection against

neurodegenerative diseases such as PD (Cook et al., 2011; Mazzio et al., 2011; Tufekci et al., 2011; van Muiswinkel & Kuiperij, 2005). Though cinnamon extract increased insulin signaling, we observed increase of stress responsive transcription factors *skn-1* (Table 2and Table 4).

The mechanism of stress responsive transcription factors and PD remain unclear, but some studies reported redox equilibrium in neuronal cell and PD development is associated. For example, we observed cinnamon extract increased the expression of genes relative to glutathione, such as *gst-4* and *gss-1*, but it is reported that reduction of glutathione expression may contribute to PD development (Martin & Teismann, 2009). Also we found that cinnamon extract increases thioredoxin expression and it is reported that thioredoxin may play important role to maintain the redox condition of neuron because thioredoxin reduces oxidative stress induced by MPP^+ (Bai et al., 2002) . These finding may explain the reasons to reduced MPP^+, LG and NDMA induced neurotoxicity by cinnamon treatment.

It is reported that diabetes mellitus is associated with the risk of PD because of the dysfunction mitochondrial and abnormality of glucose metabolism (Schernhammer et al., 2011; Xu et al., 2011). Hyperglycemia mediated by diabetes mellitus is reported to cause advanced glycation end-products (AGE), which cause mitochondria dysfunction and increase ROS (Edwards et al., 2010; A. M. Vincent et al., 2007). Thus, AGE is associated to reduce of ATP production because of the dysfunction of mitochondrial (Petersen & Dufour, 2004), and increase insulin resistance (Tan & Shiu, 2011), thus diabetes is considered as risk of developing PD. Intake of cinnamon is known to inhibit formation of AGE and reduced oxidative stress (Peng et al., 2008b; Roussel et al., 2009). This

reducing AGE property of Cinnamon may contribute our results to delaying neurotoxicity in our models.

Also, we observed proteins associated with clearance of extracellular misfolded proteins, such as *bec-1* and *ced-4* in previous chapter (Table 5).It is reported that increase of Beclin-1 decreased extracellular synaptic protein α-synuclein and decreased development of PD *in vitro* study (Spencer et al., 2009). Cinnamon is known to mimic insulin and increase insulin signaling cascade, thus, even excitation of NDMA receptor inhibits Akt signaling, and perhaps cinnamon extract may inhibit the effect of LG and NDMA on accumulation of dopamine. Also previous section we found that cinnamon increase insulin signaling, thus effect of cinnamon overcome effect of apoptosis by promoting neuronal cell growth and cell survival.

Conclusion

Our results indicate cinnamon extract and its fractions, especially cinnamon PN treatment reduced LG and NDMA induced excitotoxicity in primary chick neural retina culture, and we also observed cinnamon PA fraction delay MPP^+ induced neurodegeneration which contribute to PD like symptoms *in vivo C. elegans*.

We previously observed increasing of apoptotic signaling gene expression in response to cinnamon whole AE, but interestingly, in MPP^+ induced neurotoxicity and hyper activated NDMA receptor induced neurotoxicity, different cinnamon fractions show neuroprotective effects. It is reported cinnamon contains proapoptotic compounds and inhibit tumor growth (B.-C. Liao et al., 2008), and we observed increase of MPP^+

induced paralysis in whole and other fractions. On the other hand, cinnamon is well known to mimic insulin and increase insulin signaling cascade, and increasing cell growth and cell survival via insulin signaling, which perhaps inhibit the effect of excitotoxicity caused cell death induced by LG and NDMA accumulation. Also, the effects of cinnamon extract on increasing Nrf-2 signaling, especially glutaredoxin and thioredoxin mediated redox modulation, suggested cinnamon extract maintaining redox condition of neuronal cell by reducing oxidative stress. Whole cinnamon extract may have both pro- and anti- apoptotic properties, thus our data suggest the importance of fractionation and removing proapoptotic compounds from whole cinnamon extract. This was also clearly indicated by looking at the prevention of neuronal degradation of dopaminergic neurons with cinnamon co-treatments in future research.

GENERAL CONCLUSION

Cinnamon is reported as one of spices containing rich bioactive phytochemical compounds, and it is studied for its therapeutic potentials for many diseases. In this research we investigated the effects of cinnamon comprehensively among multiple stress response signaling pathways which are involved in neurodegenerative diseases using *in vivo C. elegans* model. In this thesis I found that cinnamon extract mimics insulin like properties and activates insulin signaling. Interestingly, I also found that cinnamon increased signaling related to redox homeostasis, heat shock proteins, anti-inflammatory, anti-apoptotic and autophagy. However, cinnamon also increased pro-apoptotic and phase I detoxification genes expression suggesting a potential toxic effect of certain bioactive compounds. These findings may suggest beneficial effects of cinnamon on neurodegenerative diseases, but also indicate that these effects may be dosage related and specific to only certain bioactives in cinnamon. I also investigated the neuroprotective effects of cinnamon and its fractions in *in vivo c. elegans* and *in vitro* primary cell culture models for neurotoxicity. For Alzheimer's disease model and Parkinson's disease model, cinnamon extract and some of its fractions had potential neuroprotective effects in both *in vitro* chick primary neural retina cell culture models and *in vivo C. elegans* model. Based on these results, a future investigation on the HPLC-MS identification of bioactive compounds in cinnamon and evaluation of its neuroprotective effect in more complex models of neurodegeneration is highly merited.

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VITA

Yuki Gomada was born in Yokohama, Japan on September 3rd, 1985, the son of Tomoya Gomada and Shitomi Gomada. After completing his degree at Kibogaoka High School, Yokohama, Kanagawa, in 2004, he entered Texas State University-San Marcos in Texas. He received the degree of Bachelor of Science from Texas State in San Marcos, Texas in December 2009. He entered the Graduate College of Texas State in January 2011.

Permanent Address: Sakonyama 16-8, 1-12-403

Asahi-ku, Yokohama city, Kanagawa Prefecture, Japan

Postal code 241-0831

This thesis was typed by Yuki Gomada.