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Neuroprotection Comparison of Different Nutraceutical Compounds Against Mechanistically Distinct Cell Death Inducing Agents

Abstract

Neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), include the progressive loss of structure and function of neurons leading to neuronal death. All of these diseases are fatal, as there is no cure for them. The causes of these diseases are unknown; however, there are many proposed mechanisms that lead to neurodegenerative diseases. Oxidative stress is the leading cause of cell death in neurodegenerative diseases, in addition to other mechanisms including endoplasmic reticulum stress, proteasome inhibition, nitrosative stress, inflammation and excitotoxicity. More understanding of the death mechanisms at work in neurodegeneration is necessary to find ways to block those mechanisms in order to slow or stop the progress of these disorders. Interest in nutraceutical compounds has increased recently because of the discovery their bioactive properties. Many studies have suggested that nutraceutical compounds have the ability to inhibit the different death pathways.

In this study, we investigated the neuroprotective efficacy of seven natural compounds in several *in vitro* models of cell death. In primary cultured rat cerebellar granule neurons (CGNs), pre-incubation with caffeic acid, which is abundant in green coffee beans, completely protected against cell death induced by several mechanisms. Caffeic acid protected against effects of sodium nitroprusside (SNP), an agent that induces nitrosative stress, glutamate /glycine which prompts excitotoxicity, hydrogen peroxide, an inducer of oxidative stress, proteasome inhibitors, 5K medium that induces caspase-dependent apoptosis, and endoplasmic reticulum stressors. Furthermore, caffeic acid reduced inflammation induced by lipopolysaccharides in BV2 mouse microglial cell line. Chlorogenic acid and ferulic acid protected from one agent. Chlorogenic acid only protected CGNs from SNP; however, ferulic acid protected the CGNs from glutamate toxicity. Quinic acid did not protect from any agents or stressors. Rosmarinic acid protected the CGNs against glutamate toxicity, and carnosic acid protected the cells from 5K medium that typically induces apoptosis. Both rosmarinic acid and carnosic acid protected the cells from SNP and subsequent nitrosative stress. We further suggest that carnosic acid protects CGNs from caspase-dependent apoptosis through activation of the phosphoinositide 3-kinase (PI3K) pro-survival pathway. Pyrroloquinoline quinone (PQQ) also showed a significant protection against oxidative stress typically induced by hydrogen peroxide and copper chloride. The cumulative data suggest that nutraceutical compounds hold promising therapeutic implications for neurodegenerative disease.

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COMPOUNDS AGAINST MECHANISTICALLY DISTINCT CELL DEATH
INDUCING AGENTS.**

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The Faculty of Natural Sciences and Mathematics

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Faten I. Taram

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CHAPTER ONE: INTRODUCTION

1.1. Mechanisms of neurodegenerative diseases

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are progressive nervous system disorders defined by loss of neurons consequent to multiple factors including, mis-folding and dysfunctional trafficking of proteins, mitochondrial dysfunction, excitotoxicity, and oxidative stress (Ferraiuolo et al., 2011; Dawson and Dawson 2003; Chakrabarti and Mohanakumar, 2016). Neurodegenerative diseases typically target elderly people between 50-70 years old. While many neurodegenerative movement disorders cause muscle atrophy, resting tremor, and, postural instability (Thomas and Beal, 2007; Ferraiuolo et al., 2011), it is also common for many of these disorders to affect patients mentally by disrupting cognitive function and causing loss memory (Anand et al., 2013). Nonetheless, the underlying cause and mechanism of neuronal cell death remains largely unknown. For instance, in the case of ALS, epidemiological studies have revealed that the disease is caused by a familial mutation in only 5-10 % of patients, while the majority of patients harbor the sporadic ALS with no known genetic component (Thomas and Beal, 2007; Ferraiuolo et al., 2011; Anand et al., 2013). It is important to unravel the

precise signaling pathways and etiology that underlie fatal neurodegenerative diseases as only few medications have been approved by US Food and Drug Administration (FDA). Further, the medications that are currently FDA-approved merely help to decrease the severity of symptoms but do not cure the underlying cause of the disease (Kumar et al., 2015). Furthermore, many of these drugs are not very effective, and a number of patients do not respond to treatment. Although each disease has its own molecular hallmark and unique etiology, it is intriguing that disruption of similar cellular processes (e.g. oxidative stress, inflammation, etc.) is believed to be common to several neurodegenerative diseases. Thus, more efforts must be made to discover drugs that target several common molecular pathways that underlie these diseases. Further understanding of the pathological mechanisms contributing to neuronal cell death may be an encouraging method to identify novel therapeutics for the treatment of these devastating neurodegenerative disorders.

1.1.1. Apoptosis.

Apoptosis, or programmed cell death (PCD), is a major cell death mechanism. Apoptosis is characterized by cytoplasmic condensation, nuclear fragmentation, cell rounding, cytoskeletal collapse, and membrane blebbing (Ghavami et al., 2014). Disruption of cellular survival and death pathways such as apoptosis are believed to play a critical role in neurodegenerative diseases. At the cellular level, the caspase family of proteins are the key executioners in apoptosis (Stroh et al., 2002). There are two types of caspases: initiator and effector caspases (Ghavami et al., 2014). The initiator caspase group includes caspase-8 and caspase-9, both of which are activated by upstream signals. Following activation of caspases-8 and caspases-9, the signal is propagated by activation

of the downstream effector caspases, caspase-3 and caspase-7 (Los et al., 1995; Fuchs and Steller, 2011; Ghavami et al., 2009b). External apoptosis is mediated by cell death molecules like TRAIL, TNF α , and Fas, which activate caspase -8. However, caspase-9 is activated by internal stress such as starvation and mitochondrial dysfunction (Hashemi et al., 2013; Los et al., 1995; Fuchs and Steller, 2011). In response to stress, cytochrome *c* is released from the mitochondria, causing the formation of the apoptosome complex, or the caspase-9 activating complex (Fuchs and Steller, 2011). There are two types of apoptotic pathways; the extrinsic apoptotic pathway and the intrinsic apoptotic pathway (Fig. 1.1). In the extrinsic pathway, implicated death receptors recruit adaptor molecules to their death domain, activate caspase-8, and subsequently activate caspase-7 and caspase-3 to cause cell death. In the intrinsic apoptotic pathway, the imbalance in the level of pro-apoptotic Bcl-2 family members (Bax, Bak, etc.) and anti-apoptotic members (Bcl-2, Mcl-1, etc.), results in the formation of Bax/Bak oligomers in the outer mitochondrial membrane, which forms a pore to release cytochrome *c*. To enhance our understanding of the intrinsic apoptosis pathway, primary cultures of cerebellar granule neurons (CGNs) provide an ideal and commonly used model for this type of study. These cells survive under high extracellular potassium concentrations, and die by apoptosis when cultured in a low level of potassium (D'Mello et al., 1993). To study apoptosis, CGNs exposed to low concentrations of extracellular K⁺ offer a good *in vitro* model (Lauritzen et al., 2003) (Fig. 1.2).

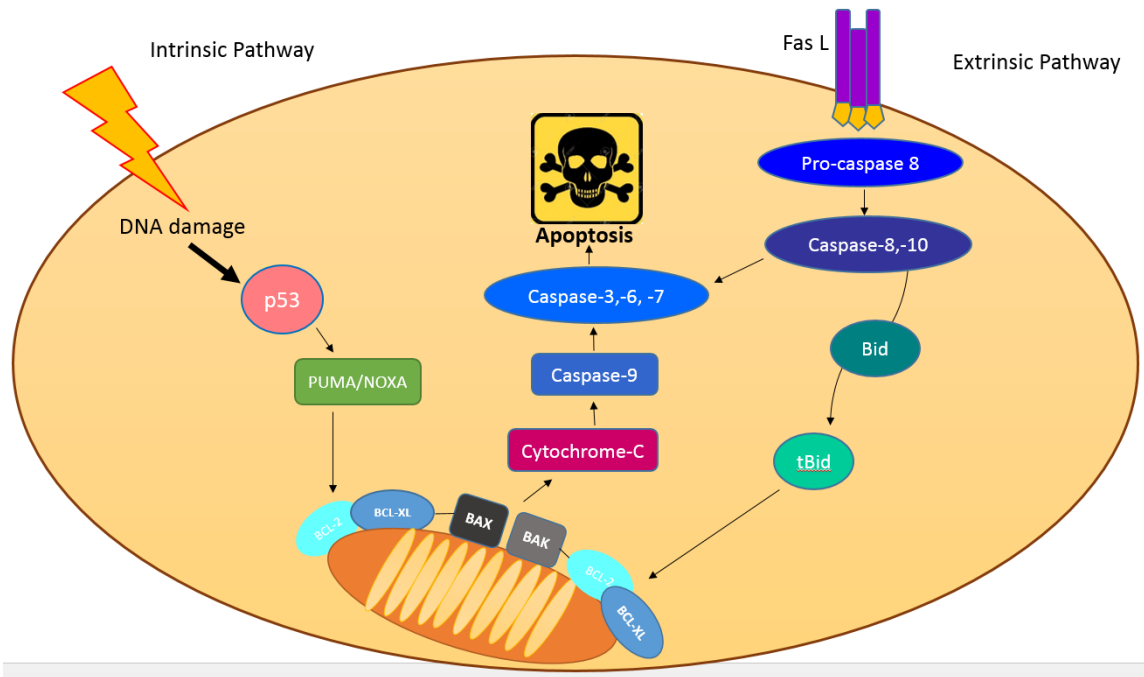


Figure 1.1. Overview of Apoptotic Pathways. The extrinsic and intrinsic pathways illustrate the binding of the death receptor Fas by the death ligand Fas L, activation of pro caspases to caspases leading to apoptosis. Cleavage of Bid to t-Bid shows a conjunction point between the extrinsic and intrinsic pathway where the inhibition of pro-survival proteins (Bcl2/Bcl-Xl) leads to the activation of Bax and Bak. Cytochrome c is released from the mitochondria leading to the activation of caspases and then apoptosis.

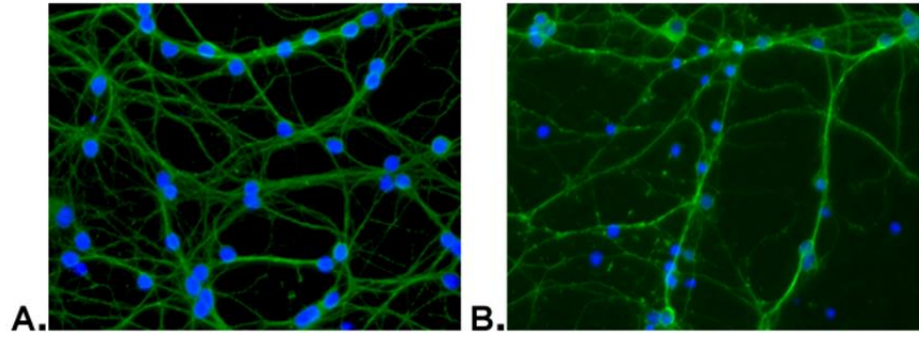


Figure. 1.2. Cerebellar granule neurons (CGNs)-a standard model system in which to study apoptosis. A. CGN culture was treated with vehicle (control). B. CGN culture was treated with H₂O₂.

1.1.2. Oxidative stress.

Strong scientific evidence suggests that oxidative stress is one of the most common known mechanisms contributing to degenerative diseases. Oxidative stress is also thought to contribute to aging, which is one of the highest risk factors for neurodegenerative diseases. Oxidative stress underlies additional factors that are believed to lead to neurodegenerative diseases, such as mitochondrial dysfunction and excitotoxicity (Duffy et al., 2011; Rao and Weiss, 2004).

Free oxygen radicals such as hydroxyl ($\text{OH}\cdot$), superoxide ($\text{O}_2^{\cdot-}$) and nitric oxide ($\text{NO}\cdot$), as well as, other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), are produced in our bodies and play an essential role in a variety of critical biological processes such as gene transcription (Uttara et al., 2009). The production of ROS is known to be regulated by antioxidants such as glutathione, polyphenols, and creatine (Uttara et al., 2009). The balance between ROS production and the availability of antioxidants is critical for determining cell fate, as imbalances between the two results in oxidative damage to many fundamental molecules (lipid, protein, and DNA), leading to oxidative stress and potentially cell death (Fridovich, 1999). Current research suggests that oxidative stress may also arise from genetic mutations. For instance, both oxidative stress and neuropathology develop in people with hemochromatosis-associated mutations (Gordeuk et al., 1992). As oxidative stress is one of the most common pathological mechanisms underlying neurodegenerative disorders, animal models demonstrating key characteristics of oxidative stress will be useful to find new therapies for these diseases. For instance, one current model treats animals with toxicants that induce oxidative stress

(i.e. paraquat) and then observes the effect of this insult. Another approach to modeling oxidative stress is a genetic one. Transgenic mice that have been manipulated to express different genes, like super oxide dismutase 1 (SOD1), super oxidative dismutase 2 (SOD2), and glutathione peroxide 1 (GPx1), provide a suitable approach to study oxidative stress. Moreover, there are a number of genes in *Drosophila melanogaster* and *Caenorhabditis elegans* have been studied to model the biological effects caused by the production of toxic ROS (Melov, 2002). Each of these model systems is helping to unravel the contribution of oxidative stress to neurodegenerative disorders.

1.1.3. Inflammation.

Inflammation is a response caused by pathogenic or irritant stimuli that results in the activation of an organism's defensive response against that stimulation. Acute inflammation is inherently protective; however, aberrant inflammation may lead to the destruction of the host cell. Strong evidence supports a role for neuroinflammation in various neurodegenerative disorders including AD, PD, and ALS.

Neuroinflammation is a process that includes activation of glia cells, including microglia and astrocytes. Microglia exert a dual role in the progression of neurodegenerative diseases, as they have the potential to cause both protection and degeneration in the central nervous system (CNS). In the short term, microglia have a major role in the inflammatory process, and protect the CNS integrity; however, when they are constitutively activated, the morphological characteristics of the cell become more phagocytic, and display increased levels of the major histocompatibility complex (MHC) antigens that recognize foreign molecules (Hayes et al., 1987). Microglia also produce inflammatory cytokines, which are responsible for enhancing the inflammatory

reaction as a protective measure in neurons exposed to an irritant stimulus. However, when chronically activated microglia may cause toxicity to neurons. This type of toxicity in the CNS is a pathogenic hallmark of several neurodegenerative disorders, and is initiated by the production of inflammatory cytokines like IL-1 β , TNF- α , and IL-6 (Glass et al., 2010). Furthermore, activated microglia can also engulf intact cells, leading to neurodegeneration (Glass et al., 2010). Studying the mechanism of neuro-inflammation is a critical way to modulate many chronic neurodegenerative disorders linked to this mechanism. There are different types of models to study neuro-inflammation including viral, drug administration, and genetic models. There are three genetic mouse models of Alzheimer's diseases that are known to cause microgliosis and astrogliosis (activated microglia and astrocytes, respectively). These models include APP/PS1, P301S, Tau, and Tg2576. Another model of neuro-inflammation is AVV- α -synuclein a common viral model of Parkinson's disease. Administration to mice of the neurotoxin MPTP, is another model of Parkinson's disease used to study the neuro-inflammatory phenotype of this disease (Jackson-Lewis and Przedborski, 2007). Lipopolysaccharide administered to animals or cultured cells provides another technique to study neuro-inflammation *in vitro* (Lee et al., 2008; Gui et al., 2012).

1.1.4. Excitotoxicity.

Excitotoxicity plays a substantial role in many human diseases such as ischemia, trauma, and neurodegenerative disease (Doble, 1999). Excitotoxicity is defined as an extreme stimulation of glutamatergic receptors by the excitatory amino acid neurotransmitter, glutamic acid. Aberrant stimulation of glutamatergic receptors leads to enhanced intracellular calcium elevation (Doble, 1999). As a result of overstimulation of

glutamate receptors due to either elevated synaptic levels of glutamate or increased sensitivity of the postsynaptic neuron to glutamate, excitotoxicity can trigger neuronal cell death (Van Damme et al., 2005).

Regulation of neuronal glutamate uptake is tightly controlled by a variety of diverse mechanisms, which may be implicated in excitotoxicity when glutamate levels are dysregulated. For instance, glutamate undergoes reuptake from the synapse by glutamate transporter, excitatory amino acid transporter 2 (EAAT2) (Ferraiuolo et al., 2011). In addition, α – Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are also implicated in glutamate excitotoxicity, as they are responsible for much of the glutamatergic neurotransmission in the CNS. The permeability of AMPA receptors to calcium is dependent on its GluR2 subunit (Williams et al., 1997), and low expression of GluR2 leads to increase Ca^{2+} influx and neuronal toxicity (Ferraiuolo et al., 2011). N-Methyl-D-aspartate (NMDA) is another glutamate receptor that is believed to contribute to both excitotoxicity and thus, neurodegenerative diseases (Zhou et al., 2013). When NMDA receptors are over activated, excessive neuronal Ca^{2+} influx can lead to enhanced excitotoxicity and subsequent neuronal cell death (Zhou et al., 2013).

Given the role of glutamate in regulating neuronal Ca^{2+} influx, it is not surprising that glutamate excitotoxicity is associated with many other pathways that have been implicated in contributing to the etiology of neurodegenerative disorders. For instance, excitotoxicity has been intimately linked to mitochondrial dysfunction, the production of toxic ROS and reactive nitrogen species (RNS), loss of mitochondrial membrane potential, and apoptotic cell death (Prentice et al 2015; Didier et al., 1996; Finiels et al., 1995).

In order to understand the contribution of excitotoxicity to neurodegeneration, several suitable models to study this phenomenon have been designed, such as the lurcher mouse. These transgenic mice possess a mutation in an excitatory amino acid receptor delta2 (GluRdelta2) that causes excitotoxicity and is a relevant model for excitotoxicity cell death (Philips, 1960). However, more work is needed to test the specific involvement of excitotoxicity in the pathogenesis of various neurodegenerative disorders. Further, since this mechanism is implicated in various other pathological pathways, it is important to develop drugs that block excitotoxicity in order to develop promising therapeutics for the treatment neurodegenerative diseases. Interestingly, the only drug that is currently approved by the FDA for the treatment of ALS, riluzole, targets glutamatergic receptors. However, riluzole only extends the survival of ALS patients a mere three months on average (Zoccolella et al., 2007), underscoring the need to precisely delineate the involvement of excitotoxicity in the progression of various neurodegenerative diseases such as ALS.

1.1.5. Reactive nitrogen species

Another possible factor underlining the etiology of neurodegenerative diseases is the overproduction of reactive nitrogen species (RNS). For example, the generation of nitric oxide may accumulate as a consequence of excessive activation of the glutamatergic receptor that increases the entry neuronal Ca^{2+} levels. In turn, this leads to the activation of neuronal NO synthase (nNOS) (Garthwaite et al., 1988; Bredt et al., 1991). In addition to the generation of nNOS, mitochondria have a well-described role in generating free radicals during respiration, such as super-oxide anion, which reacts with

nitric oxide to form the toxic product peroxynitrite (Beckman et al., 1990; Lipton et al., 1993). Nitrosative stress has also been demonstrated to contribute to the misfolding of proteins, which has been described in many neurodegenerative diseases (Nakamura and Lipton, 2007). Yu and Chuang (1997) established that treatment with sodium nitroprusside (SNP), a nitric oxide donor (NO), is a suitable model to generate nitrosative stress *in vitro*. Thus, treating cell cultures with specific concentration of SNP is a well-established model to study nitrosative stress and to understand the pharmacological pathways regulating RNS-induced neuronal death.

1.1.6. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an essential organelle responsible for many cellular processes including the synthesis, folding, modification, and transport of proteins (Paschen and Frandsen, 2001; Breckenridge et al., 2003; Rao et al., 2004). Nevertheless, under some genetic and environmental conditions, ER stress can lead to activation of the unfolded protein response (UPR). It is well documented that activation of the UPR can enhance aggregation of misfolded proteins, and subsequently promote cell death (Lindholm et al., 2006). The involvement of the UPR to the progression of neurodegenerative diseases has been studied in a variety of contexts (Scheper et al., 2015), and one mechanism by which ER stress may contribute to neuronal cell death is through activation of mitochondrial apoptosis (Lindholm et al., 2006). Given this important involvement of ER stress in maintaining cellular survival, it is not surprising that dysregulation of the UPR is one of the mechanisms that scientists are investigating as a promising target for such diseases.

To study ER stress, Iwawaki et al. (2004) established the ER stress-activated indicator (ERAI) in living cells. In addition, ER stress may also be studied *in vitro* by using different insults like tunicamycin, thapsigargin, and Brefedin A, depending on the type of the cell culture. In future studies, these tools will be important for elucidating the specific involvement of ER stress in neuronal cell death.

1.1.7. Proteasome dysfunction.

Every cellular system survives by maintaining a balance between the production and the degradation of proteins, which determines on the lifespan of the protein. The proteasome is a multi-subunit enzyme that plays a fundamental role in protein degradation by degrading all abnormal proteins and expired proteins (Tanaka et al., 2014). However, dysfunctional proteasomes can lead to protein mis-folding and aggregation of abnormally modified proteins, thereby promoting in cell death. MG132 (carbobenzoxy-Leu-Leu-Ieucinal) is one tool used to study proteasome inhibition. This aldehyde can block proteasome activity, causing the cell to switch to other mechanisms that play a role in neurodegenerative disorders, including apoptosis and mitochondrial dysfunction (Wu et al., 2002; Ling et al., 2003; Qiu et al., 2000). In addition, there are several animal models to study proteasome inhibition through ablation of particular proteasome subunits, such as deletion of all of the $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits (Kincaid et al., 2012). Moreover, deletion of the thymo-proteasome subunit $\beta 5t$ provides an additional model of proteasome dysfunction (Murata et al., 2007).

Modulation of each of the mechanisms discussed above offers promising approaches to identify novel molecular pathways as targets the therapeutic treatment of devastating neurodegenerative diseases with no known cause (Fig. 1.3).

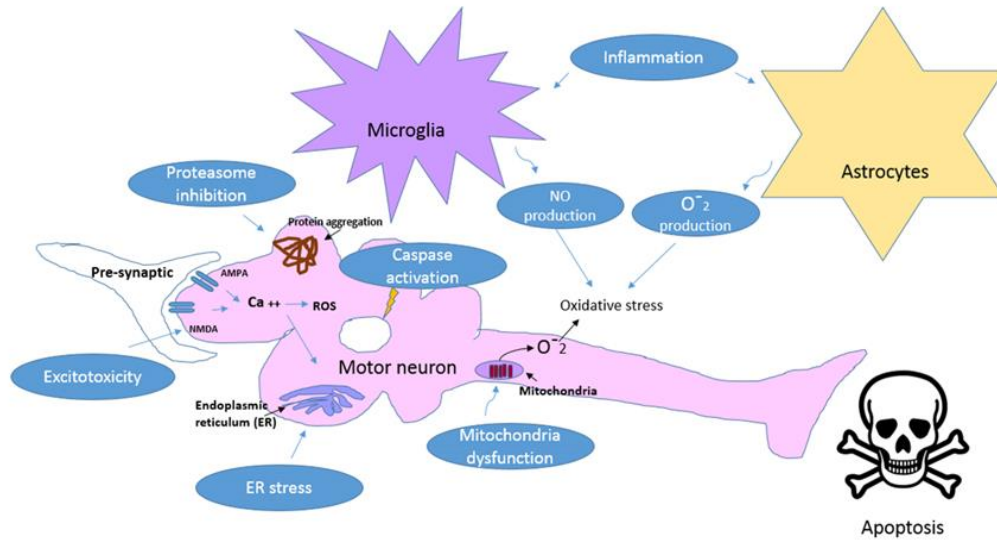


Figure. 1.3. Overview of the common pathogenic mechanisms of neurodegenerative diseases. Neuron death is the main cause of neurodegenerative diseases. Many mechanisms contribute to neuron death including activated astrocytes and microglia, oxidative stress, mitochondrial dysfunction, excitotoxicity, proteasome inhibition, and endoplasmic reticulum stress.

1.2. Nutraceutical compounds

After many clinical studies failed aimed to treat neurodegenerative disorders, with the long duration necessary to approve new drugs, and the increasing number of patients with neurodegenerative disorders, scientists have begun to investigate novel therapeutics for these diseases based on the underlying disease mechanisms. New pathways of exploration include patient lifestyle choices and a new examination of traditional and/or holistic therapies that may modulate the several of the common molecular pathways implicated in these disorders. In particular, oxidative stress and neuro-inflammation are two specific pathways which are implicated in several distinct diseases including PD, AD, and ALS. To block these pathways, scientists are beginning to study natural compounds (nutraceuticals) with antioxidant and anti-inflammatory properties.

Since many nutraceuticals are not regulated by the FDA, they are not subject to the same regulatory hurdles as pharmaceutical drugs. The term “nutraceutical” is created from two root words; nutrition and pharmaceutical. This term was originally coined by Stephen De Felice, the chairman of the Foundation for Innovation in Medicine (FIM), Cranford NJ in 1989 (Maddi et al., 2007; Brower 1998). According to Dr. Felice, the nutraceuticals cover any food or part of a food that possess healthy properties which can provide benefit against diseases. Nutraceuticals can be extracted as a product of food, and can be taken as a powder or supplement (Wildman, 2001; Bull, 2000). Many studies have investigated the medical benefits of a number of nutraceuticals, including those against cold and cough, sleeping disorders, cancer, depression, diabetes, and various neurodegenerative diseases (Pandey et al. 2010; Bhullar and Rupasinghe, 2013). In

addition to the bioactivities of nutraceutical compounds, many studies indicate that some of these compounds have the ability to cross the blood brain barrier and gain access to specific regions of the brain to provide beneficial effects (Choi et al., 2012). Thus, there is great interest in identifying new nutraceutical compounds with therapeutic effects for the treatment of neurodegenerative diseases. One class of these compounds that has gained significant attention is polyphenols.

1.2.1. Polyphenols.

Polyphenols are compounds produced as secondary metabolites, and are found in many plants, fruits, vegetables spices, oils, herbals, teas, and wine (Fig.1.4). These compounds possess significant antioxidant properties through their ability to either scavenge free radical species or increase the levels of antioxidants in organisms (Choi et al., 2012). The neuroprotective effect of polyphenols is due to their intrinsic antioxidant activity and their ability to enhance the capacity of endogenous cellular antioxidant molecules via activation of the Nrf2/ARE pathway. In turn, Nrf2 activates and modulates many antioxidants like γ -glutamylcysteine ligase, the rate-limiting enzyme for glutathione synthesis and an essential antioxidant compound (Dumont and Beal, 2011; Moskaug et al., 2005). In addition to the antioxidant properties of polyphenols, many *in vitro* studies have identified polyphenols as important modulators of other cellular process such as apoptosis, gene expression, and intracellular signaling (Duthie et al., 2003).

Anti-inflammatory activities of polyphenols have also been reported (González et al., 2011), therefore increasing insight into their ability to reduce the risk of many age-related disorders, such as neurodegenerative disorders. Many studies demonstrate that the

anti-inflammatory effects of polyphenolic compounds lead to a reduction the production or the level of many cytokines and transcription factors which are related to the inflammation process. For example, one *in vivo* study found that polyphenolic compounds can suppress multiple inflammatory biomarkers (Parelman et al., 2012). Moreover, diets containing fruits that are rich in polyphenols have been shown to reduce the level of Interleukin 6 (IL-6), a major pro-inflammatory cytokine (Joseph et al., 2016). In addition, nitric oxide (NO), one of the inflammatory mediators, is reduced by several different polyphenolic compounds (Soliman and Mazzio, 1998).

An additional inflammatory marker shown to be modulated by polyphenols is the nuclear transcription factor (NF- κ B). Under normal conditions, this transcription factor is found in the cytoplasm, but once activated by free radicals or inflammation, it translocates to nuclei and induces transcription of many genes involved in cellular process such as inflammation (Butcher and Denkers 2002). Different polyphenolic compounds have been shown to significantly inhibit the activity of NF- κ B transcription factor. Several studies suggest that a diet comprised of a high intake of polyphenol-rich foods improves cognitive performance in elderly people and reduces risk of dementia (Nurk et al., 2010; Polidori et al., 2009; Hughes et al., 2010; Morris et al., 2006). It has been reported that elderly people who are at the highest risk for degenerative disorders, demonstrate decreases in important antioxidants, which leads to the accumulation of ROS, an underlying pathophysiologic mechanism that many contribute to the onset or progression of neurodegenerative diseases. Thus, polyphenolic rich diets are one promising area being investigated as a mechanism to reduce neurodegeneration.

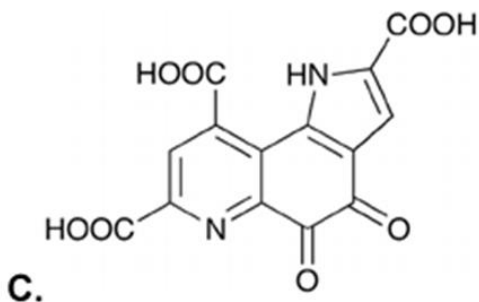
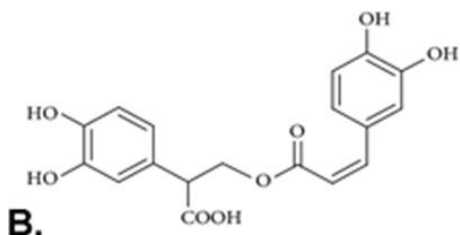
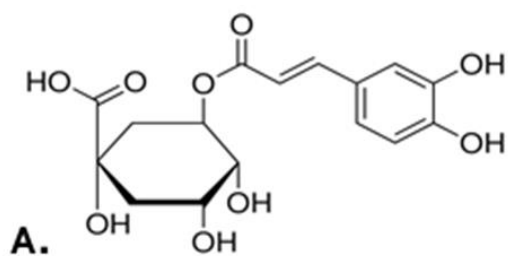


Figure 1. 4. Common polyphenols and their natural sources. A. Chemical structure of chlorogenic acid and one of its major sources, green coffee beans. B. Chemical structure of rosmarinic acid and rosemary herb, one of its main sources. C. Chemical structure of pyrroloquinoline quinone (PQQ) and one of its major sources green tea.

1.2.1.1 Coffee

Coffee is the second most consumed drink in the world (Bae et al., 2014). Coffee contains different chemicals including caffeine, carbohydrates, lipids, vitamins, minerals, alkaloids, and polyphenols (Spiller, 1984). Many studies have demonstrated that coffee intake may have pharmacological benefits for the treatment of several chronic diseases. For example, in a study examining the effect of coffee on type 2 diabetes, coffee intake significantly reduced the risk of type 2 diabetes in women (Van Dam et al., 2006). Moreover, coffee consumption was demonstrated to decrease the risk of liver disease by controlling the level of enzymes that are well known to elevate liver damage (Homan and Mobarhan 2006). Coffee consumption has also been shown to reduce the risk of different type of cancer (Cano-Marquina et al., 2013). Finally, coffee intake has been demonstrated to mitigate the development of neurodegenerative diseases. Indeed, two studies demonstrated that drinking coffee on a regular basis lowered the risk of developing either Parkinson's disease or Alzheimer's diseases (Trevitt et al., 2009; De Mendonca et al., 2010).

One the major polyphenolic component in coffee is chlorogenic acid (Figure. 1.5) (Sato et al., 2011; Upadhyay and Mohan Rao, 2013). Chlorogenic acid is formed by the esterification of cinnamic acids, such as caffeic acid and ferulic acid, with quinic acid (Fang et al., 2016). The benefits of chlorogenic acid are attributed to several biological effects, such as anti-inflammatory and antioxidants activities (Shi et al., 2016). The antioxidant activity of chlorogenic acid is related to its chemical structure, which contains a phenolic hydroxyl group known to scavenge both the hydroxyl radical and superoxide anion radical (Bohn et al., 2014; Gallus et al., 2002; Padol and Hunt, 2004; Phan et al.,

2003). Chlorogenic acid can also increase the expression of nuclear Nrf2, which is a known transcriptional regulator of many pro-survival antioxidant genes (Shi et al., 2016). Furthermore, chlorogenic acid suppresses the NF- κ B-dependent transcription of pro-inflammatory signaling pathway components and reduces the expression of pro-inflammatory genes, including cytokines, chemokines, and adhesion molecules (Shen et al., 2012). In addition, chlorogenic acid also has been reported to exhibit significant pro-survival biological activities including anti-carcinogenic (Rakshit et al., 2010), anti-obesity (Cho et al., 2010), anti-microbial (Lou et al., 2011), anti-tumor (Granado-Serrano et al., 2007), anti-lipidemic activity, and anti-diabetic effects (Ong et al., 2013). Recent research indicates that chlorogenic acid may also inhibit the activity of HIV-1 RNase (Naso et al., 2014). In an *in vivo* study, chlorogenic acid reduced infarct volume and rescued sensory motor function in a rat model of transient middle cerebral artery occlusion (Lee et al., 2012). Chlorogenic acid has been suggested to reduce the risk of many health issues like type 2 diabetes and cardiovascular disease (Ranheim and Halvorsen 2005; Salazar-Martinez et al., 2004).

Many findings indicate a promising effect of chlorogenic acid on neuronal survival. For instance, chlorogenic acid directly protects neurons *in vitro* from cytotoxicity and death that are induced by glutamate, perhaps by controlling the entry of Ca²⁺ influx (Mikami and Yamazawa 2015). New evidence has also supported the positive effects of chlorogenic acid on the progression of specific neurodegenerative disorders, such as AD. In the Oboh et al. (2013) study, the authors demonstrated that chlorogenic acid inhibits acetylcholinesterase (AChE) and butyrylcholinesterase (BChE),

the key enzymes associated with the development of AD. Thus, chlorogenic acid is a nutraceutical with significant promise for neurodegenerative disease therapy.

More recently, a growing interest examining the pharmaceutical effects of the chlorogenic acid-derivative, caffeic acid, is emerging. Caffeic acid is a polyphenol compound that is abundant in many agriculture products such as fruits, vegetables, teas, and coffee (Moridani et al., 2001; Scalbert et al., 2005). Caffeic acid has a wide variety of health benefits including anti-inflammatory, anti-cancer, anti-thrombosis, anti-hypertensive, anti-fibrosis, anti-viral, anti-oxidative, and anti-tumor properties (Touaibia et al., 2011; Rehman and Sultana, 2011; Roos et al., 2011; Scapagnini et al., 2011). A study on human peripheral blood mononuclear cells showed that cells pretreated with caffeic acid, and subsequently exposed to H₂O₂, are protected from the apoptotic damage that is induced by this ROS-inducing compound. The authors further demonstrated that the protection against ROS may have been afforded by the free radical scavenging properties of caffeic acid. Moreover, this study demonstrated that caffeic acid inhibits lipid peroxidation and protects DNA from oxidative damage (Khanduja et al., 2006).

Caffeic acid also has been shown to have promising effects in animal models of AD. In one study, Kim et al. (2015) found that administration of caffeic acid to mice injected with A β 25-35 peptide protected against both A β toxicity and oxidative stress, which are two of the most common hallmarks of AD (Kim et al., 2015). In the methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model of PD, several inflammatory factors are induced including interleukin (IL)-1beta, IL-6, and tumor necrosis factor (TNF)-alpha. However, administration of caffeic acid to mice inhibited pro-inflammatory the effect of MPTP treatment (Tsai et al., 2011). Caffeic acid was also demonstrated to

prevent neurotoxicity induced by acrolein *in vitro*. This insult has the ability to mimic many mechanisms that underlie neurodegenerative pathways, such as those activated in AD. Indeed, pretreating cells with caffeic acid decreased neurotoxicity, ROS, and also preserved the essential endogenous antioxidant, glutathione (Huang et al., 2013). Thus, similar to chlorogenic acid, caffeic acid is neuroprotective in multiple models of neuronal toxicity and neurodegeneration.

Ferulic acid is another polyphenol compound found in coffee that is also a chlorogenic acid derivative. Recently, scientists have focused their attention on the potential of ferulic acid to treat many disorders given its anti-oxidant activity and anti-inflammatory activities. Ferulic acid modulates many anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), as well as the chaperone heat shock protein (Hsp)-70 (Mancuso and Santangelo, 2014). Ferulic acid also has the ability to scavenge free radicals, and it is a promising therapy for various diseases that depend on the downregulation of NF-kB and enhancing the expression of antioxidant enzymes, such as cancer, cardiovascular diseases, and diabetes, (Mancuso and Santangelo, 2014).

The pharmacological activities of ferulic acid also suggest that it may be a novel therapy for neurodegenerative diseases, such as AD. In a transgenic mouse line fused with A β peptide, pretreatment with ferulic acid considerably reduced memory loss and decreased the level of neuro-inflammation, reactive gliosis, and interleukin 1 beta (IL-1B) production (Yan et al., 2001). Further, a study by Cho et al. (2005) demonstrated that long-term administration of ferulic acid reduced astrocyte activation induced by h-amyloid peptide (1–42) *in vivo*. Higher doses of ferulic acid led to greater reductions of the inflammation bio-markers *in vivo* (Jin et al., 2005), and ferulic acid was also shown to

attenuate the microglia activated by LPS in a rat model of chronic neuro-inflammation (Wenk et al., 2004). Moreover, in a similar manner, an *in vitro* study showed that ferulic acid reduces several biomarkers of inflammation that are induced by LPS (Sakai et al. 1997).

Quinic acid is another chlorogenic acid metabolite, yet unlike caffeic acid and ferulic acid, quinic acid does not possess a phenolic hydroxyl group. Interestingly, the phenolic hydroxyl group is the main structural basis for the antioxidant properties of these compounds (Pandey and Rizvi 2009). Thus, perhaps it is not surprising that there are not currently many studies examining the neuroprotective effects of quinic acid. Nonetheless, limited evidence has demonstrated that quinic acid reduces NF-KB activation and displays anti-viral effects *in vitro* (Akesson et al., 2005; Wang et al., 2009).

Further, several studies described the bioactivities of quinic acid derivatives: 5-O-trans-o-coumaroylquinic acid methyl ester, chlorogenic acid methyl ester, 4, 5-dicaffeoyl quinic, and macranthoin G (Hur et al., 2001). 4,5-dicaffeoyl quinic acid, one of its derivatives showed a significant neuroprotective effect against A β - induced toxicity *in vitro* (Hur et al., 2001). (2)3, 5-dicaffeoyl-muco-quinic acid, another derivative has the ability to enhance neurite outgrowth of PC12 cells (Hur et al., 2001). Thus, despite evidence demonstrating that quinic acid may be able to modulate neuronal survival, studies do not suggest that quinic acid mediates neuronal survival through significant antioxidant and/or anti-inflammatory properties, as do other chlorogenic acid metabolites containing a phenolic hydroxyl group.

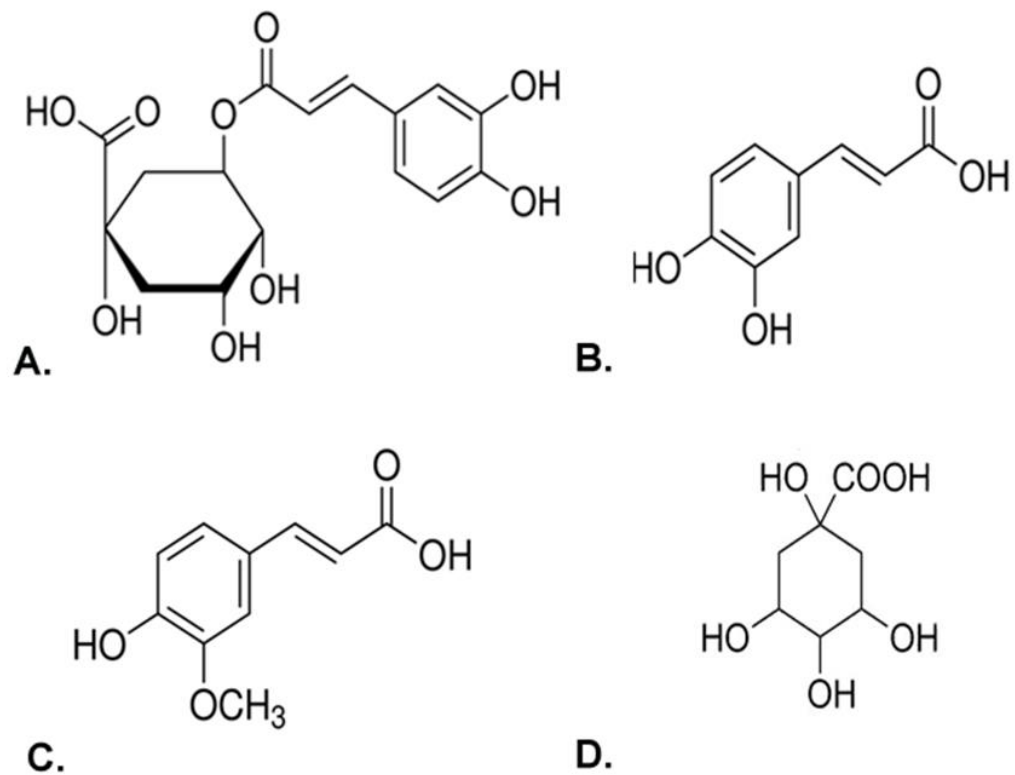


Figure. 1. 5. Chemical structure of chlorogenic acid and its three major metabolites. A. Chemical structure of chlorogenic acid. B. Chemical structure of caffeic acid. C. Chemical structure of ferulic acid. D. Chemical structure of quinic acid.

1.2.3. Rosemary.

Medical plants have been used as a major source of various pharmacological agents for many decades. Rosemary is a Mediterranean herb that is considered a traditional therapy for many health conditions such as rheumatic diseases (Bellumori et al., 2015). Many studies have shown numerous bioactivities of rosemary extract including antioxidant, free radical scavenging, anti-inflammatory, antimicrobial, antipyretic, and antidiabetic properties (Erkan et al., 2008; Basaga et al., 1997; Arranz et al., 2015; Laham et al., 2013; Al attar and Shawush, 2015; Bakirel et al., 2008). All of these activities may be related to its major polyphenol components, which are carnosic acid and rosmarinic acid (Ribeiro et al., 2016) (Fig. 1.6). Rosmarinic acid, one of the major rosemary polyphenol constituents, has been reported in several studies to possess several pharmaceutical properties like anti-inflammatory, anti-oxidant, antitumor, anti-viral, and antimicrobial effects (Bulgakov et al. 2012; Wu and Wang, 2012).

The promising properties of rosmarinic acid prompted scientists to investigate the effect of this compound on human health disorders including, AD (Vladimir-Knezevic et al., 2014), cardiovascular disease (Karthik et al. 2011), and allergic inflammation (Sanbongi et al. 2004). Rosemary extract produced a dramatic inhibition of astrogliosis in a rat model of repetitive mild traumatic brain injury by decreasing the levels of the neuroinflammatory biomarkers, IL-1 β , IL-6, and TNF- α (Song et al., 2016). The protective effects of rosmarinic acid have also been investigated in a mouse model of ALS. Administration of rosmarinic acid to ALS mice expressing a mutant SOD1 transgene, increased the life span, improved spinal cord morphology, prevented body weight loss, and increased motor performance (Shimojo et al., 2010).

Rosmarinic acid has also been demonstrated to protect skin cells from the exposure of solar ultra-violet radiation based on its antioxidant and anti-inflammatory activities (Kim et al., 2015). The anti-tumor effect of rosmarinic acid established in the Kim et al. (2015) study was based on the compound enhancing TNF- α -induced-apoptosis in human leukemia U937 cells. The antioxidant effects of rosmarinic acid were mediated via its free radical scavenging and this led to its protection from oxidative stress (Perez-Fons et al., 2010). Suppression of ROS, intracellular glutathione depletion, and suppressing different inflammatory cytokines has been demonstrated by treating human cells with rosmarinic acid (Zdarilova et al., 2009). Altogether, increasing evidence suggests that rosmarinic acid suppresses many pathways that contribute to neurodegenerative diseases.

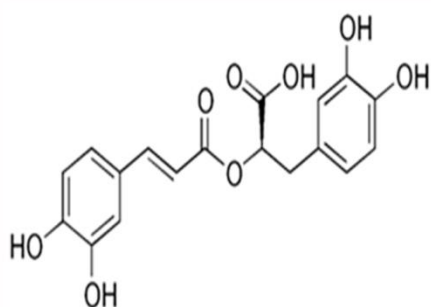
Carnosic acid is another polyphenolic compound abundant in rosemary (*Rosmarinus officinalis* L.), and it has been proposed that the majority of the antioxidant activity of rosemary extract may be attributed to carnosic acid (Aruoma et al., 1992). The chemical structure of carnosic acid captured the attention of researchers due to its similarity to caffeic acid. Intriguingly, carnosic acid contains the same phenolic hydroxyl group that is proposed to contribute to oxygen radical scavenging activity (Nakamura et al., 2003). Numerous health benefits have been observed with carnosic acid such as, antioxidant, anti-inflammatory, anti-carcinogenic, and anti-obesity (Hadad and Levy, 2012; Yesil-Celiktas et al., 2010; Park and Sungb, 2015). Furthermore, carnosic acid enhances the expression of many detoxifying enzymes like glutathione S-transferase (GST) and NADH-quinone oxidoreductase 1 (NQO1) (Debersac et al., 2001). The stimulation of

these types of enzymes may contribute to the ability of carnosic acid to protect cells from ROS and RNS, for instance, in RAW264.7 macrophages (Xiang et al., 2012).

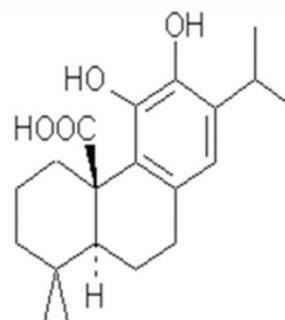
Several studies suggest different intracellular mechanisms to explain the neuroprotective effects of carnosic acid, and many of these mechanisms implicate modulation of the Nrf2 pathway. In this scenario, carnosic acid binds to Keap1, the suppressor of Nrf2, and the latter translocates from the cytoplasm to the nucleus to bind the ARE of various genes. Lastly, the induction of transcription of phase 2 enzymes is activated due to the previous binding (Sato et al., 2008). The anti-viral properties of carnosic acid have also been demonstrated in a study by Shin et al., (2013), which demonstrated that human respiratory syncytial virus (hRSV) replication and infection is inhibited *in vitro* by treatment of human adenocarcinoma alveolar basal epithelial cell line A549 cells with carnosic acid. Further, in an *in vivo* study, carnosic acid showed an anti-inflammatory action by inhibiting the cytokines, cyclooxygenase-2 (COX-2), IL-1 β , and TNF- α , which are known to mediate responses to inflammation in different cells (Mengoni et al., 2011).

The neuroprotective effects of carnosic acid have also been examined in different disease models, both *in vitro* and *in vivo*. In a PD study, the authors demonstrated that pretreatment of either SH-SY5Y neuroblastoma cells or a rat model of PD with carnosic acid, protected against 6-hydroxydopamine (6-OHDA)-induced toxicity. The protection observed was related to the anti-oxidant activity of carnosic acid through its ability to increase protein expression of γ -glutamyl-cysteine ligase catalytic subunit, γ -glutamyl-cysteine ligase modifier subunit, superoxide dismutase, and glutathione reductase. Further, the anti-apoptotic properties of carnosic acid were also observed,

such as blocking the reduction of Bcl-2/Bax ratio and inhibiting 6-OHDA-induced caspase-3 cleavage (Wu et al., 2015). In an *in vitro* model of AD-related toxicity, it was demonstrated that carnosic acid protects cells from A β -induced apoptosis (Meng et al., 2015). Thus, many lines of evidence suggest that carnosic acid may be beneficial for the treatment of neurodegenerative diseases caused, in part, by the production of toxic ROS and/or apoptosis, such as PD and AD.



A Rosmarinic acid



B Carnosic acid

Image credit: <http://www.health-ingredients.com/cosmetic-ingredients/rosemary-extract.html>

Figure 1. 6. Chemical structure of major compounds in rosemary. A. Chemical structure of rosmarinic acid. B. Chemical structure of carnosic acid.

1.2.4. Pyrroloquinoline quinone

Pyrroloquinoline quinone (PQQ) is a bioactive compound that is found naturally in various foods, but with particularly high concentrations in fermented soy beans, green tea, kiwi, green peppers, and papaya (Kumazawa et al., 1995) (Fig. 1.7). Recently, PQQ has received attention due to its multiple bioactivities including antioxidant, anti-inflammatory, anti-diabetic and anti-cancer properties (Yang et al., 2014; Harris et al., 2013; Akagawa et al., 2015; Min et al., 2014). However, it is distinguished from the previous compounds discussed in this thesis by its ability to stimulate mitochondrial biogenesis (Stites et al., 2006). PQQ also influences many essential physiological processes in the cell, which were demonstrated in *in vivo* nutritional studies where mice were given a diet lacking in PQQ. PQQ-deficient mice showed attenuated in growth, immune response, and reproductive performance (Akagawa et al., 2015). Also, a PQQ-deficient diet was shown to lead to reduced mitochondrial quantity, and deficiencies in both lipid and energy metabolism in rats (Bauerly et al., 2011). Human studies have also demonstrated that dietary PQQ is essential for maintaining endogenous antioxidant levels and diminishing pro-inflammatory mediators (Harris et al., 2013). Furthermore, PQQ showed a substantial neuroprotective effect in multiple *in vivo* and *in vitro* studies.

Pretreatment with PQQ prior to exposure to various oxidative stressors, demonstrated a noteworthy neuroprotection in SH-SY5Y neuroblastoma cells (Hara et al., 2007; Nunome et al., 2008). A similar protective effect was observed *in vivo* in rats with dietary PQQ.

Addition of PQQ to the diet markedly improved learning ability when compared to control rats (Ohwada et al., 2008).

The protection afforded by PQQ occurs primarily through modulation of the NF- κ B pathway, resulting in the suppression of numerous inflammatory mediators like TNF- α , iNOS, and IL-1 β , that are enhanced *in vivo* and *in vitro* by LPS treatment (Yang et al., 2014). The mechanism underlying the neuroprotective effects of PQQ against glutamate-induced cell death have also been investigated. PQQ protects neurons from excitotoxicity in a PI3K/Akt-dependent manner that leads to stimulation of Nrf2 and regulation of antioxidant genes (Zhang et al., 2012). Another protection pathway activated by PQQ has been demonstrated by Yang et al. (2015), and involves control of the mitochondria pathway. In their study, PQQ induced the expression of pro-survival Bcl-2, and also decreased the Bax-dependent release of mitochondrial cytochrome c, therefore inhibiting apoptosis. As we mentioned above, PQQ also stimulates mitochondrial biogenesis, which was demonstrated by Chowanadisai et al. (2010) to occur through phosphorylation of cAMP response element-binding protein (CREB), the regulator of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α). The latter, in turn, induces mitochondrial biogenesis by activating nuclear respiratory factors. One important point is that PQQ showed a remarkable protective effect against diverse mitochondrial inhibitors (Chowanadisai et al., 2010). Thus, PQQ is a potential therapy for many diseases that are caused by mitochondrial deficiency, such as PD, which is often referred to as a mitochondrial disorder. In this context, Zhang et al. (2014) studied the signaling pathways that are involved in the protective effects of PQQ against a PD model *in vitro*, elucidating a role for PQQ protecting against rotenone- induced injury via modulation of the expression of Bcl-2 and Bax, and by activation of the pro-survival ERK1/2 pathway. In another *in vitro* model based on the aggregation and amyloid fibril formation pathway

implicated in AD and PD, PQQ decreased both mechanisms and reduced cytotoxicity (Kim et al., 2010). It has also been suggested that PQQ is a promising therapy for AD and dementia. It is thought that reducing the expression of nerve growth factor (NGF) may contribute to AD and PD through hindrance of cholinergic neuron survival. Pretreatment with PQQ induced the level of NGF *in vivo* and *in vitro* (Yamaguchi et al., 1993).

The neuroprotective effects of PQQ have been further investigated in diverse models of neurological disorders. Significant improvement in the recovery from traumatic brain injury in rats was observed with treatment of PQQ (Zhang et al., 2012). In addition, β -amyloid aggregation is one of the pathological hallmarks of AD and is a cause of apoptosis. Interestingly, PQQ significantly protects human neuroblastoma SH-SY5Y cells from death induced by β 25-35 peptide through modulation of Bcl-2, Bax, and caspase-3 (Zhang et al., 2009). In another study, a dramatic reduction in infarct size from focal cerebral ischemia, and improved behavior, have been demonstrated in rats after pretreatment or co-treatment with PQQ (Zhang et al., 2006). This neuroprotective compound may also have relevance as a potential therapy for stroke as a significant reduction of infarct size was demonstrated following PQQ treatment in an *in vivo* model of cerebral hypoxia/ischemia (Jensen et al., 1994). In summary, PQQ protects from several death pathways in neurons through not only antioxidant and anti-inflammatory effects, but also through its capacity to modulate mitochondrial biogenesis, intrinsic apoptosis, and growth factor signaling.

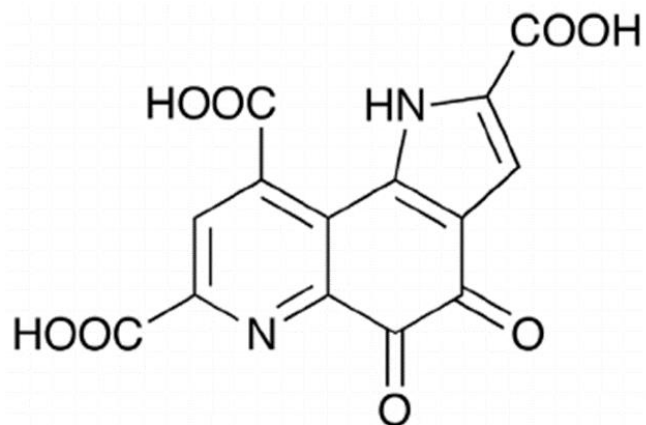


Image credit:

https://www.researchgate.net/publication/264799310_Discovery_of_a_Eukaryotic_Pyrroloquinoline_Quinone

Figure. 1.7. Chemical structure of pyrroloquinoline quinone (PQQ).

1.3. Models to study neuronal cell death and neuroinflammation

1.3.1. Cerebellar granule neurons (CGNs)

Finding a good model to study the molecular and cellular mechanisms of neurodegenerative diseases was a major challenge for scientists for decades. Primary cultures of cerebellar granule neurons (CGNs) have been recognized to be a standard model to study various fields of development, function, and neurobiology *in vitro*. While numerous studies have utilized various cells to study the pathways of neuronal cell death, CGNs have been proposed as the most reliable *in vitro* model to study survival and apoptotic pathway implicated in neuroprotection and neurodegeneration. One of the remarkable characteristics of this primary cell culture system is that some of the conditions and pathways that control neuronal survival and apoptosis *in vivo* can also be stimulated in culture in CGNs (Contestabile 2002). This is a very important component of properly investigating the neuroprotective effects of drugs or natural compounds as it allows for the design of appropriate experiments and manipulations of the cell death pathways.

Multiple molecular pathways that are implicated in neurodegenerative diseases were originally investigated utilizing CGNs as a model to study apoptosis the capacity of small molecules to reverse these effects. For example, ROS-induced cell death is one of the most common death pathways implicated in neurodegenerative diseases that was initially characterized in *in vitro* CGNs cultures (Behl et al., 2000). As expected, the apoptotic death induced by ROS in CGNs can be reversed by using antioxidants and free radical scavenging compounds (Contestabile, 2001). In addition to ROS-induced cell death, excessive glutamate exposure and proteasome inhibition have also been

characterized in CGNs (Dessi et al., 1994; Canu et al., 2000). ER stress and nitrosative stress also induce neuronal death, and have also been tested in CGNs have been used to understand more about the pathological pathways in degenerative diseases and to find some way to manipulate them pharmacologically (Kosuge et al., 2006; Lin et al., 2001). CGNs require depolarizing condition by culturing in specific extracellular concentration of potassium which is approximately 25 mM (Balazs et al., 1988). The reduction from 25 mM K⁺ to 5 mM K⁺ leads to CGNs apoptosis (Balazs et al., 1988). This paradigm of potassium deprivation is a well-characterized method to initiate and study survival signaling and intrinsic apoptosis. Thus, CGNs are a good *in vitro* model system to study multiple pathways of neuronal cell death *in vitro*.

1.3.2. BV2 cells

Accumulating evidence has linked neuroinflammation to neurodegenerative disorders. This molecular pathway has been investigated in order to identify novel therapies for such degenerative disorders. Microglia are the principle immune cells of the CNS, and they play a pivotal role in neuroinflammation. During the pathological progression of a neurodegenerative disorder, inflammation occurs following microglial activation (Cunningham, 2013). Henn et al. (2009) suggested the mouse BV2 cell line is a suitable alternative model for primary microglia, in order to reduce the experimental preparation time and animal use. Indeed, the similarity of inducing inflammation mediators in both primary microglial and BV2 cells has been demonstrated previously (Henn et al. 2009). BV2 cells have also been utilized in pharmacological studies. For example, LPS is a recognition factor known to activate both microglia and BV2 cell lines to induce neuroinflammation (Fig. 1.8) (Onasanwo et al., 2016). To enhance

understanding of this death pathway, stimulation of BV2 microglia cells with LPS was investigated to study the anti-inflammatory effect of different compounds (Onasanwo et al., 2016). Thus, BV2 cells are an appropriate *in vitro* model to study neuroinflammation and will be useful to identify novel compounds that are capable of inhibiting this particular cell death pathway.

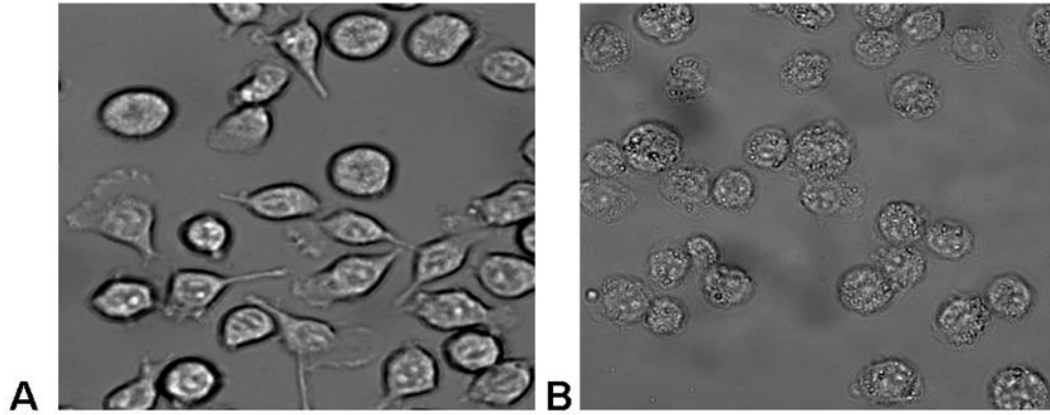


Figure. 1.8. BV2 mouse microglial cell line a standard model to study inflammation. A. BV-2 culture was treated with vehicle (control). B. BV-2 culture was treated with lipopolysaccharide (LPS).

CHAPTER TWO: NEUROPROTECTION COMPARISON OF CHLOROGENIC ACID AND ITS METABOLITES AGAINST MECHANISTICALLY DISTINCT CELL DEATH-INDUCING AGENTS IN CULTURED CEREBELLAR GRANULE NEURONS AND BV2 CELL LINE.

2.1. Abstract

Although the number of patients diagnosed with neurodegenerative disorders like Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease is increasing, there are currently no effective treatments that significantly limit the neuronal cell death underlying these diseases. Chlorogenic acid (CGA), a polyphenolic compound found in high concentration in coffee, is known to possess antioxidant and free radical scavenging activity. In this study, we investigated the neuroprotective effects of CGA and its major metabolites in primary cultures of rat cerebellar granule neurons and BV2 the mouse microglia cell line. We show that CGA and caffeic acid displayed a dramatic protective effect against the nitric oxide donor, sodium nitroprusside. In marked contrast, ferulic acid and quinic acid had no protective effect against this nitrosative stress. While CGA and quinic acid had no protective effect against glutamate-induced cell death, caffeic acid and ferulic acid significantly protected neurons from excitotoxicity. Finally, caffeic acid was the only compound that displayed significant protective activity against hydrogen peroxide, proteasome inhibition, caspase-dependent intrinsic apoptosis, and endoplasmic reticulum stress in CGNs, and it protected BV2 cell from the inflammation induced by

LPS. These results indicate that caffeic acid displays a much broader profile of neuroprotection against a diverse range of stressors than its parent polyphenol, CGA, or the other major metabolites, ferulic acid and quinic acid. We conclude that caffeic acid is a promising candidate for testing in pre-clinical models of neurodegeneration.

2.2. Introduction

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, are defined by the progression of nervous system dysfunction attributed to multiple factors including but not limited to, misfolding of proteins, mitochondrial dysfunction, excitotoxicity, and oxidative stress. Therapeutic strategies that focus on these underlying factors are promising approaches for the treatment of neurodegenerative diseases. Oxidative stress has been proposed as a common pathological mechanism of essentially all major neurodegenerative diseases, and typically results from excessive production of reactive oxygen species (ROS) consequent to mitochondrial injury or dysfunction (Carrì et al, 2015; Moon and Paek, 2015; Simoncini et al., 2015).

As a means of protecting against oxidative stress, cells are equipped with free radical scavenging defenses that prevent accumulation of ROS. In addition to enzymes that detoxify free radicals (e.g., superoxide dismutase and catalase), antioxidants are chemical substances that either directly scavenge ROS or indirectly induce the expression or activity of free radical scavenging systems that protect cells from oxidative stress. Based on their neuroprotective effects, antioxidants have been suggested as a viable approach to slow or halt the progression of neuronal cell loss in various

neurodegenerative diseases. However, clinical trials with many common antioxidants (e.g., vitamin E or vitamin C) have met with limited success, indicating that utilization of additional antioxidants with diverse mechanisms of action, or targeted intracellular delivery of these compounds (e.g., mitochondrial-targeted antioxidants), may be necessary to reveal their full therapeutic potential (Polidori and Nelles 2014; Jin et al. 2014). Therefore, dietary intake of natural antioxidants that can directly scavenge free radicals, as well as induce endogenous antioxidant defenses, may be one useful approach to treat neurodegenerative diseases.

Polyphenols are an abundant class of micronutrients found in many plants and are typically regarded as safe when taken as dietary supplements (e.g., epigallocatechin 3-gallate (EGCG) and resveratrol). They have the impressive ability to scavenge ROS and reactive nitrogen species (e.g., nitric oxide). In addition, polyphenols have been shown to modulate apoptosis, inflammation, ion channels, signal transduction, and neurotransmitter release – all processes which are implicated in a number of neurodegenerative diseases (Bhullar and Rupasinghe, 2013). Therapies that implement dietary supplementation with these compounds have demonstrated their neuroprotective potential (Bhullar and Rupasinghe, 2013; Bigford and Del Rossi, 2014; Daglia et al., 2014; Virmani et al., 2013). Although polyphenols directly scavenge ROS and nitric oxide, many studies have suggested that the antioxidant capacity of polyphenols is also dependent on their indirect activation of pathways like nuclear factor E2-related factor 2 (Nrf2)/heme oxygenase-1 (Bhullar and Rupasinghe, 2013). Others have shown that polyphenols inhibit many pathways that cause neuronal cell death through

neuroinflammation, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and pro-inflammatory molecules like tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), and interleukin (IL)-1 β , IL-6, IL-17A, IL-22. (Spencer et al., 2012). Polyphenols can also upregulate free radical detoxifying enzymes, including catalase, superoxide dismutase, and glutathione peroxidase (Bhullar and Rupasinghe, 2013). Finally, polyphenols have been shown to modulate the expression of pro-survival and pro-apoptotic pathway components, such as Bcl-2 and Bax (Kelsey et al., 2010).

Coffee, the second most consumed drink in the world, has been shown to have protective effects from diverse inflammatory diseases associated with oxidative stress (Andersen et al., 2006). Coffee consists of many antioxidant compounds including its main element caffeine, polyphenols like CGA, and volatile aromatic compounds (Gonthier et al., 2003; Ludwig et al., 2014). Several studies have shown that oxidative stress biomarkers are inversely correlated with the consumption of coffee (Hori et al., 2014; Kempf et al., 2010; Yoshida et al., 2008). Furthermore, coffee consumption enhances antioxidant capacity *in vivo* (Corrêa et al., 2012). In the context of neurodegeneration, several studies have documented that coffee intake is associated with a reduced risk of developing Parkinson's disease (Ross et al., 2000; Sääksjärvi et al., 2008; Costa et al., 2010).

Chlorogenic acid is a major polyphenolic component in many plants and is abundant in coffee - particularly in green coffee beans that contain from 5% to 12% CGA by weight (Farah and Donangelo 2006; Farah et al., 2008). It is an ester of trans-cinnamic acids (including caffeic acid, ferulic acid, p-coumaric acid) and quinic acid

(Clifford, 1999). Chlorogenic acid has diverse health benefits such as cardioprotective effects, lipid peroxidation inhibitory activity, anti-tumor effects, and antioxidant activity (Namba and Matsuse 2002; Wan et al., 2013).

Caffeic acid is a derivative of CGA that also has antioxidant properties, a function dependent on its chemical structure (Rice-Evans et al., 1996). The neuroprotective effects of CGA and caffeic acid have been revealed through *in vitro* studies using rat brain, where both polyphenolic compounds have been shown to inhibit acetylcholinesterase activity and lipid peroxidation induced by various pro-oxidants, suggesting a possible beneficial effect for Alzheimer's disease (Oboh et al., 2013). Caffeic acid has also been shown to reduce intracellular ROS produced by H₂O₂ and protects neuronal cells *in vitro* from this oxidative insult (Jeong et al., 2011).

Ferulic acid, a component of green coffee beans and another metabolite of CGA, has also been found to be an effective free radical scavenger. This antioxidant action is related to its phenolic hydroxyl group that donates electrons in order to render free radicals inert (Srinivasan et al., 2007). In addition to its intrinsic free radical scavenging activity, *in vivo* studies have established that administration of ferulic acid inhibits astrocyte activation induced by beta-amyloid peptide in mice (Cho et al., 2005).

The neuroprotective effects of quinic acid, another metabolite of CGA, have been investigated through *in vitro* studies. Although not typically regarded as an antioxidant, various chemical derivatives of quinic acid have been shown to inhibit the toxicity induced by beta-amyloid peptide in PC12 cells (Hur et al., 2001).

In order to investigate the neuroprotective effects of CGA and some of its metabolites against a number of diverse mechanisms that contribute to neurodegenerative diseases, we pre-incubated cerebellar granule neurons (CGNs) with CGA, caffeic acid, ferulic acid, or quinic acid (Fig. 2.1), and then exposed them to different stressors that result in neuronal cell death. Cell viability was measured by MTT assay, immunocytochemistry was used to visualize the microtubule network, Hoechst stain was used to visualize nuclear morphology, and nitrite level was assessed by the Griess assay. The results demonstrate that CGA, caffeic acid, and ferulic acid each show some neuroprotective effects; however, caffeic acid displays a much broader neuroprotective profile against a diverse range of neurotoxic stressors than CGA or ferulic acid. Based on its broad neuroprotective profile, caffeic acid is a viable therapeutic candidate for testing in pre-clinical models of neurodegeneration.

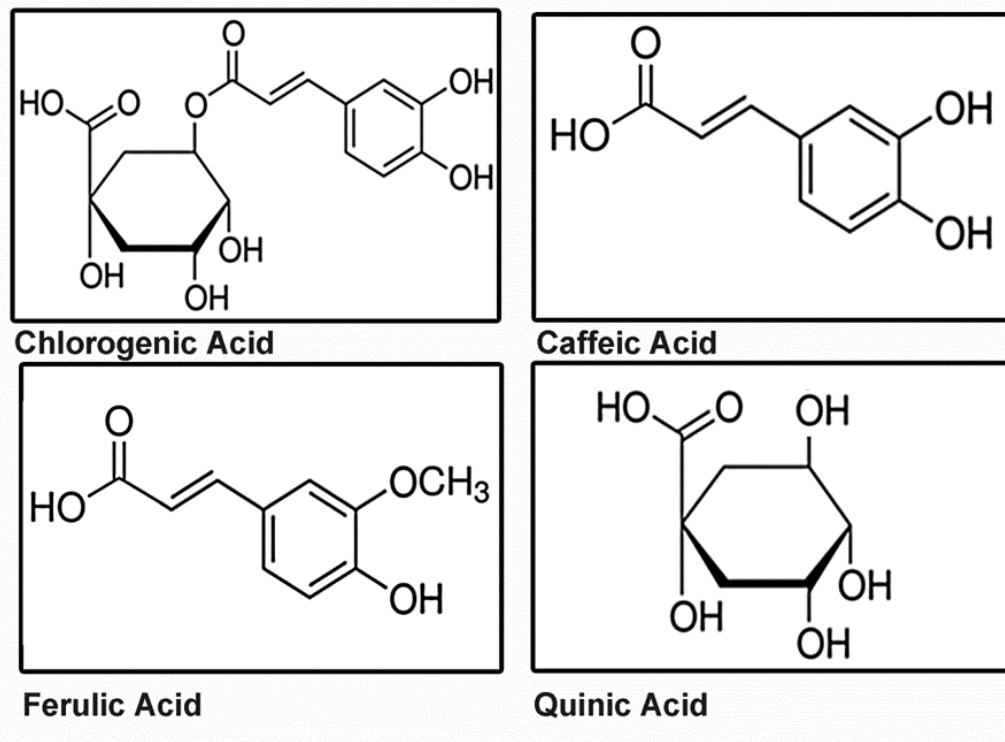


Figure 2.1. Structural comparison of chlorogenic acid, caffeic acid, ferulic acid, and quinic acid

2.3. Materials and methods

2.3.1. Materials.

Hoechst dye, monoclonal antibody to β -tubulin, paraformaldehyde, glutamic acid, and glycine were obtained from Sigma Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody for immunofluorescence was from Jackson ImmunoResearch Laboratories (West Grove, PA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent was obtained from Life Technologies (Grand Island, NY) and made up in 1X phosphate buffered saline (PBS, pH 7.4). Brefeldin A was obtained from Cell Signaling (Danvers, MA). PS-341 was purchased from Bio Vision (Mountain View, CA). Sodium nitroprusside (SNP) was obtained from Calbiochem (San Diego, CA). Chlorogenic acid, caffeic acid, ferulic acid, and quinic acid were obtained from Sigma Aldrich (St. Louis, MO). Caffeic acid was solubilized as stock solutions in DMSO and other compounds were made in sterile water. Griess reagent assay was obtained from Thermo Scientific (Waltham, MA). Lipopolysaccharide obtained from Sigma Aldrich (St. Louis, MO). Stock solution of glutamate/glycine was made in sterile water. Brefeldin A and PS-341 were solubilized as stock solutions in DMSO.

2.3.2. Cell culture.

2.3.2.1. Cerebellar granule neurons (CGNs)

Cerebellar granule neurons (CGNs) were isolated from 7-day-old Sprague-Dawley rat pups (15-19g) of both sexes as described previously (Linseman et al., 2001). Briefly, neurons were plated on poly-L-lysine-coated culture plates at a density of

2.0x10⁶ cells/ml in Basal Modified Eagle's medium (BME) containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, penicillin (100units/ml)-streptomycin (100µg/ml) (Life Technologies, Inc., Gaithersburg, MD). Cytosine arabinoside (final concentration of 10µM) was added after 24h to limit the growth of non-neuronal cells. With this protocol, the purity of granule neurons was ~95-99%. After 6 or 7 days in culture, CGNs were rinsed two times in serum-free culture medium containing 25mM potassium (25K-Serum) and maintained in the same medium for pre-incubation with either chlorogenic acid (10µM), caffeic acid (50µM), ferulic acid (50µM), or quinic acid (10µM), individually for 24h. Apoptosis was induced either by removal of depolarizing extracellular potassium (5mM KCl; 5K apoptotic condition), or by the direct addition of the following stressors: (1) the nitric oxide donor, sodium nitroprusside (SNP; 50µM), (2) 100µM glutamate and 10µM glycine (Glu/Gly) to induce excitotoxicity, (3) hydrogen peroxide (H₂O₂; 50µM), (4) the proteasome inhibitor PS-341 (100 µM), or (5) the endoplasmic reticulum (ER)-Golgi transport inhibitor, Brefeldin A (15 µM). Pre-treated cells were treated overnight for approximately 16-18h with each stressor.

2.3.2.2 BV2 cell line

BV-2 cells were seeded on uncoated culture plates and maintained in DMEM supplemented with 10% fetal bovine serum, and penicillin (100units/ml)-streptomycin (100µg/ml) at 37° C in 5 % CO₂. Next day we removed 1 ml of the medium from each well and pretreat cells with the same concentration from the four compounds that we used with CGNs for 24h. Then we added LPS (1µg/ml) for an additional 17 h. After that we measured nitrite production by the Griess assay.

2.3.3. MTT assay.

After overnight incubation with the appropriate stressor, 100 μ l of MTT reagent was added to each well followed by incubation at 37°C for an additional 4h. Two ml of DMSO was added to each well to dissolve formazan crystals and the medium containing the dissolved crystals was read immediately in a microplate reader (BIO-RAD microplate reader-550) at 570 nm. A blank containing 25K-Ser medium, MTT reagent, and DMSO was prepared in parallel, and incubated without cells. The data were plotted as the percentage of cell viability relative to the control mean absorbance for each independent experiment. A minimum of n=4 experiments with duplicate wells for each treatment group were performed for each stressor.

2.3.4. Immunocytochemistry and fluorescence imaging.

CGNs were washed once in 1X PBS and then fixed with 4% paraformaldehyde for 45min at room temperature. Cells were then blocked and made permeable at room temperature for 1h using a solution of 5% BSA and 0.2% Triton X-100 in 1X PBS. Then they were incubated with a primary antibody to β -tubulin in 2% BSA and 0.2% Triton X-100 in 1X PBS, overnight at 4°C. The next day, cells were washed 5 times with 1X PBS and then incubated for 1h at room temperature with FITC-conjugated secondary antibody diluted 1:250 in 2% BSA and 0.2% Triton X-100 in 1X-PBS. To stain the nuclei, Hoechst staining (1:500) was added to the secondary antibody solution. The following day, cells were washed 5 times with 1 X PBS, and anti-quench solution prepared from 1X PBS and 1-5 mg of *p*-phenylenediamine was added to the cells before imaging. Images were

captured using a 40X objective on a Zeiss Axiovert-200M inverted fluorescence microscope.

2.3.5. Nitrite Assay

After pretreated cells with the four compounds individually and add LPS, nitrite level was assessed by the Griess assay. Sodium nitrite was used as standard to determine nitrite concentration, and absorbance was measured at 550nm.

2.3.6. Statistical Analysis.

Experiments were performed using duplicate or triplicate wells for each treatment condition. Graphic data represent the means \pm SEM for the number (n) of independent experiments performed. Statistical analysis was performed using either one-way analysis of variance (ANOVA) and a post hoc Tukey's test or unpaired t test. Differences between treatment groups were considered statistically significantly different at a value of $p < 0.05$.

2.4. Results

2.4.1. CGA and caffeic acid protect CGNs from nitrosative stress induced by the nitric oxide donor, sodium nitroprusside (SNP).

CGNs were pre-incubated with CGA (10 μ M), caffeic acid (50 μ M), ferulic acid (50 μ M), or quinic acid (10 μ M) for 24h, and then treated in the presence or absence of SNP (50 μ M) for an additional 16-18h. To assess neuronal injury induced by SNP in CGNs, we first examined the integrity of the microtubule network and nuclear morphology. Compared to control cells, SNP caused a dramatic disassembly of the

microtubule network in CGNs as shown by immunostaining for β -tubulin (Fig. 2.2A). CGA and caffeic acid each substantially protected the microtubule cytoskeleton from damage induced by SNP. Nuclei were considered as apoptotic if fragmented or condensed as assessed by Hoechst staining. De-colored (black & white) Hoechst staining images are shown in Fig. 2.2A and enlarged (2.5X) in Fig 2B to clearly show differences in nuclear size and morphology. Control cells displayed large intact nuclei. In contrast, SNP- treated CGNs showed very small and condensed nuclei (Fig 2.2B). However, nuclear condensation was decreased markedly by pre-incubation with either CGA or caffeic acid. Neither ferulic acid nor quinic acid had any effect on SNP-induced nuclear condensation or microtubule disassembly. In agreement with these morphological findings, quantitative measurement of cell viability by MTT assay confirmed that only CGA and caffeic acid significantly protected CGNs from nitrosative stress induced by SNP. The data from four independent experiments are shown in Fig. 2.2C as the (means \pm SEM) percentage of viable CGNs relative to controls.

It should be noted that similar results were obtained when CGA and each of its metabolites were used at equivalent (50 μ M) concentrations for pre-treatment of CGNs. Because CGA and quinic acid each displayed a small amount of basal cytotoxicity at 50 μ M in CGNs, for all subsequent experiments we utilized these two compounds at a concentration of 10 μ M. Regardless, for each stressor used, similar results were obtained to those shown when CGA and its metabolites were each used at equivalent (50 μ M) concentrations (data not shown).

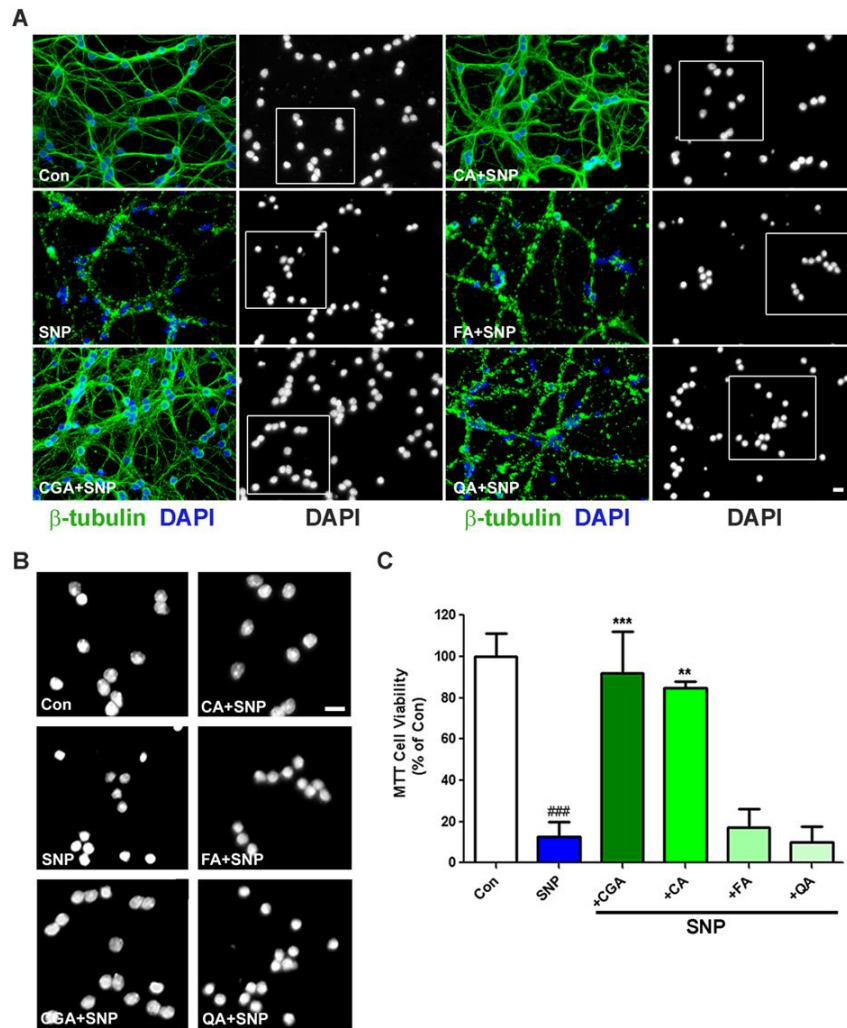


Figure 2.2. Chlorogenic acid and caffeic acid protect CGNs from nitrosative stress. (A) CGNs were pre-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con) alone or with chlorogenic acid (CGA; 10 μ M), caffeic acid (CA; 50 μ M), ferulic acid (FA; 50 μ M), or quinic acid (QA; 10 μ M). Cells were then incubated for an additional 16-18h with the nitric oxide donor, sodium nitroprusside (SNP; 50 μ M). Following incubation, CGNs were fixed and stained for β -tubulin (FITC; green) and nuclei were visualized with Hoechst staining (blue). Decolorized (black & white) panels are shown to emphasize nuclear morphology. (B) The areas demarcated by the boxes in (A) were magnified 2.5X to show differences in nuclear size. (C) Quantification of cell viability following SNP exposure using an MTT assay. Results are expressed as a % of Con viability (set to 100%) and are shown as mean \pm SEM (n= 4 experiments performed in duplicate). ### p<0.001 vs. Con; ***p<0.001, **p<0.01 vs. SNP; as assessed by one-way ANOVA with a post hoc Tukey's test.

2.4.2. Caffeic acid and ferulic acid protect CGNs from excitotoxicity induced by glutamate/glycine.

Next, we used glutamate/glycine co-treatment to trigger Ca^{2+} -dependent excitotoxicity in CGNs and investigate the protective effects of CGA and its metabolites. CGNs were pre-incubated with each of the four compounds as indicated above and then treated for a further 16-18h with glutamate/glycine (final concentrations; 100 μM /10 μM). The integrity of the microtubule network and nuclear morphology were assessed by immunocytochemistry for β -tubulin and Hoechst staining, respectively. Compared with untreated control cells, glutamate/glycine caused a marked disassembly of the microtubule network in CGNs (Fig. 2.3A). Furthermore, nuclei were fragmented or condensed following exposure to glutamate/glycine (Fig. 2.3A and enlarged 2.5X in Fig. 2.3B). Pre-incubation with either caffeic acid or ferulic acid significantly reduced excitotoxic damage to the microtubule cytoskeleton and diminished the appearance of apoptotic nuclei (Figs. 2.3A, 2.3B). In contrast, neither CGA nor quinic acid protected CGNs from these morphological effects of excitotoxicity (data not shown). As an additional measure of neuroprotection, cell viability was measured by MTT assay. The data from four independent experiments are shown in Fig. 2.3C as the percentage of viable CGNs relative to controls (means \pm SEM). Glutamate/glycine caused a nearly 75% reduction in CGN viability that was unaffected by pre-incubation with CGA. However, caffeic acid and ferulic acid each provided significant protection from excitotoxicity-induced cell death. Cells treated with quinic acid trended towards increased viability but the results did not reach statistical significance. These data show that only caffeic acid

and ferulic acid are capable of protecting CGNs from glutamate/glycine-induced excitotoxicity.

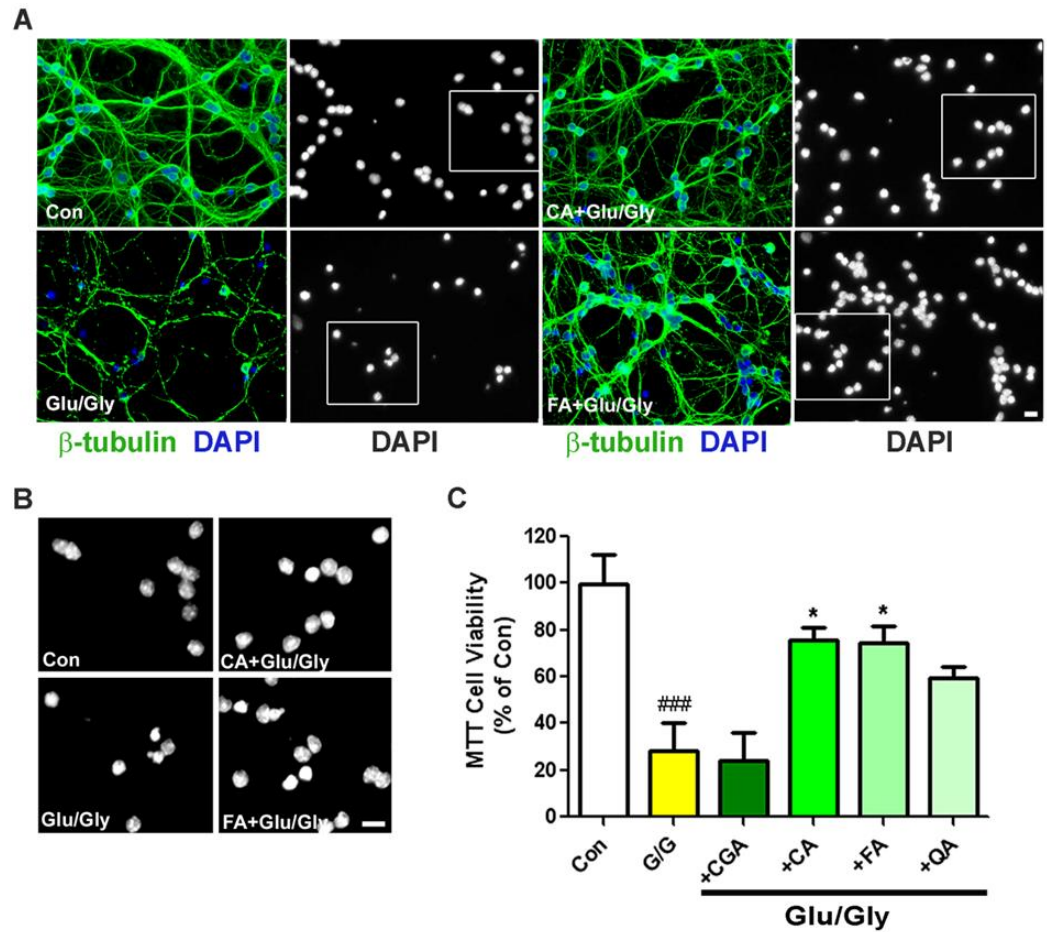


Figure 2.3. Caffeic acid and ferulic acid protect CGNs from excitotoxicity induced by glutamate/glycine. (A) CGNs were pre-incubated for 24h with CGA or its metabolites as described in the legend to Figure 2A, and then exposed to glutamate/glycine (100 μ M/10 μ M; final concentrations) for 16-18h. Cells were stained for β -tubulin and nuclear morphology as described in Figure 2.2 (B) The areas demarcated by the boxes in (A) were magnified 2.5X to show differences in nuclear size. (C) Quantification of cell viability following glutamate/glycine (Glu/Gly or G/G) exposure using an MTT assay. Results are expressed as a % of Con viability (set to 100%) and are shown as mean \pm SEM (n= 4 experiments performed in duplicate). ###p<0.001 vs. Con; *p<0.05 vs. Glu/Gly; as assessed by one-way ANOVA with a post hoc Tukey's test

2.4.3 Caffeic acid uniquely protects CGNs from oxidative stress induced by hydrogen peroxide.

Oxidative stress is a major factor underlying the neuronal cell death in diverse neurodegenerative disorders. Common ROS that cause oxidative stress include superoxide radical and hydrogen peroxide (H_2O_2). To examine the neuroprotective effects of CGA and its metabolites against oxidative stress, CGNs were pre-incubated with each of the four compounds as indicated above and then treated for a further 16-18h with H_2O_2 ($50\mu\text{M}$). In contrast to untreated control cells, CGNs exposed to H_2O_2 displayed a notable alteration of the microtubule network, characterized primarily by a loss of smaller interconnecting processes (Fig. 2.4A). In addition, cells treated with H_2O_2 demonstrated substantial nuclear condensation indicative of an apoptotic-like death (Figs. 2.4A, 2.4B). Both of these effects were largely prevented by pre-incubation with caffeic acid. Consistent with these morphological findings, MTT assay results showed that only caffeic acid provided significant protection of CGNs (Fig. 2.4C). Incubation with H_2O_2 decreased cell viability by approximately 80% while pre-incubation with caffeic acid prevented this reduction. These data demonstrate that among the compounds tested, caffeic acid uniquely and significantly protects CGNs from oxidative stress induced by H_2O_2 .

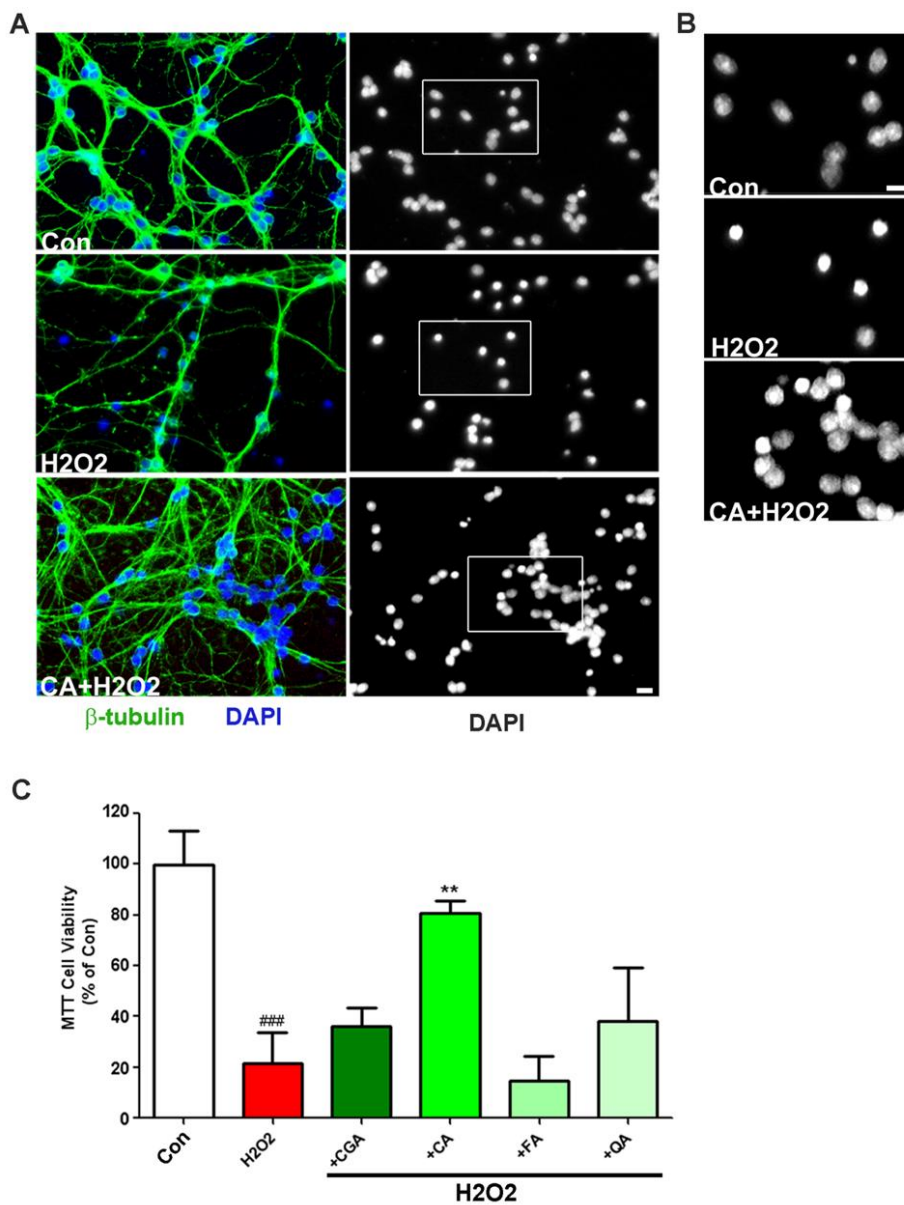


Figure 2.4. Caffeic acid protects CGNs from the oxidative stress induced by hydrogen peroxide. (A) CGNs were pre-incubated for 24h with CGA or its metabolites as described in the legend to Figure 2.2A, and then exposed to H₂O₂ (50 μ M) for an additional 16-18h. Cells were stained for β -tubulin and nuclear morphology as described in Figure 2.2. (B) The areas demarcated by the boxes in (A) were magnified 2.5X to show differences in nuclear size. (C) Quantification of cell viability following H₂O₂ exposure using an MTT assay. Results are expressed as a % of Con viability (set to 100%) and are shown as mean \pm SEM (n= 4 experiments performed in duplicate). ###p<0.001 vs. Con; **p<0.01 vs. H₂O₂; as assessed by one-way ANOVA with a post hoc Tukey's test.

2.4.4. Caffeic acid exclusively protects CGNs from an inhibitor of ER-Golgi transport, proteasome inhibition, and caspase-dependent apoptosis.

Additional mechanisms leading to neuronal cell death, besides those discussed above (nitrosative stress, excitotoxicity, and oxidative stress), include ER stress, proteasome inhibition, and caspase-dependent apoptosis, amongst others. In the next series of experiments, we evaluated the neuroprotective effects of CGA and its metabolites against Brefeldin A, an inhibitor of ER-golgi transport, a proteasome inhibitor (PS-341), and 5K-induced (caspase-dependent) CGN apoptosis. Brefeldin A suppresses transport between the ER and Golgi, eliciting inositol 1, 4, 5 triphosphate (IP3) receptor-dependent Ca^{2+} toxicity and caspase-9-mediated apoptosis in CGNs (Brewster et al., 2006). Proteasome inhibition induces CGN apoptosis by causing an accumulation of c-Jun and pro-apoptotic Bim (Butts et al., 2005). CGNs require a high extracellular potassium-regulated depolarization stimulus for survival in culture, and removal of this depolarization stimulus (by switching the culture medium from 25mM KCl to 5mM KCl; 5K apoptotic condition) induces CGN apoptosis via the intrinsic or mitochondrial apoptotic pathway (D'Mello et al., 1993; Watson et al., 1998; Linseman et al., 2002). When CGN survival was assessed by MTT assay, each of these stressors (Brefeldin A, PS-341, and 5K apoptotic medium) induced at least a 60% loss of cell viability (Figs. 2.5A-C). In cells pre-incubated with CGA and its metabolites, only those CGNs treated with caffeic acid displayed a significant enhancement of survival upon exposure to each of these diverse stressors. Thus, caffeic acid exclusively protects CGNs

from ER stress, proteasome inhibition, and caspase-dependent apoptosis, demonstrating its broad neuroprotective profile in this cell system.

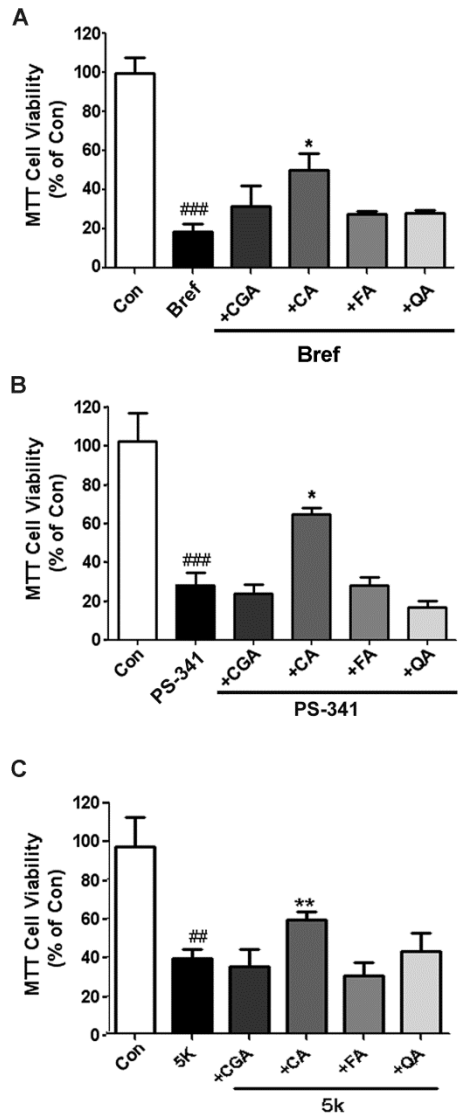
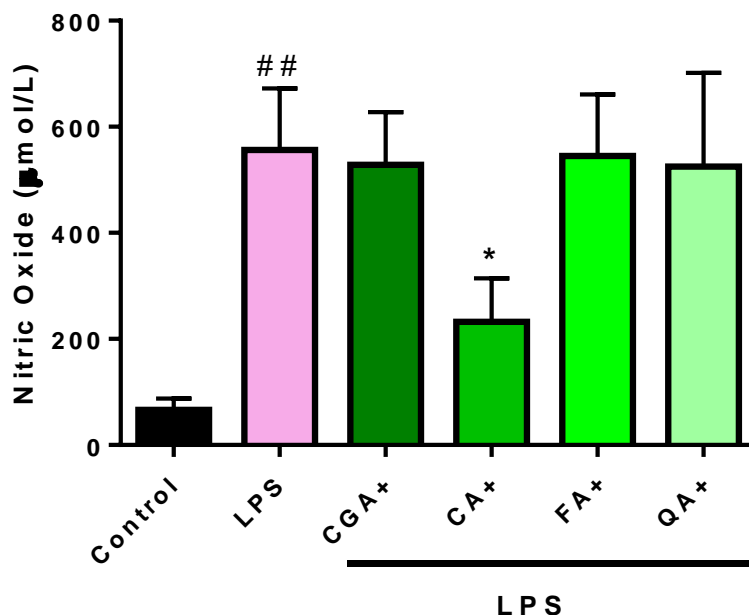


Figure 2.5. Caffeic acid protects CGNs from toxicity induced by Brefeldin A, the proteasome inhibitor PS-341, and the caspase-dependent apoptosis inducer, 5K medium. CGNs were pre-incubated for 24h with CGA or its metabolites as described in the legend to Figure 2.2A, and then exposed to Brefeldin A (Bref), PS-341, or 5K apoptotic medium for an additional 16-18h. (A), (B), and (C) Quantification of cell viability (MTT assay) following incubation with Bref, PS-341, or 5K apoptotic medium, respectively. Results are expressed as a % of Con viability (set to 100%) and are shown as mean±SEM (n=4-6 experiments performed in duplicate). ###p<0.001, ##p<0.01 vs. Con; **p<0.01; *p<0.05 vs. Bref, PS-341, or 5K; as assessed by one-way ANOVA with a post hoc Tukey's test

2.4.5. Caffeic acid protects BV2 from inflammation induced by lipopolysaccharide (LPS) the inflammatory stimulator.

Inflammation is another mechanism implicated in neurodegenerative diseases that been investigated in this study. BV2 the mouse microglia cell line has been used as a model to study inflammation. Lipopolysaccharide (LPS) was added to enhance the production of pro-inflammatory mediators, including nitric oxide (NO). BV2 were pre-incubated with CGA (10 μ M), caffeic acid (50 μ M), ferulic acid (50 μ M), or quinic acid (10 μ M) for 24h, and then treated in the presence or absence of LPS (1 μ G) for an additional 16-18h. On the next day, NO levels were measured by Griess assay. Caffeic acid decreased the level of NO induced by LPS. Hence, caffeic acid inhibits the inflammatory effect of LPS with BV2 (Fig. 2.6).



2.6. Caffeic acid protects BV2 from inflammation induced by lipopolysaccharide (LPS) the inflammatory stimulator. Quantitative assessment of nitric oxide production by untreated BV2 microglia, and microglia stimulated by LPS alone or with chlorogenic acid (CGA; 10µM), caffeic acid (CA; 50µM), ferulic acid (FA; 50µM), or quinic acid (QA; 10µM) pre-incubated for 24h. Data are represented as mean ± SEM (n= 3 experiments for nitric oxide assay). ###p<0.001 vs. Con; *p<0.001, **p<0.01 vs. LPS; as assessed by one-way ANOVA with a *post hoc* Tukey's test.**

2.5. Discussion

Coffee contains multiple antioxidant compounds including the polyphenol CGA. Coffee intake is associated with an enhanced antioxidant status and reduced risk of some neurodegenerative disorders, suggesting that coffee constituents, like CGA, are neuroprotective. In the current study, we compared the neuroprotective effects of CGA and its major metabolites against diverse stressors in cultured rat CGNs, an established model to study neuronal cell death (Maycotte et al., 2010), we also stimulated BV2 microglia cells with LPS to investigate the anti-inflammatory effects of these compounds (Onasanwo et al., 2016). An investigation of seven different cell stressors demonstrated that CGA and ferulic acid each protected from one stressor, nitric oxide and glutamate, respectively. In contrast, caffeic acid showed a very broad range of neuroprotective activity against multiple stressors (Fig. 2.7). Although some previous studies have investigated the neuroprotective effects of CGA and its metabolites *in vitro*, this has not previously been explored in a systematic manner within a common cell system. Here, we systematically compared the neuroprotective effects of these compounds in primary CGNs and BV2 cell line challenged with diverse neurotoxic stressors.

Similar to our results showing that CGA and caffeic acid each protect CGNs against the nitric oxide donor SNP, these compounds have also been shown to reduce lipid peroxidation induced by SNP in rat brain *in vitro* (Oboh et al., 2013). The protective effects of CGA and caffeic acid against nitric oxide-induced neurotoxicity are likely due to direct free radical scavenging and appear to be dependent on the presence of a catechol moiety which is common to these two compounds (Fig. 6) (Kono et al., 1997).

In the case of excitotoxicity induced by glutamate, caffeic acid and ferulic acid each significantly preserved the viability of CGNs. These results are consistent with a previous report indicating that caffeic acid protects primary cultures of rat cortical neurons from the excitotoxic effects of glutamate (Koo et al., 2006). Also in the same study, caffeic acid attenuated excessive calcium influx and reduced ROS generation caused by glutamate. In a similar manner, ferulic acid administration to pregnant mice protected the brains of the developing fetuses, possibly through the action of ferulic acid acting as an NMDA receptor antagonist (Yu et al., 2006).

Similar to our results showing that caffeic acid protects CGNs from H₂O₂, pre-incubation with caffeic acid also protected PC12 cells from this oxidative stress (Pavlica and Gebhardt, 2005). In addition, a study in rats showed that caffeic acid protects the brain against H₂O₂ *in vivo* (Pereira et al., 2006). The present study also revealed the unique protective effects of caffeic acid against CGN apoptosis induced by low potassium medium. Although we have not investigated the mechanism of this anti-apoptotic effect, it seems plausible that caffeic acid may modulate pro-apoptotic or pro-survival gene expression to suppress caspase activity in this neuronal system. For example, caffeic acid has been shown to decrease neurotoxicity induced by amyloid beta by modulating Akt signaling in HT22 mouse hippocampal cells (Huang et al., 2013). Caffeic acid also attenuated cell death of CGNs induced by the ER stressor Brefeldin A. On the other hand, one prior report showed that in SH-SY5Y neuroblastoma cells, caffeic acid did not inhibit cell death induced by tunicamycin, another ER stressor that acts through a distinct mechanism to that of Brefeldin A (Izuta et al., 2008). While Brefeldin

A acts by inhibiting transport between the ER and Golgi apparatus, tunicamycin inhibits protein glycosylation in the Golgi apparatus (Colanzi et al., 2013; Izuta et al., 2008).

Therefore, the differences in the protective action of caffeic acid against Brefeldin A and tunicamycin likely reflects modulation of factors involved in ER-Golgi transport rather than protein glycosylation. Finally, caffeic acid also protected CGNs from cell death caused by the proteasome inhibitor PS-341. To our knowledge, there are no prior reports of caffeic acid protecting neurons from proteasome inhibition. One potential mechanism through which caffeic acid could mediate protection is by modulation of pro-apoptotic protein, Bim. Chronic exposure of CGNs to proteasome inhibition is shown enhance expression of Bim, inducing apoptosis (Butts et al., 2005). Caffeic acid is known to activate Akt, a known inhibitor of Bim signaling, which may promote cellular survival under conditions of proteasome inhibition (Huang et al., 2013).

Finally, caffeic acid protected BV-2 cell from the inflammation induced by LPS. Our data are in agreement with those that found caffeic acid decreased NO production induced by LPS in macrophage cells (Búfalo et al., 2013). That protection and inhibition of NO accumulation may be related to caffeic acid ability to block different pathway implicated in forming NO like NF-kB, and MAPK (Búfalo et al., 2013). Pretreating BV-2 with other compounds did not present any suppression of NO induced by LPS. Our data were in contract with Shen et al. In their study chlorogenic acid decrease NO production that promote by LPS in microglia cells. That contrast may because we used lower concentrations of chlorogenic acid than in the Shen et al. study

In summary, based on our systematic comparison of the neuroprotective effects of CGA and its major metabolites in a single cell system, primary CGNs, we conclude that CGA protects only from nitrosative stress and ferulic acid protects only from glutamate excitotoxicity. In contrast, caffeic acid displays a broad neuroprotective profile against oxidative and nitrosative stress, excitotoxicity, intrinsic apoptosis, ER stress, proteasome inhibition and inflammation (Fig. 2.7). These findings suggest that caffeic acid is a promising candidate for pre-clinical testing in mouse models of neurodegeneration. In this context, caffeic acid phenethyl ester (CAPE) has been shown to limit dopaminergic neurodegeneration and dopamine loss in mouse and rat models of Parkinson's disease, and CAPE also extends survival in a mouse model of amyotrophic lateral sclerosis (Fontanilla et al., 2011; Fontanilla et al., 2012; Barros et al., 2013). It is noteworthy that CAPE is rapidly hydrolyzed *in vivo* and its major metabolite is caffeic acid (Celli et al., 2007). These facts may warrant a comparison of CAPE and caffeic acid in pre-clinical disease models.

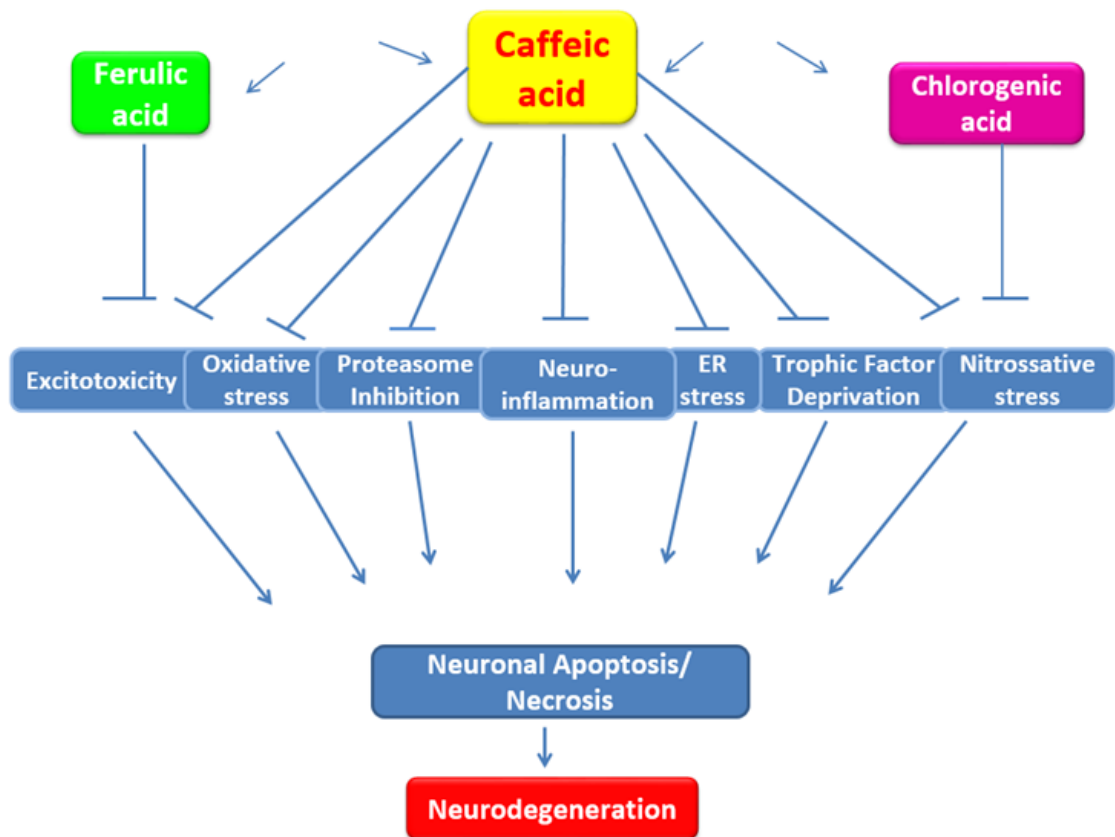


Figure 2.7. The neuroprotective effects of chlorogenic acid and its metabolites in CGNs. The schematic shows the broad neuroprotective effect of caffeic acid against oxidative and nitrosative stress, glutamate excitotoxicity, proteasome inhibition, ER stress, caspase depended apoptosis, and inflammation. In contrast, CGA protects only from nitrosative stress and ferulic acid protects only from glutamate excitotoxicity. Quinic acid did not display any significant neuroprotective activity in CGNs and is omitted from the figure.

CHAPTER THREE: THE NEUROPROTECTIVE EFFECTS OF ROSMARINIC AND CARNOSIC ACID ON PRIMARY CEREBELLAR GRANULE NEURONS

3.1. Abstract

Neurodegeneration in the brain is the main cause of many devastating diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's, and Parkinson's diseases. All of these diseases are characterized by the progressive loss of neurons in specific regions of the brain and/or spinal cord through either apoptosis or necrosis. Oxidative and nitrosative stress, along with excitotoxicity and caspase activation, have been implicated as the major underlying causes of cell death. Nutraceutical products have recently been shown to have neuroprotective effects in a variety of *in vitro* and *in vivo* disease models. The two nutraceuticals under investigation in this study are rosmarinic and carnosic acid both of which are found at substantial concentrations in rosemary. In this study, these two compounds' ability to attenuate damage to cultured cerebellar granule neurons (CGNs) was observed and recorded. Both rosmarinic and carnosic acid have been shown to mitigate these effects and reduce cell death when nitrosative stress is induced. Rosmarinic acid also notably protected neurons against glutamate-induced excitotoxicity. Furthermore, carnosic acid has the unique ability to protect CGNs from caspase-dependent intrinsic apoptosis induced by removal of serum and depolarizing extracellular potassium in the cell culture medium (i.e., 5K apoptotic condition). We additionally

revealed that carnosic acid protects CGNs from 5K conditions specifically by activating the phosphoinositide 3-kinase (PI3K) pro-survival pathway. The aim of this study was to test the neuroprotective effects of these two compounds against these various different kinds of cell death in order to determine their usefulness in attenuating neurodegenerative diseases.

3.2. Introduction

Neurodegenerative diseases, or diseases of the brain with marked neuronal death and consequential loss of brain matter, are increasing in prevalence. Some of the most common neurodegenerative diseases include amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's diseases. According to a study published by Environmental Health Perspectives in 2005, 50 million Americans a year are affected by a neurodegenerative disease (Brown et al., 2005). Because most neurodegenerative diseases are primarily sporadic in nature, an effective method of treatment would be to prevent or slow the dying of neurons in affected brain regions. Inflammation, misfolded proteins, mitochondrial dysfunction, oxidative and nitrosative stress and excitotoxicity are all thought to be major mechanisms in the pathology of various neurodegenerative diseases. Thus, therapeutic agents that target multiple disease aspects may be the most viable treatment options.

Since ancient times, plants have been used as natural treatments for acute and chronic disorders (Ghaffari et al., 2014). Over the last few decades, natural compounds which possess medical benefits (or nutraceuticals) have been proposed as promising treatment options for many diseases due to their intrinsic antioxidant abilities in scavenging reactive oxygen and reactive nitrogen species. The activities of many of these

compounds have been extensively investigated both within *in vitro* and *in vivo* models (Ghaffari et al., 2014). Manufactured or commercially synthesized drugs often have more activity than natural compounds. However, natural compounds have long term benefits and generally produce fewer side effects (Espin et al., 2007). Most of these nutraceuticals are abundant in potent phenolic antioxidant compounds, like phenolic acids, flavonoids, tannins, and lignans (Cai et al., 2004).

Rosemary is a shrub found originally in different Mediterranean countries, and now it is found abundantly throughout the world. In addition to its common use as a spice in food, rosemary has been used in alternative and complementary medicine due to its antioxidant activity (Rašković et al., 2014). In folk medicines, rosemary has been used as a therapy for different diseases including headaches, inflammatory diseases, and stomach problems (Rašković et al., 2014). Rosemary has significant intrinsic antioxidant activity which is due to its molecular constituents, such as carnosol, carnosic acid, ursolic acid, rosmarinic acid, and caffeic acid.

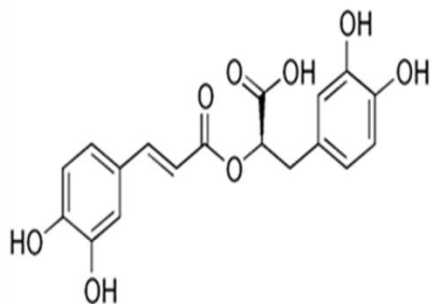
Rosmarinic acid (Figure 3.1A) is a polyphenol derivative of caffeic acid which has antioxidant capabilities (Bhatt et al., 2013). It is naturally present in a number of plants traditionally used in folk medicinal practices such as *Orthosiphon diffusus*, *Orthosiphon staminus*, *Rosmarinus officinalis*, *Artemisia capillaris* and *Calendula officinalis* (Scarpati and Oriente, 1958). It possesses many biological properties including antioxidant (Lee et al., 2008), anti-inflammatory (Erkan et al., 2008), anti-mutagenic (McKay and Blumberg, 2006), anti-angiogenic (Huang and Zheng, 2006), hepatoprotective, anti-neurodegenerative (Lee et al., 2008), and NF- κ B down-regulating (Osakabe et al., 2004). Moreover, rosmarinic acid has been demonstrated to protect

dopaminergic neuronal cells by inhibiting nitric oxide (NO) production from activated glial cells (Lo et al., 2002; Park et al., 2008).

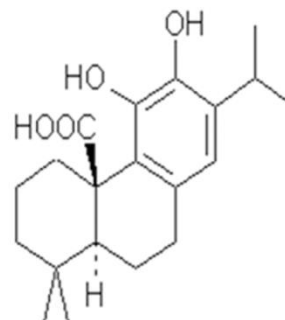
Carnosic acid (Figure 3.1B) also possesses antioxidant activity that may be related to its ability to up-regulate endogenous free radical scavenging enzymes via activation of the Nrf2 transcriptional pathway (Sato et al., 2008). In another study, carnosic acid showed a neuroprotective effect *in vitro* in human, induced pluripotent stem cells (hiPSC)-derived neurons and *in vivo* in a mouse model against cyanide-induced brain damage (Zhang et al., 2015). It also has antimicrobial properties (Birtic et al., 2015). In addition, both compounds have demonstrated neuroprotective effects in different neurodegenerative diseases. Rosmarinic acid has been shown to attenuate motor neuron death in a mouse model of familial ALS (Shimojo et al., 2010; Seo et al., 2015), and carnosic acid has been shown to have similar effects in a mouse model of Alzheimer's disease (Azad et al., 2011).

The purpose of the present study was to directly compare the neuroprotective effects of these two compounds against oxidative damage caused by nitrosative stress, excitotoxicity, and caspase activation in a single cell model of primary rat cerebellar granule neurons culture. Further, we have defined the signaling pathway that carnosic acid activates under 5K apoptosis conditions which rescues neurons from caspase activation. Apoptosis was measured morphologically by immunocytochemistry to visualize the microtubule network and Hoechst staining to visualize nuclear size and architecture. To investigate the pro-survival pathway that carnosic acid activates when demonstrating its neuroprotective effects, we used several different inhibitors including wortmannin to inhibit PI3K, PD98059 to block MEK/ERK, and AKT inhibitor to

attenuate AKT pro-survival pathway signaling (Figure 3.2). Our data demonstrate that these compounds show overlapping and unique neuroprotective effects against different stressors that cause cell death. The unique neuroprotective mechanism of these two polyphenols has direct implications for designing future preclinical studies to evaluate the therapeutic potential of these compounds for neurodegenerative diseases.



A Rosmarinic acid



B Carnosic acid

Image credit: <http://www.health-ingredients.com/cosmetic-ingredients/rosemary-extract.html>

Figure 3.1. Structural comparison of rosmarinic acid (A) and carnosic acid (B).

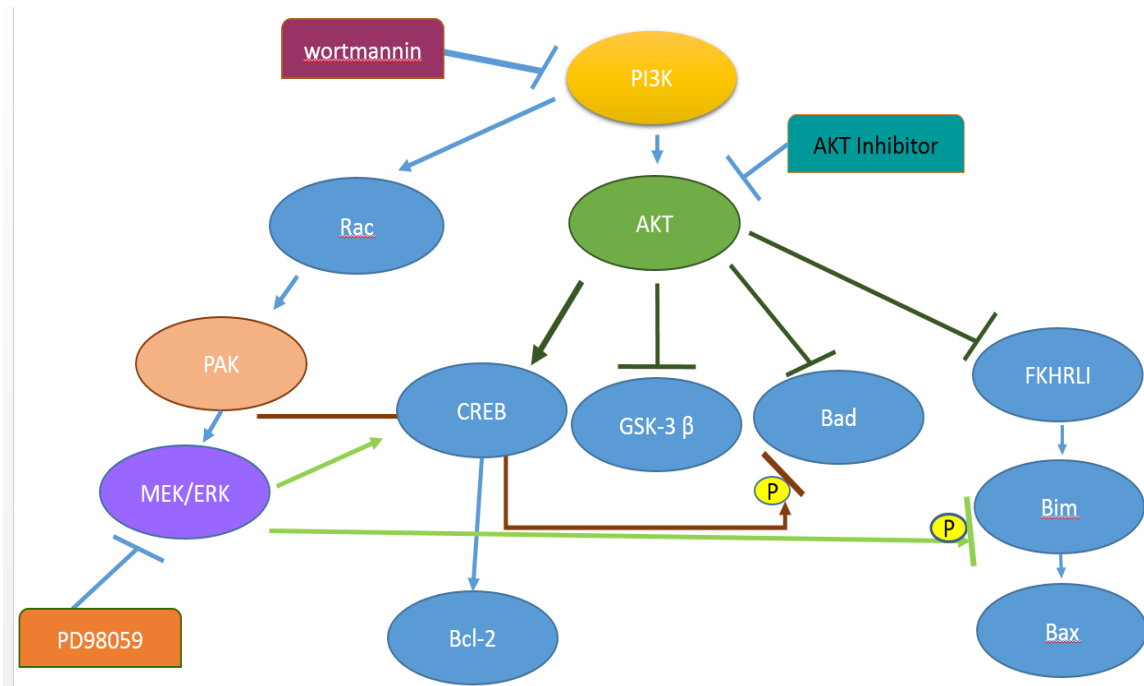


Figure 3.2. Overview of the pro-survival pathways that carnosic acid may activate when demonstrating its neuroprotective effects. Different inhibitors include wortmannin to inhibit PI3K, PD98059 to block MEK/ERK, and AKT inhibitor to inhibit AKT pro-survival pathway signaling.

3.3. Materials and methods

3.3.1. CGN Culture

Cerebellar granule neurons (CGNs) were isolated as previously described (Linseman et al., 2001) from seven day-old Sprague Dawley rat pups. Cells were plated on poly-L-lysine coated six-well plates (35mm-diameter), with a density of approximately 2×10^6 cells/well in Basal Medium Eagle's supplemented with 25mM potassium chloride, 2mM L-glutamate, 10% fetal bovine serum, and 2mM penicillin-streptomycin (100U/mL/100 μ g/mL). 10 μ M cytosine arabinoside was added to the culture medium 24 hours after plating to inhibit the growth of non-neuronal cells. Cultures were ~95% pure for granule neurons. The resulting CGNs were then incubated at 37°C in 10% CO₂ for six to seven days in culture prior to experimentation. All animal manipulations were performed in accordance with and under approval of the University of Denver Institutional Animal Care and Use Committee.

3.3.2. Reagents

Sodium nitroprusside (SNP) and PD98059 were obtained from Calbiochem (San Diego, CA). Carnosic acid (4aR,10aS)-5, 6-Dihydroxy-1,1-dimethyl-7-propan-2-yl-2,3,4,9,10,10a-hexahydrophenanthrene-4a-carboxylic acid) and rosmarinic acid ((R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl) lactic acid, 3,4-Dihydroxycinnamic acid (R)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester) were obtained from AG Scientific (San Diego, CA). Glutamic acid was purchased from MP Biomedical (Santa

Ana, CA). Glycine, Hoechst, and wortmannin were obtained from Sigma Aldrich (St. Louis, MO). AKT inhibitor was purchased from Calbiochem (San Diego, CA).

3.3.3 Treatment protocols.

3.3.3.1. Protocol for Treatment with SNP/glutamate+glycine

CGNs were co-treated with either carnosic or rosmarinic acid with the stressors SNP or glutamate-glycine (100 μ M/10 μ M), for 24 before fixation and Hoechst staining for quantification of cell death. For all experiments, an untreated control and an SNP or glutamate-glycine only control were used to compare cell death/protection. Prior to treatment, cell medium was removed and replaced with serum-free medium containing 25 mM KCl to prevent any potential protective effects of the serum.

3.3.3.2. Protocol for Treatment with 5K

CGNs were co-treated with either carnosic or rosmarinic acid, and 5K medium. Prior to treatment, cell culture medium was aspirated, and the cells were washed once with 5K medium to remove any leftover serum. 5K medium contains of Basal Medium Eagle's supplemented with 25mM potassium chloride, 2mM L-glutamate, and 2mM penicillin-streptomycin (100U/mL/100 μ g/mL). After the first wash with 5K, the medium was removed and replaced with fresh 5K medium. Wells whose medium was never replaced, and wells with only 5K medium were used as controls. In the remaining experimental wells, the appropriate concentration of rosmarinic or carnosic acid was added. The cells were left in an incubator at 37°C for 24 hours before fixation and staining with Hoechst for quantification of apoptosis.

3.3.3.3 Protocol for treatment with inhibitors

CGNs were co-treated with varying concentrations of carnosic acid, and then either 5K medium, or 5K plus 10 μ M PD-98059, 100nM wortmannin, or 10 μ M AKT inhibitor. Cells whose medium was never replaced, and cells with only 5K were used as controls. Cells containing 5K plus the wortmannin, PD-98059, and AKT inhibitor without carnosic acid were also included as controls. Cells were treated and then left in an incubator for 24 hours before fixation, Hoechst staining, and quantification were carried out.

3.3.4. Fixation and Hoechst Staining

Following treatment, CGNs were washed once with phosphate buffered saline (PBS; pH=7.4) and fixed for one hour at room temperature in 4% paraformaldehyde. Cells were then washed again with PBS and stained with Hoechst (10 μ g/mL). After a minimum of one hour, the cells were imaged using a Zeiss Axiovert-200M epi-fluorescence microscope. Five images were taken per well to assess apoptosis, with either duplicate or triplicate wells per experiment. Cells were counted and scored as either living or apoptotic based on nuclear morphology using images showing decolorized Hoechst fluorescence. CGNs having condensed or fragmented nuclei were counted as apoptotic.

3.3.5. Data Analysis

Each experiment was performed using either duplicate or triplicate wells for each treatment, with each experiment being performed at least three times. Data were represented as the means \pm SEM of the total number of experiments. Data was analyzed

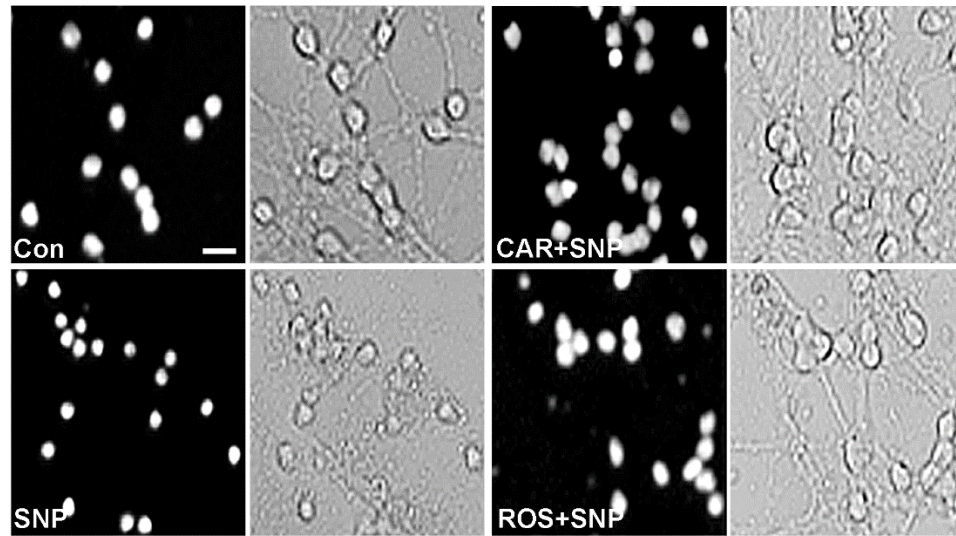
using a one-way ANOVA with a post hoc Tukey's test. A p-value of <0.05 was considered statistically significant.

3.4. Results

3.4.1 Carnosic acid and rosmarinic acids each protect CGNs from nitrosative stress

SNP, a nitric oxide donor, has been shown to induce nitrosative stress, causing cell death through the formation of reactive nitrogen species (RNS) (Eu et al., 2000). Here, the efficacy of rosmarinic acid and carnosic acids against nitrosative stress was examined by assessing the extent to which these two compounds prevented cell death when cells were exposed to SNP. Cells death was determined by examining nuclear morphology, and the state of the neuronal processes under bright field imaging. It was found that SNP was very toxic to CGNs, as observed through their condensed and fragmented nuclei as compared to the nuclei of untreated control cells (Figure 3.2A). It is also evident through the bright field images that the neuronal processes are essentially destroyed by SNP. These effects were largely reduced by 50 μM rosmarinic acid, and to a slightly lesser but still significant extent by 10 μM carnosic acid (Figure 3.3A). Quantification of these results is shown in Figure 3.2B as the percentage of apoptotic cells counted using decolorized Hoechst fluorescence (Figure 3.3B) to assess nuclear morphology. By this method, it was found that rosmarinic acid significantly decreased cell death at doses of 50 μM and 100 μM . Carnosic acid demonstrated a more potent protective effect, at dose of 10 μM and 20 μM . Moreover, at concentrations of over 20 μM and 100 μM of carnosic acid and rosmarinic acid, respectively, the compounds were somewhat toxic on their own to CGNs (data not shown).

A



B

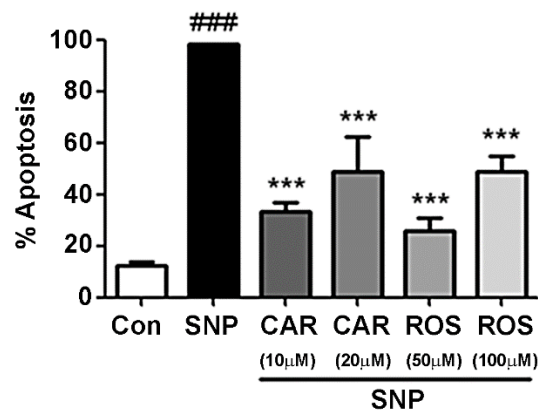


Figure 3.3. Rosmarinic acid and carnosic acid each protect CGNs from nitrosative stress. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25 mM KCl (Control; Con) alone, or with rosmarinic acid (ROS; 50 μ M) or carnosic acid (CAR; 10 μ M) and sodium nitroprusside (SNP; 100 μ M), or SNP alone. Following incubation, CGNs were fixed and stained with Hoechst staining to visualize the nuclei. Decolorized (black & white) panels are shown to emphasize nuclear morphology (DAPI), and gray panels show the bright field images of the same fields. Scale bar indicates 10 μ m. **(B)** Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 100 μ M SNP, and 100 μ M SNP plus 50 or 100 μ M ROS, or 10 or 20 μ M CAR. Cells were quantified by counting as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates p<0.001 compared to control, *** indicates p<0.001 compared to SNP alone as determined using one-way ANOVA with a post hoc Tukey's test.

3.4.2. Rosmarinic acid protects uniquely CGNs against excitotoxicity.

Glutamate and its co-agonist glycine, when introduced to cells, are known to cause excitotoxicity through an influx of calcium. The molecules bind to ionotropic glutamate receptors, allowing extracellular calcium to enter and greatly depolarize the cell. This increase in intracellular calcium can activate calpains, form reactive oxygen species (ROS) and RNS, and more generally cause the neuron to fire more action potentials, causing further depolarization and increased frequency of the aforementioned issues with high concentrations of intracellular calcium (Arundine and Tymianski 2003). Rosmarinic acid notably protected CGNs against the insult of glutamate-glycine (100 μ M/10 μ M); however, carnosic acid appended to worsen the effects of glutamate-toxicity (Figure 3.3). To assess the extent to which CGNs were damaged by the insult with glutamate and glycine, the cells were imaged and quantified by looking at decolorized Hoechst images for nuclei stability and integrity (Figure 3.4A). The percentage of apoptotic cells was determined for each treatment and the quantitative results are shown in Figure 3.4B. Rosmarinic acid significantly protected CGNs from excitotoxicity at 20 μ M and 50 μ M concentrations. In contrast, carnosic acid at doses of 10 μ M and 20 μ M enhanced the CGNs cell death in the presence of glutamate, though this effect did not reach statistical significance.

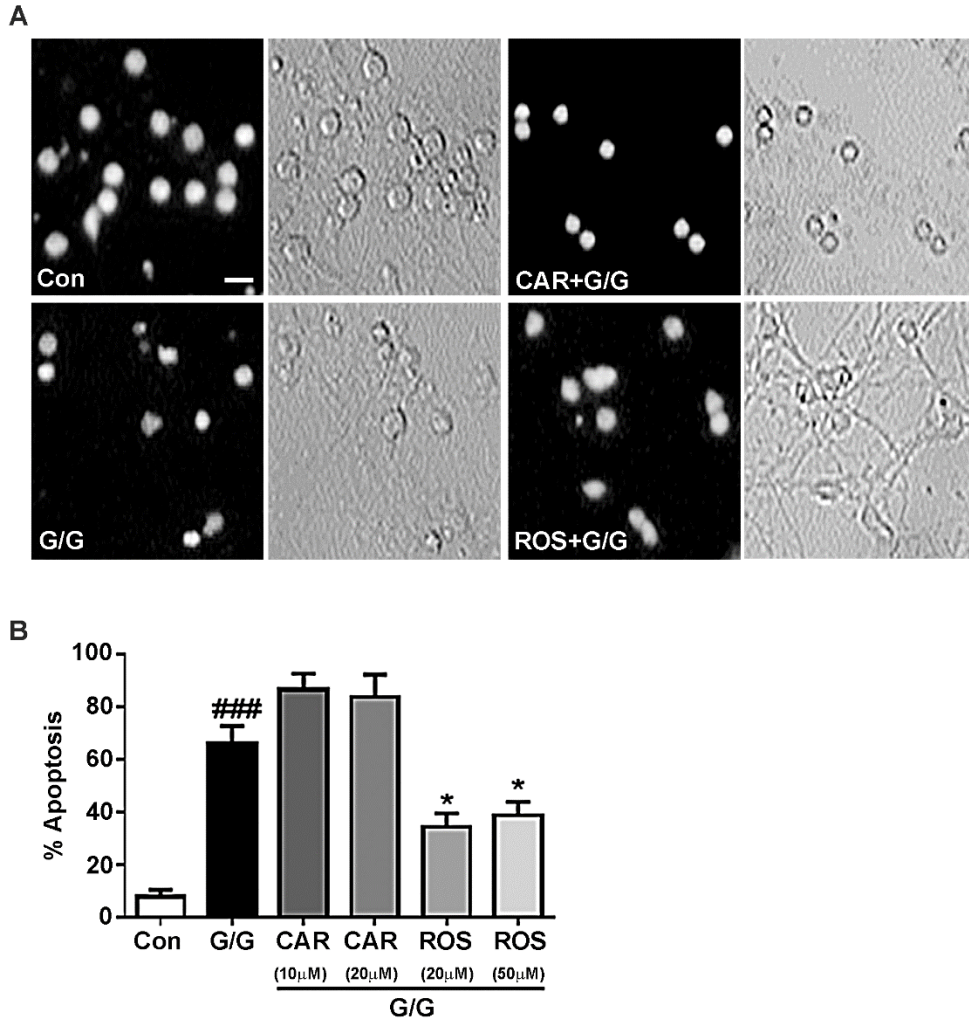


Figure 3.4. Rosmarinic acid, but not carnosic acid, protects CGNs from excitotoxicity. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25 mM KCl (Control; Con) alone or with rosmarinic acid (ROS; 20 μ M) or carnosic acid (CAR; 10 μ M) and glutamate/glycine (G/G; 100 μ M/10 μ M final concentrations), or G/G alone. Following incubation, CGNs were fixed and stained with Hoechst staining to visualize the nuclei. Decolorized (black & white) panels are shown to emphasize nuclear morphology (DAPI), and the gray panels show the bright field images of the same fields. Scale bar indicates 10 μ m. (B) Quantitative assessment of cellular apoptosis for CGNs in untreated controls, G/G (100 μ M/10 μ M) alone, and G/G plus 20 or 50 μ M ROS, or 10 or 20 μ M CAR. Cells were quantified as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates p<0.001 compared to control, *indicates p<0.05 compared to G/G alone as determined using one-way ANOVA with a post hoc Tukey's test.

3.4.3. Carnosic acid uniquely protects CGNs against caspase activation

5K is a non-depolarizing, low potassium medium that causes caspase activation in CGNs. Caspases cause what is known as programmed cell death when activated. When the cell undergoes stress, such as the low potassium conditions seen with this insult, pro-apoptotic proteins are ultimately activated. There are several pro-survival cell signaling pathways that can be inactivated by 5K medium to allow caspase activation and subsequent death of the cell. Stimulation of these pathways under 5K conditions can therefore lead to cell survival and neuronal protection. The specific pathways we tested in subsequent experiments were the pro-survival PI3K, MEK/ERK, and AKT pathways. Carnosic acid significantly protected CGNs against 5K-induced apoptosis, whereas rosmarinic did not (Figure 3.5). These results were again quantified from decolorized Hoechst images (Figure 3.5B), and the percentage of apoptotic cells was determined. Conversely to the results observed with glutamate, carnosic acid significantly protected against the 5K insult at 10 μ M and 20 μ M. Additionally, 5K treatment alone damaged processes to a lesser extent than either SNP or glutamate treatment (Figure 3.5A); however, treatment with carnosic acid did improve the overall integrity of neuronal processes. Rosmarinic acid offered no protect from 5K-induced apoptosis (Figure 3.5B)

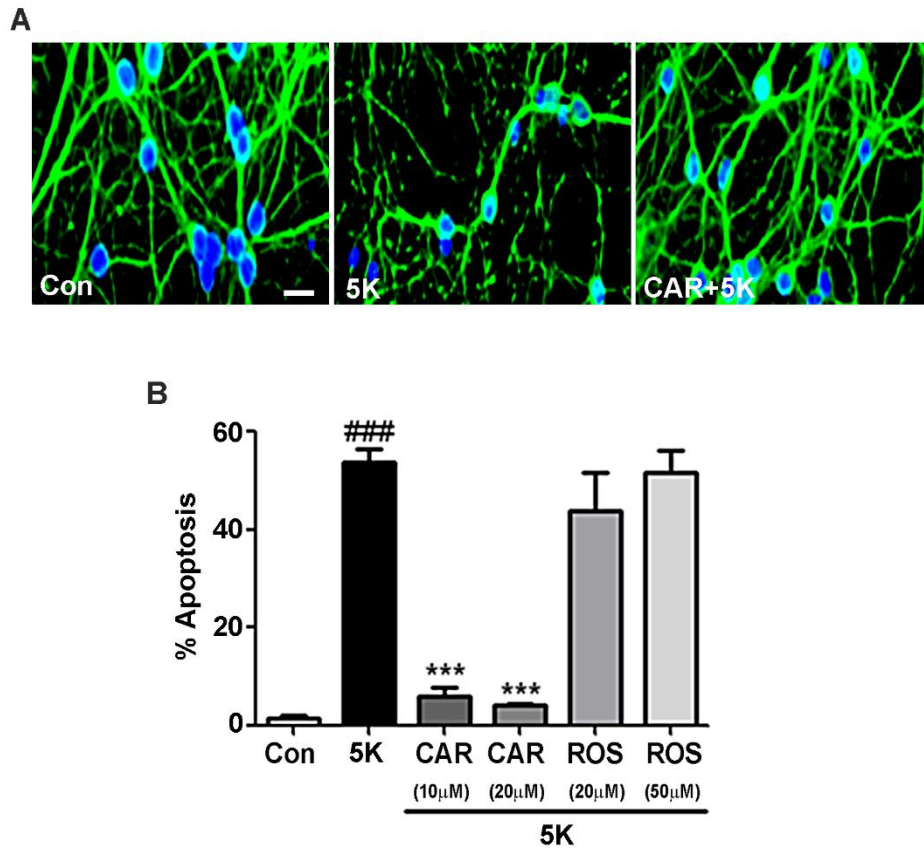


Figure 3.5. Carnosic acid, but not rosmarinic acid, protects CGNs from caspase activation. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con). Treated cells had 25 mM KCl medium replaced with a non-depolarizing, low potassium medium (5K medium) either alone or with carnosic acid (CAR; 20 μ M). Following incubation, CGNs were fixed and stained with Hoechst (blue) and β -tubulin antibody (green) staining to visualize the nuclei and tubulin respectively. Scale bar indicates 10 μ m. (B) Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 5K, and 5K plus 20 or 50 μ M ROS, or 10 or 20 μ M CAR. Cells were quantified as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates p<0.001 compared to control, *** indicates p<0.001 compared to 5K media alone as determined using one-way ANOVA with a post hoc Tukey's test.

3.4.4. Carnosic acid protects CGNs from 5K-induced apoptosis through PI3K activation, but not AKT or MEK/ERK signaling.

Once it was determined that carnosic acid protected CGNs against 5K-induced and caspase activation, we attempted to determine if these protective effects were due to activation of specific pro-survival pathways that would inhibit caspase activation. First, the PI3K pathway was investigated using wortmannin, a known PI3K inhibitor (Wymann et al. 1996). It was determined that PI3K inhibition with wortmannin counteracted the protective effects of carnosic acid (Figure 3.6). Decolorized Hoechst images for this experiment are shown in Figure 6A, and were quantified to determine the percentage of apoptotic cells (Figure 3.6B). The results showed that cells containing 5K medium, carnosic acid, and wortmannin looked like the cells containing 5K medium alone. Thus wortmannin effectively reversed the protective effect of carnosic acid seen under 5K apoptosis conditions (Figure 3.6B). These findings demonstrated that the protective effects of carnosic acid against 5K-induced apoptosis are PI3K dependent.

The next pathway investigated was the AKT pro-survival pathway, one of the pathways activated downstream of PI3K. We blocked this pathway by using an AKT inhibitor. The protective effect of carnosic acid was sustained even in the presence of the AKT inhibitor. Decolorized Hoechst images for this experiment are shown in Figure 7A and were quantified to determine the percentage of apoptotic cells (Figure 3.7B). The results demonstrate that carnosic acid protects CGNs from 5K-induced apoptosis independently of the AKT pro-survival pathway.

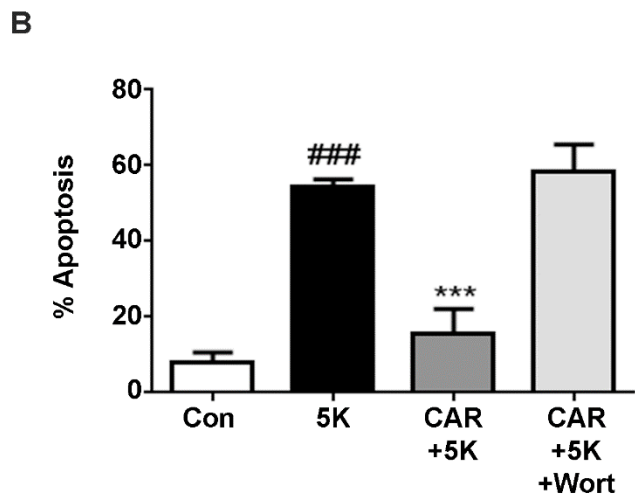
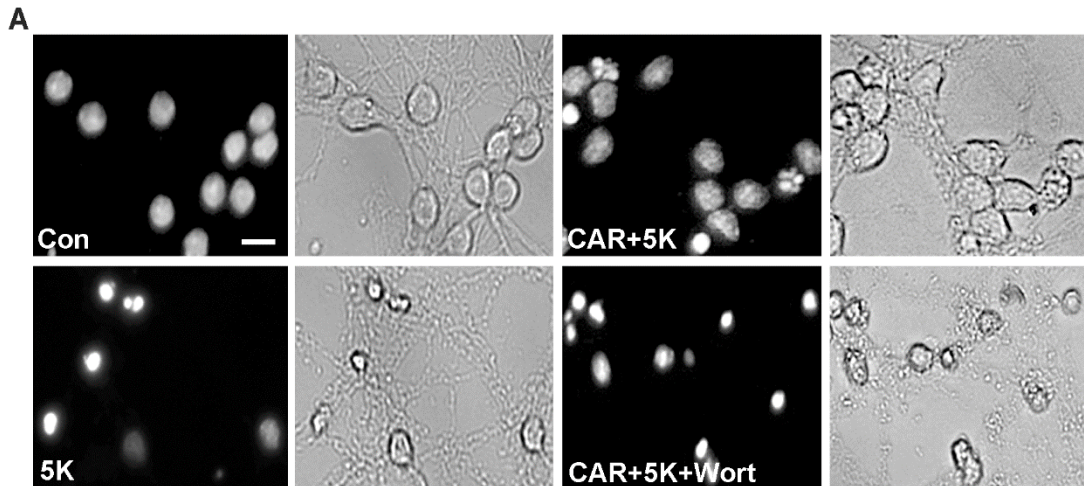


Figure 3.6. Carnosic acid protects CGNs from caspase activation through the PI3K pro-survival pathway. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25 mM KCl (Control; Con). Treated cells had 25K mM KCl medium replaced with a non-depolarizing, low potassium medium (5K medium) either alone, with carnosic acid (CAR; 20 μ M), with wortmannin (Wort; 100 nM), or with Wort and CAR. Following incubation, CGNs were fixed and stained with Hoechst staining to visualize the nuclei. Decolorized (black & white) panels are shown to emphasize nuclear morphology (DAPI), and the gray panels show the bright field images of the same fields. Scale bar indicates 10 μ m. (B) Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 5K, 5K plus Wort, 5K plus 20 μ M CAR, and 5K plus Wort and 20 μ M CAR. Cells were quantified by counting as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates p<0.001 compared to control, * indicates p<0.001 compared to 5K media alone as determined using one-way ANOVA with a post hoc Tukey's test.**

Finally, we investigated the MEK/ERK pathway by using PD98059, a known MEK/ERK inhibitor that works through binding to inactive MEK-1 and prevents subsequent downstream activation of pro-survival ERK signaling (Crews et al., 1992). Carnosic acid significantly protected CGNs from 5K-induced apoptosis even in the presence of PD98059 (Figure 3.8A, 3.8B). This suggests that carnosic acid prevents caspase activation through a mechanism independent of MEK/ERK signaling.

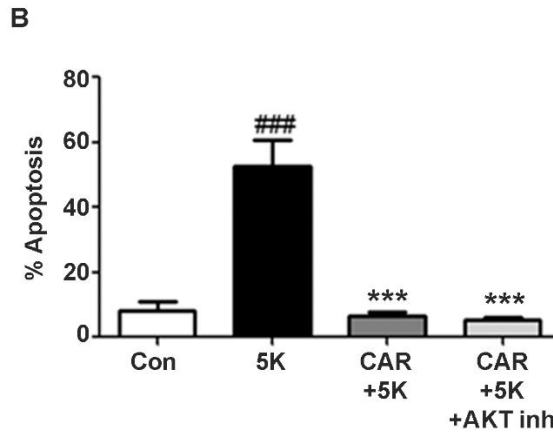
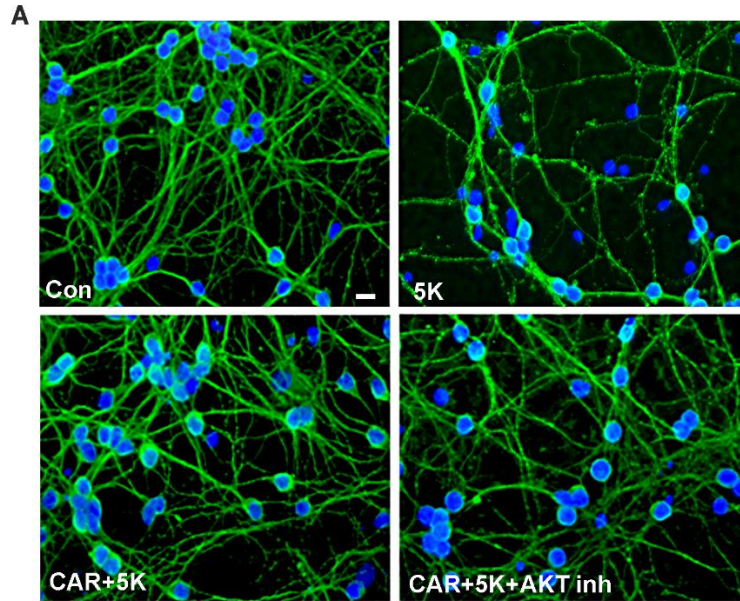


Figure 3.7. Carnosic acid protects CGNs from caspase activation independently of the AKT pro-survival pathway. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con). Treated cells had 25 mM KCl medium replaced with a non-depolarizing, low potassium medium (5K medium) either alone, with carnosic acid (CAR; 15 μ M), with AKT inhibitor (AKT inh; 10 μ M), or AKT inh and CAR. Following incubation, CGNs were fixed and stained with Hoechst (blue) and β -tubulin antibody (green) staining to visualize the nuclei and tubulin, respectively. Scale bar indicates 10 μ m. **(B)** Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 5K, 5K plus AKT inh, 5K plus 15 μ M CAR, and 5K plus AKT inh and 15 μ M CAR. Cells were quantified by counting as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates $p < 0.001$ compared to control, *** indicates $p < 0.001$ compared to 5K media alone as determined using one-way ANOVA with a post hoc Tukey's test.

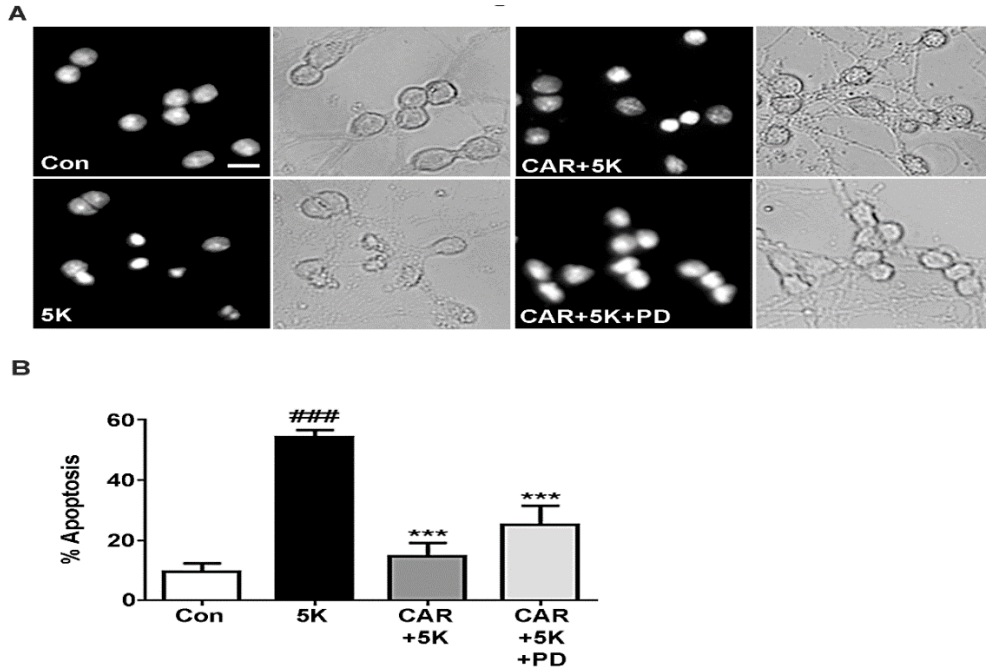


Figure 3.8. Carnosic acid protects CGNs from caspase activation independently of the MEK/ERK pro-survival pathway. (A) CGNs were co-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con). Treated cells had 25 mM KCl medium replaced with a non-depolarizing, low potassium medium (5K medium) either alone, with carnosic acid (CAR; 10 μ M), with PD98059 (PD; 10 μ M), or PD and CAR. Following incubation, CGNs were fixed and stained with Hoechst staining to visualize the nuclei. Decolorized (black & white) panels are shown to emphasize nuclear morphology (DAPI), and the gray panels show the bright field images of the same fields. Scale bar indicates 10 μ m. **(B)** Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 5K, 5K plus PD, 5K plus 10 μ M CAR, and 5K plus PD and 10 μ M CAR. Cells were quantified by counting as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. ### indicates p<0.001 compared to control, *** indicates p<0.001 compared to 5K media alone as determined using one-way ANOVA with a post hoc Tukey's test.

3.4. Discussion

Rosmarinic acid and carnosic acid have many bioactivities, including acting as antioxidants, that may make them viable therapeutic options to diminish the underlying pathophysiology of various neurodegeneration diseases. In the current study, we compared the neuroprotective effects of rosmarinic acid and carnosic acid against different stressors in cultured rat CGNs, a well-established *in vitro* model to examine neuronal cell death (Maycotte et al., 2010). Moreover, we studied diverse mechanisms that may underlie the protective effects of carnosic acid against intrinsic apoptosis.

Our data show that both rosmarinic acid and carnosic acid protected neurons from an *in vitro* model of nitrosative stress induced by sodium nitroprusside (SNP). This compound is a nitric oxide (NO) donor which can form highly toxic reactive nitrogen species (RNS) such as peroxynitrite (Tsang and Chung 2009). When these toxic RNS form, they cause extensive cellular damage, often resulting in death (Tsang and Chung 2009). The neuroprotective effects of rosmarinic acid are potentially linked to its abilities to down regulate NF- κ B and inhibit NO production from activated glial cells (Osakabe et al., 2004; Lo et al., 2002; Park et al., 2008). However, rosmarinic acid may also directly scavenge RNS like NO. Moreover, both compounds share a common catechol moiety that acts as a hydrogen donor to free radicals and uses oxygen as an electron acceptor (Oliveira et al., 2011). Furthermore, rosmarinic acid has previously been shown to have both anti-inflammatory and antioxidant effects, and to attenuate motor neuron death in a familial mouse model of ALS (Petersen and Simmonds 2003; Shimojo et al., 2010). Our results are consistent with a previous study, in which rosmarinic acid inhibited NO

production in RAW2647 mouse macrophages (Huang et al., 2009). Carnosic acid has also been shown to effects of NO level that was induced by LPS in microglial cells (Yanagitai et al., 2012). Thus, both rosmarinic acid and carnosic acid appear to mitigate nitrosative stress via two mechanisms. First, these compounds blunt NO production from macrophages and microglia. Second, they can act as direct scavengers of RNS like NO.

To understand the capacity of these compounds to mitigate different death pathways that are involved in neurodegenerative diseases, we investigated their protective effects in other models of neuronal cell death. Excitotoxicity has been shown to play a role in these diseases, and therefore was tested with these agents to determine their neuroprotective effects (Meredith et al., 2009). To replicate excitotoxicity in vitro, CGNs were exposed to glutamate and glycine. When NMDA receptors are activated by phosphate and open, these calcium channels are over stimulated. Neuronal nitric oxide synthase (nNOS) is subsequently activated and produces NO, and nitrosative and oxidative stress effects can be observed, in addition to calcium overload and calpain activation (Tsang and Chung 2009). We found that rosmarinic acid showed significant protection against this particular type of cell death. Carnosic acid conversely enhanced cell death caused by excitotoxicity. The opposite effects of these compounds on excitotoxic cell death are striking and are not readily explained. Additional studies are necessary to define the mechanisms underlying these results. Rosmarinic acid showed a significant and unique neuroprotective effect against excitotoxicity, which has been substantiated in another study performed in human neuroblastoma cells (Fallarini et al., 2009).

Our study found that carnosic acid specifically worsened the effects of excitotoxicity in CGNs, while other studies have found it to antagonize intracellular calcium mobilization in leukocytes (Poeckel et al., 2008). Another study suggested that carnosic acid has anti-excitotoxic effects in vivo and in vitro neuronal models (e.g., HT22 hippocampal cells) (Satoh et al., 2008). The conflicting finding between our study and these prior reports may be due to the different cell types utilized. Regardless of these effects, carnosic acid protected CGNs against 5K apoptosis conditions, whereas rosmarinic acid did not. This protocol subjects the cells to low potassium conditions, subsequently causing caspase activation independent of nitrosative or oxidative stress, resulting in programmed cell death (PCD) or intrinsic apoptosis.

To further characterize how carnosic acid protected against this specific type of cell death, different inhibitors were used to test the involvement of specific cell survival pathways. One of the pathways examined was the PI3K pro-survival pathway, which was inhibited by wortmannin. Wortmannin suppressed the protective effects of carnosic acid seen in 5K medium, suggesting that the neuroprotective effects of this compound are dependent upon activation of the PI3K signaling pathway. A previous study reported that pretreating IMR-32 cells with wortmannin decreases Nrf2 nuclear translocation (Lee et al., 2001). Based on this result, the authors proposed that Nrf2 is activated downstream of PI3K. Satoh et al. have reported that carnosic acid activates Nrf2 in vivo and in vitro, and activates the transcriptional antioxidant response element (ARE) of phase-2 genes that modulate the cellular redox state (Satoh et al., 2008, Satoh et al., 2008b). Thus, the dependence of carnosic acid in neuroprotection against 5K-

apoptosis in CGNs on PI3K may involve subsequent activation of Nrf2. However, this result seems somewhat unlikely, since 5K-induced apoptosis in CGNs occurs largely independent of oxidative stress.

We also examined the AKT pathway, another pro-survival signaling pathway that commonly act downstream of PI3K. To block this pathway, we added an AKT inhibitor to CGNs along with carnosic acid and 5K media. Carnosic acid protected against 5K-induced apoptosis even in the presence of the AKT inhibitor, suggesting that the PI3K-dependence of carnosic acid neuroprotection is not due to activating AKT.

The effects of MEK/ERK were then investigated using PD98059. This compound is a MEK/ERK inhibitor, another cell signaling pathway involved in cell survival. MEK/ERK can be activated downstream of PI3K activation of Ras. However, carnosic acid protected against 5K medium even in the presences of PD98059. This result showed that carnosic acid protects from apoptosis independently of the MER/ERK pro-survival pathway.

Since we have tested two pro-survival cell signaling pathways activated downstream of PI3K (AKT and MEK/ERK), and neither was implicated in the protective effect of carnosic acid, this indicate that, carnosic acid protects CGNs through another PI3K-dependent pathway (Nakaso et al., 2003). One of these other PI3K-dependent pathways could potentially be Nrf2 activation. Carnosic acid has been reported as an activator of Nrf2. So we considered that carnosic acid could protect against caspase activation by activation of PI3K, and subsequently Nrf2 activation. However, as indicated above, this seems unlikely since CGN apoptosis induced by 5K does not have a

significant oxidative stress component. Another possibility is a PI3K-dependent activation of Ras GTPase and subsequent activation of P21-activated kinase (PAK) (Marinissen and Gutkind 2001). Indeed, Ras and PAK signaling has been shown to be an important pro-survival pathway for CGNs (Stankiewicz et al., 2015). Future studies will examine if carnosic acid activates Rac/Pak in CGNs.

Rosmarinic acid has demonstrated neuroprotective effects in numerous different studies, as well as it has been shown to protect against types of cell death not examined in this study. For instance, other studies have shown rosmarinic acid to protect against A β insults (commonly seen in Alzheimer's disease) by mitigating similar types of cell death as were examined in this paper (Iuvone et al. 2006). A β toxicity can cause cell death through the generation of reactive oxygen species (ROS), lipid peroxidation, DNA fragmentation and caspase activation (Iuvone et al. 2006). Interestingly, we found that rosmarinic acid did not protect from caspase activation induced by 5K medium. The experiments completed in this study, as well as results from other studies, find rosmarinic acid to be an effective neuroprotective agent, though the method by which it attenuates certain types of cell death (e.g., A β -induced apoptosis) is still largely unknown. This is the next step that should be investigated. If the method by which this compound protects against cell death could be discovered, it could have vast implications for future neurodegenerative disease therapies.

Carnosic acid has also been shown in multiple studies, through its electrophilic properties, to activate the transcription factor Nrf2 (Sato et al. 2008; Takahashi et al. 2009). Various NRF-2- specific enzymes, for example heme oxygenase-1 (HO-1), are

antioxidant molecules that provide efficient cell protection through regulating the intracellular redox state (Sato et al. 2008). In this regard, the protective abilities seen by carnosic acid could be in part due to its electrophilic capabilities, combined with its antioxidant properties. This finding is interesting because it could have an impact on how neuroprotective compounds are regarded in the future. If electrophilic molecules also attenuate cell death as Sato et al. 2008 suggest, the research of neurodegenerative diseases could be expanded and perhaps even shifted. If electrophiles prove to be more effective at preventing cell death, there are potentially many more compounds that could demonstrate such effects.

Rosmarinic and carnosic acid, when taken together, could have many important and beneficial uses. Both compounds showed a protective effect against the NO donor SNP. Rosmarinic acid uniquely protected CGNs from excitotoxicity, and carnosic acid uniquely protected CGNs from intrinsic apoptosis induced by 5K medium. Both have been shown to have impressive antioxidant and neuroprotective effects. These effects nicely complement each other, and add a potential future combination therapy for devastating, sporadic neurodegenerative diseases.

CHAPTER FOUR: PQQ PROTECTS CGNS FROM OXIDATIVE STRESS

4.1. Abstract

Neurodegenerative disorders are devastating and fatal disorders of the nervous system that remain a critical focus of scientific research today. The underlying etiological features of neurodegenerative diseases consist of many mechanisms known to cause neuronal death, including oxidative stress, mitochondrial dysfunction, excitotoxicity, and nitrosative stress. Pyrroloquinoline quinone (PQQ) is a polyphenolic compound that is found in high concentrations in fermented soybeans, parsley, and green tea. Recently, PQQ has received significant attention as a protective compound given its antioxidant and anti-inflammatory bioactivities. Moreover, PQQ has recently been tested in Parkinson's diseases models, where it was demonstrated to attenuate dopaminergic neuronal cell death. In this study, we examined the neuroprotective effects of PQQ in primary cultures of rat cerebellar granule neurons. We showed that PQQ displayed a protective effect against hydrogen peroxide- and copper chloride-induced oxidative stress. Conversely, we report that PQQ did not exhibit any protection against excitotoxicity, nitrosative stress, and caspase-dependent intrinsic apoptosis in cultured neurons. These data indicate that PQQ has a dramatic neuroprotective effect, which is specific to oxidative stress. This is an important finding as oxidative stress believed to be a common underlying factor of several neurodegenerative diseases. We conclude that

PQQ is a promising candidate for testing in preclinical models of neurodegeneration and may represent a novel therapy for the treatment of neurodegenerative disorders for which oxidative stress has been implicated as a causative factor.

4.2. Introduction

Neurodegenerative is a term from two words; neuro referring to nerves cells and degenerative referring to progressive damage of the function and structure of neurons. Neurodegenerative diseases include Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS). Age associated with increased risk of neurodegenerative diseases. These diseases have an average onset of 50-70 years (Martin, 1999). However, patients with the familial forms of neurodegenerative disorders may suffer from a much earlier onset of diseases (Martin, 1999). In many cases patients present with a sporadic form of the degenerative disorder with no known genetic component (e.g 90-95% sporadic in ALS; Thomas and Beal, 2007; Ferraiuolo et al., 2011; Anand et al., 2013). In both familial and sporadic disorders, neuronal cell death can occur through several different pathways, including mitochondrial dysfunction, oxidative stress, excitotoxicity, and/or misfolding of proteins (Ferraiuolo et al., 2011; Chakrabarti and Mohanakumar et al, 2016). Because of the complexity of the mechanisms involved, there is growing scientific interest in elucidating alternative approaches to cure these diseases. For example, several plants and their derivatives have displayed a remarkable ability to improve the health of patients who suffer from neurodegenerative diseases (Howes et al., 2003). Recent research indicates that the health benefits conferred by plant therapy may be related to the pharmacological activities of their constituents, such as

antioxidant, anti-inflammatory, pro-survival signaling pathways (i.e., enhancement of the Nrf2 and CREB; transcription factor NF-E2-related factor 2 (Nrf2) that regulates the expression of antioxidant genes and also cAMP-response element binding protein (CREB) the pro-survival protein) (Wu et al., 2010).

One of the most abundant types of molecules that is found in plants are polyphenols. Polyphenols have received a lot of attention due to their bioavailability and recent prospective studies which have shown their benefits *in vitro* and *in vivo* (Del Rio D et al., 2010). For instance, polyphenols display anti-apoptotic, antioxidant and anti-cancer activities (Mercer et al., 2005; Ferguson et al., 2004). In addition, patients with neurological disorders have demonstrated an increase in life span after following diet rich in polyphenols. Further, long-term treatment with polyphenols delayed the symptoms of aging related disorders like ALS (Xu et al., 2006). Since natural compounds have different pharmacological properties and may be able to attenuate several common causes of neuronal cell death, it is possible that consuming these compounds may represent a strong potential therapy for neurodegenerative illness.

Pyroloquinoline quinone (PQQ) is a polyphenolic compound which is found naturally in many plants, but with particularly high concentrations in fermented soybeans, parsley, and green tea (Kumazawa et al., 1995). Research has provided considerable insight into the multiple pro-survival properties of PQQ, and the importance of a PQQ-rich diet has been demonstrated in numerous studies. A PQQ-deprived diet leads to many physiological problems, such as defects in growth, reproduction, and immune system performance (Steinberg et al., 2003; Rucker et al., 2009).

In addition to its physiological activities, PQQ has several biomedical activities. For example, PQQ reduced the amount of damage in an animal model of cardiac ischemia (Rucker et al., 2009). The neuroprotective effects of PQQ have also been investigated in countless studies due to its implication in several pro-survival pathways. Neuro-inflammation is one of the prominent mechanisms that is thought to contribute to neurodegeneration. The anti-inflammatory effect of PQQ has been investigated *in vitro*. Yang et al. (2014) showed that PQQ treatment inhibited the production of many inflammatory mediators which were induced by LPS such as, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin 6 (IL-6). Moreover, PQQ showed protective effects against oxidative stress, another molecular pathway that is consistently implicated in the etiology of neurological disorders (Nunome et al., 2008). This effect of PQQ may be related to its ability to act as a free radical scavenger or its ability to enhance Nrf2, thereby upregulating antioxidant genes (Zhang et al., 2012; Nunome et al., 2008). In addition, aggregation of β -amyloid (A β) is potentially one of the critical causes of Alzheimer's disease (AD), and has been alleviated by treatment with PQQ (Zhang et al., 2009). Interestingly, PQQ can also induce mitochondrial biogenesis, and diets lacking PQQ have been shown to decrease the number of mitochondria (Chowanadisai et al., 2010). In models of PD and stroke, PQQ attenuated neuronal death (Qin et al., 2015; Jensen et al., 1994).

The purpose of the present study is to investigate the neuroprotective effect of PQQ against different insults which activate neuronal death pathways. We pre-

incubated cerebellar granule neurons (CGNs) with PQQ (Fig. 4.1), and subsequently treated the neurons with different toxic insults. The percentage of apoptosis was measured by immunocytochemistry designed to visualize the microtubule network and nuclear morphology. Our data demonstrate that PQQ displays significant neuroprotective effects against two different neuronal insults which induce cell death via oxidative stress. Conversely, PQQ failed to protect against insults that cause nitrosative stress, apoptosis, and excitotoxicity. Based on our findings, PQQ may be a promising therapy for neurodegenerative disorders for which oxidative stress is a major factor underlying disease etiology.

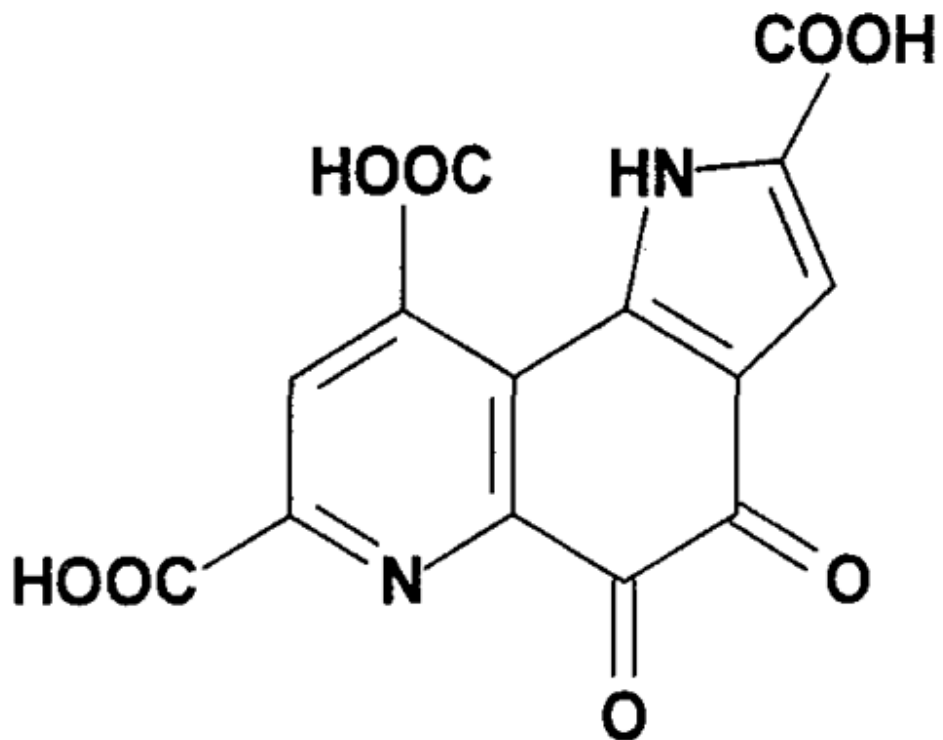


Image credit:
https://www.researchgate.net/publication/264799310_Discovery_of_a_Eukaryotic_Pyrroloquinoline_Quinone

Figure 4.1. Structural comparison of pyrroloquinoline quinone (PQQ).

4.3. Method

4.1. Materials.

Hoechst dye, paraformaldehyde, glutamic acid, and glycine were purchased from Sigma Aldrich (St. Louis, MO). Sodium nitroprusside (SNP) was obtained from Calbiochem (San Diego, CA). Stock solutions of glutamate/glycine were made in sterile water. Pyrroloquinoline quinone (PQQ) and copper chloride (CuCl_2) were obtained from Sigma Aldrich (St. Louis, MO).

4.2. Cell culture.

Cerebellar granule neurons (CGNs) were isolated from 7-day-old Sprague-Dawley rat pups (15-19g) of both sexes as described previously (Linseman et al., 2001). Briefly, neurons were plated on poly-L-lysine-coated culture dishes at a density of 2.0×10^6 cells/mL in Basal Modified Eagle's medium (BME) containing 10% fetal bovine serum, 25 mM potassium chloride (KCl), 2 mM L-glutamine, penicillin (100units/ml)-streptomycin (100 μ g/ml; Life Technologies, Inc., Gaithersburg, MD). Cytosine arabinoside (AraC, final concentration of 10 μ M) was added after 24h to limit the growth of non-neuronal cells. With this protocol, the purity of cerebellar granule neurons was ~95-99%. After 6 or 7 days in culture, CGNs were rinsed two times in serum-free culture medium containing 25mM depolarizing potassium (25K-Serum) and maintained in the same medium for pre-incubation with pyrroloquinoline quinone (PQQ; 10 nM, 30nM, 100nM, 300nM, and 1 μ M) for 24h. Apoptosis was induced either by removal of depolarizing extracellular potassium (5mM KCl; 5K apoptotic condition), or by the direct

addition of the following stressors: (1) sodium nitroprusside (SNP; 50 μ M) to induce nitrosative stress, (2) 100 μ M glutamate and 10 μ M glycine (Glu/Gly) to induce excitotoxicity, (3) hydrogen peroxide (H₂O₂; 1mM), or (4) copper chloride (CuCl₂) (70 μ M). Pre-treated cells were treated overnight for approximately 16-18h with each stressor.

4.3. Fixation and Hoechst Staining

Following treatment, CGNs were washed once with phosphate buffered saline (PBS; pH=7.4) and fixed for one hour at room temperature in 4% paraformaldehyde. Cells were washed again with PBS and Hoechst stained at a concentration of 10 μ g/ml. After a minimum of one hour, cells were imaged using a Zeiss Axiovert-200M epi-fluorescence microscope. Five images were taken per well to assess apoptosis, with either duplicate or triplicate wells per experiment. Cells were counted and scored as either living or apoptotic based on nuclear morphology using images showing decolorized Hoechst fluorescence. CGNs displaying condensed or fragmented nuclei were counted as apoptotic.

4.4. Statistical Analysis.

Every experiment was performed using either duplicate or triplicate wells for each treatment, with each experiment being performed at least three times. Data are represented as the means \pm SEM of the total number of experiments. Data have analyzed using a one-way ANOVA with a post hoc Tukey's test. A p-value of <0.05 was considered statistically significant.

4.4. Result:

4.1. PQQ protects CGNs from oxidative stress induced by hydrogen peroxide.

Oxidative stress occurs as a consequence of an imbalance between free radical and antioxidant molecules (Uttara et al., 2009), resulting in neuronal cell death.

Treatment with hydrogen peroxide (H_2O_2) is a common model to study oxidative stress (Fatokun et al., 2007). To assess the neuroprotective effects conferred by PQQ, CGNs were pre-incubated with different concentrations of PQQ (10 nM, 30nM, 100nM, 300nM, and 1 μ M) for 24h. Next, pre-treated CGNs were treated with or without H_2O_2 (1 mM) for an additional 16-18h. To evaluate neuronal injury induced by H_2O_2 in CGNs, we first examined the nuclear morphology. Compared to control cells, H_2O_2 caused dramatic damage to nuclei. Nuclei were considered as apoptotic if fragmented or condensed as assessed by Hoechst staining (Fig 4.2A left panels). It is also evident through the bright field images that the neuronal processes were also destroyed by H_2O_2 . Importantly, these effects were largely reduced by PQQ in a concentration dependent manner.

Quantification of these data presented as the percentage of apoptotic cells counted using decolorized Hoechst fluorescence (Fig. 4.2B) to assess nuclear morphology. By this method, it was found that PQQ significantly decreased cell death in a dose-dependent manner, particularly at a concentration of 100nM, 300nM, and 1 μ M. However, lower concentration did not show significant protective effects.

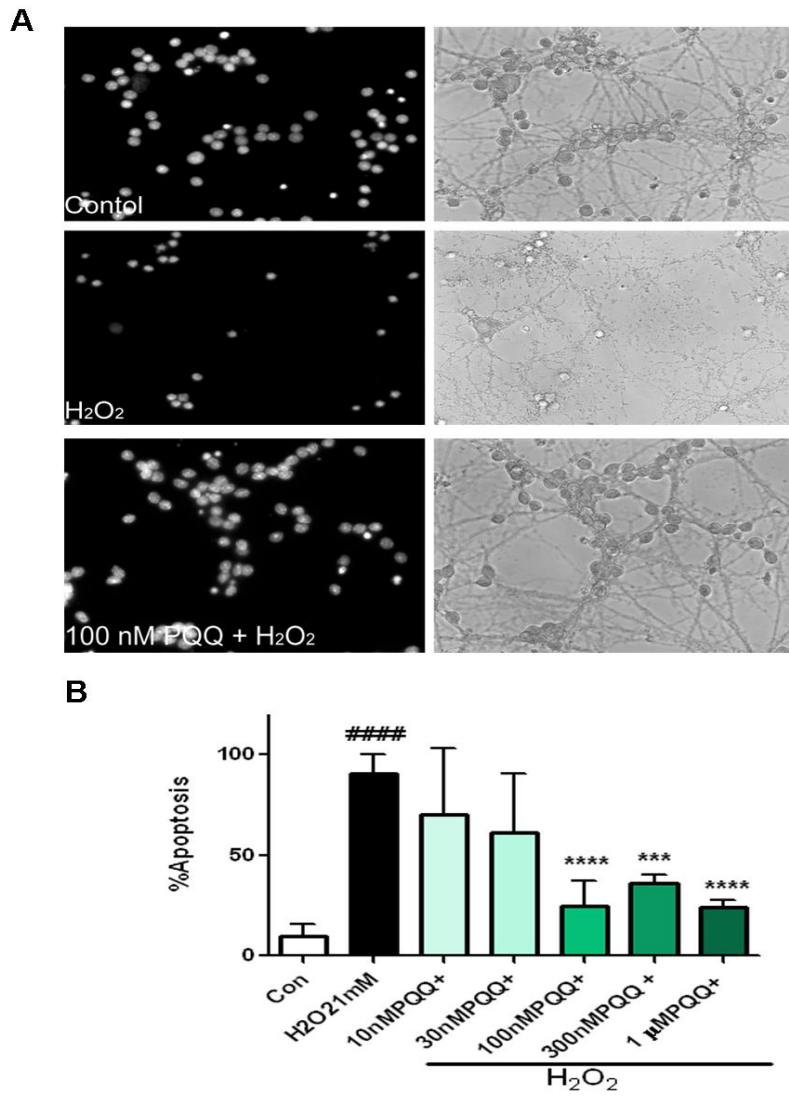


Figure 4.2. PQQ protects CGNs from oxidative stress induced by hydrogen peroxide. (A) CGNs were pre-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con) alone or with one of five different concentrations of PQQ (10nM, 30 nM, 100nM, 300nM, and 1 μ M) individually. Cells were then incubated for an additional 16-18h with hydrogen peroxide (H₂O₂; 1 mM). Following incubation, CGNs were fixed and Hoechst stained. (B) Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 1 mM H₂O₂ concentration, and 1mM H₂O₂ plus 10nM, 30 nM, 100nM, 300nM, or 1 μ M PQQ. Cells were quantified by counting them as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. *** indicates p<0.001 compared to control, #### indicates p<0.001 compared to H₂O₂ alone, and # indicates p<0.05 compared to H₂O₂ alone as determined using one-way ANOVA with a post hoc Tukey's test.

4.2. PQQ protects CGNs from oxidative stress induced by copper chloride.

In addition to H₂O₂, we also tested the oxidative stressor, copper chloride (CuCl₂) (Hu et al., 2016). CGNs were pre-incubated with PQQ (100nM, 300nM, and 1 μM) and subsequently treated for a further 16-18h with CuCl₂ (final concentration; 70μM). Nuclear morphology was assessed by Hoechst staining. Nuclei were fragmented or condensed following exposure to CuCl₂ (Fig. 4.3A). However, pre-incubation with each concentration of PQQ significantly reduced oxidative damage and diminished the appearance of apoptotic nuclei (Fig. 4.3A, left panels). These data show that PQQ is capable of protecting CGNs from CuCl₂-induced oxidative stress. The percentage of apoptotic cells was determined for each treatment, and the quantitative results are shown in Figure 3B. All doses of PQQ significantly protected against CuCl₂.

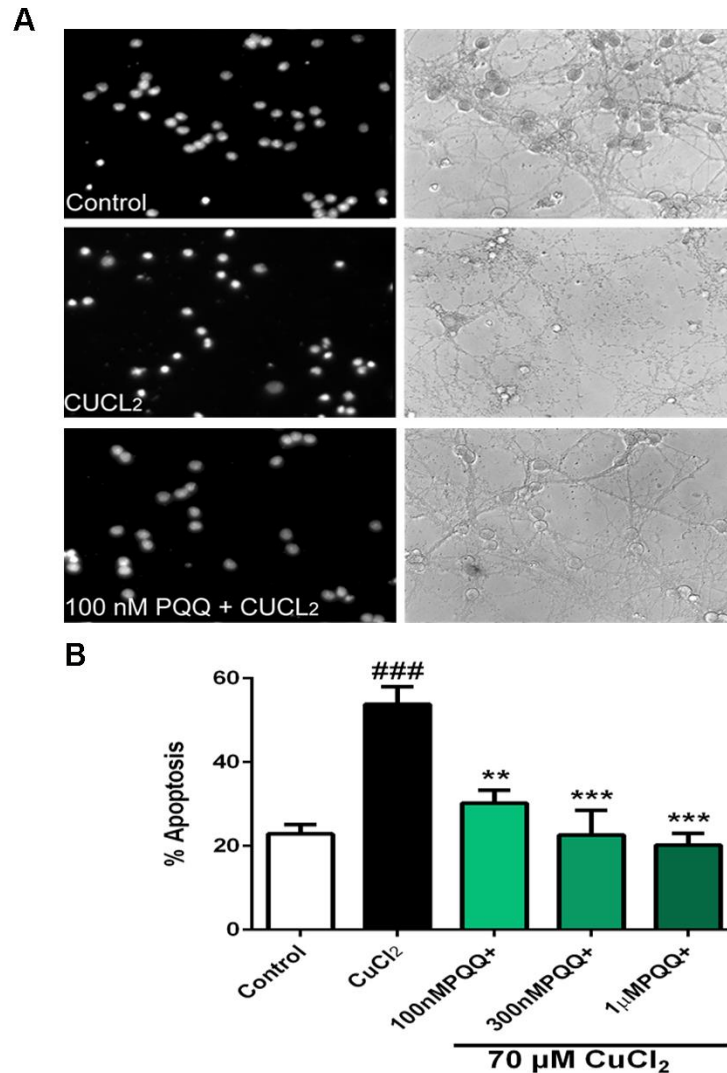


Figure 4.3. PQQ protects CGNs from oxidative stress induced by copper chloride. (A) CGNs were pre-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con) alone or with one of five different concentrations of PQQ (10nM, 30 nM, 100nM, 300nM, or 1 μ M). Cells were then incubated for an additional 16-18h with copper chloride (CuCl₂; 70 μ M). Following incubation, CGNs were fixed and Hoechst stained. (B) Quantitative assessment of cellular apoptosis for CGNs in untreated controls, 70 μ M CuCl₂, and 70 μ M CuCl₂ plus 10nM, 30 nM, 100nM, 300nM, and 1 μ M PQQ. Cells were quantified by counting them as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. *** indicates p<0.001 compared to control, ### indicates p<0.001 compared to CuCl₂ alone, and # indicates p<0.05 compared to CuCl₂ alone as determined using one-way ANOVA with a post hoc Tukey's test.

4.3. PQQ did not protect CGNs from excitotoxicity, nitrosative stress, or caspase activation.

Next, we examined the neuroprotective effect of PQQ against other death pathways induced by glutamine/glycine, sodium nitroprusside (SNP), and 5K medium. Extracellular glutamate mediates excitotoxicity by allowing calcium to enter and depolarize the cell. This prolonged increase in intracellular calcium leads to the formation of ROS and in turn, neuronal death (Arundine and Tymianski 2003). To modulate the previous death pathway, we added glutamine/glycine (100 μ M/10 μ M). We induced nitrosative stress by adding SNP (50 μ M), the nitric oxide donor. Moreover, we enhanced caspase activation by modifying the potassium level in CGN medium. CGNs were pre-incubated with PQQ (1 μ M) and then treated for a further 16-18h with glutamine/glycine (100 μ M/10 μ M), SNP (50 μ M), or exposure to 5K apoptotic medium consisting of low potassium conditions (5K medium). Unexpectedly, pretreatment with PQQ (1 μ M) did not show any protection against the previous insults and stressors. The percentage of apoptotic cells was determined for each treatment and analyzed in Fig 4.4.

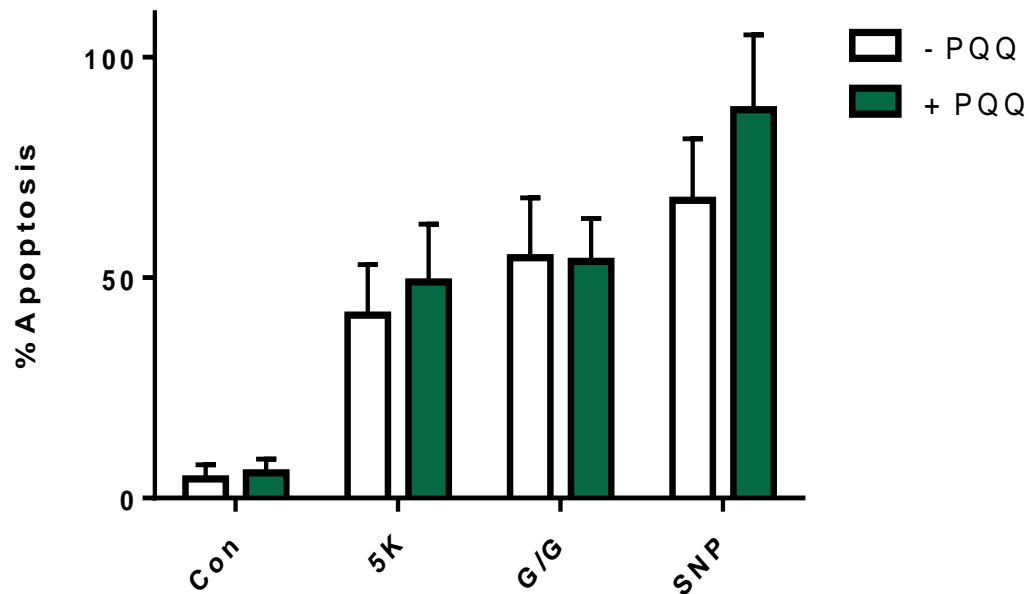


Figure 4.4. PQQ did not protect CGNs from excitotoxicity, nitrosative stress, or caspase activation by glutamate/glycine, SNP, or 5K medium, respectively. CGNs were pre-incubated for 24h in serum-free culture medium containing 25mM KCl (Control; Con) alone or with one of five different concentrations of PQQ (10nM, 30 nM, 100nM, 300nM, or 1 μ M). Cells were then incubated for an additional 16-18h with glutamate/glycine (100 μ M/10 μ M), SNP (50 μ M), or the low potassium medium was removed. Quantitative assessment of cellular apoptosis for CGNs in untreated controls, glutamate/glycine, SNP and 5K medium, and glutamine/glycine, SNP and 5K medium plus 10nM, 30 nM, 100nM, 300nM, and 1 μ M PQQ. Cells were quantified by counting them as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are expressed as the mean \pm SEM, n=4. * indicates $p < 0.001$ compared to control, ### indicates $p < 0.001$ compared to glutamine/glycine, SNP and 5K medium alone, and # indicates $p < 0.05$ compared to glutamine/glycine, SNP and 5K medium alone as determined using one-way ANOVA with a post hoc Tukey's test.**

4.5. Discussion

PQQ has many pro-survival properties such as antioxidant, anti-cancer, and anti-inflammatory activities. In addition, PQQ has a unique ability for inducing mitochondrial biogenesis. In the present study, we assessed the neuroprotective effects of PQQ against different molecular cell death pathways that we modulated by exposure to several stressors in cultured rat CGNs, an established model to study neuronal cell death (Maycotte et al., 2010). An examination of five different cell stressors revealed that PQQ showed a significant protection against insults that are known to induce oxidative stress (H_2O_2 and $CuCl_2$). However, PQQ failed to protect CGNs from other mechanisms that prompt neuronal death including nitrosative stress, excitotoxicity, and caspase activation. Oxidative stress is one of the mechanisms that leads to neuronal death, disturbing the balance between ROS (e.g., H_2O_2) and antioxidants, ultimately underlying biological aging (Yang et al., 2015).

The neuroprotective effects of PQQ against H_2O_2 are hypothetically interrelated with its ability to scavenge free radicals, in addition to its other properties, such as upregulation of the pro-survival protein Bcl-2, which is known to prevent the release of mitochondrial cytochrome *c* that promotes cell death (Yang et al., 2015). Our findings are similar to Yang et al. (2015), who demonstrated that PQQ alleviated the oxidative stress induced by H_2O_2 and that PQQ modulated mitochondria to protect cells from death (Yang et al., 2015). Taken together, our present findings in conjunction with previous studies, suggest that PQQ possesses a neuroprotective effect against oxidative

stress and may represent a novel therapy for the treatment of neurodegenerative disorders.

There is not a lot of research examining the effects of PQQ on CuCl₂; however, other studies have used another insult to induce oxidative stress which is 6-hydroxydopamine (6-OHDA) (Nunome et al. 2008). Similar to our data utilizing another oxidative stress promoter, PQQ protected cells from this oxidative stressor (Nunome et al. 2008). The neuroprotective effects of PQQ against nitrosative stress induced by SNP, the NO donor have been examined. Our data indicate that PQQ does not protect cells from nitrosative stress. Currently, other studies corroborating our findings are lacking. Since it has been confirmed that excitotoxicity plays an important role in neurodegenerative disorders, we also examined the protective effects of PQQ against insults known to induce excitotoxic neuronal cell death. To induce excitotoxicity, we treated cells with glutamate/glycine following pretreatment of CGNs with PQQ. However, treatment with PQQ did not show any protection. These data are in contrast with a study by Aizenman et al., demonstrating that PQQ attenuates the activation of NMDA receptor to mediate in excitotoxicity in both cortical neurons and astrocytes (Aizenman et al., 1992). This difference in experimental results may be correlated to using different cell types or to the higher concentration of glutamate/glycine used in their study.

The anti-apoptotic properties of PQQ have also been demonstrated in a study by Lu et al. (2015), demonstrating that pretreatment with PQQ in an intracerebral hemorrhage rat model, decreased caspase activation. The authors also reported that PQQ

protected rats from apoptosis by increasing the ratio of the pro-survival protein Bcl-2 to the pro-apoptotic protein Bax.

As an additional stressor to evaluate the protective effects of PQQ, we activated the intrinsic apoptotic pathway by treating with 5K apoptotic medium, leading to potassium deprivation by removing the optimum level of potassium required by CGNs. Under that condition of deprivation (5K medium), PQQ didn't protect CGNs from apoptosis. Thus, it appears that PQQ is specifically protective against oxidative stress in CGNs.

On the whole, our data support the neuroprotective effect of PQQ against two distinct inducers of oxidative stress. Nevertheless, PQQ did not show any neuroprotection against other neuronal death pathways (i.e., nitrosative stress, excitotoxicity, or intrinsic apoptosis). This finding provides substantial vision into PQQ as a potential therapy for neurodegenerative disorders. After the successful result by testing PQQ in pre-clinical disease models of Parkinson disease (Qin et al., 2015), it's noteworthy to consider PQQ as a novel therapeutic option to treat patients with neurodegenerative diseases that may be caused by oxidative stress.

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

Caffeic acid is a broad compound among chlorogenic acid and the two other metabolites (Ferulic acid and quinic acid) that showed protection against multiple insults and stressors *in vitro*. Results show promising therapeutic possibilities and pre-clinical testing, as caffeic acid phenethyl ester (CAPE) has been shown to delay and decrease the hallmark symptoms in mouse and rat models of Parkinson's disease (Fontanilla et al., 2011). CAPE also delays the death in a mouse model of amyotrophic lateral sclerosis (Fontanilla et al., 2012). Since CAPE hydrolyzes to caffeic acid, and is the principle CAPE metabolites, further research on caffeic acid would give similar results as CAPE. Therefore, it was decided to not invest in more studies with caffeic acid.

Rosmarinic acid and carnosic acid showed a significant protective effect from nitrosative stress. Moreover, rosmarinic acid and carnosic acid protected CGNs from excitotoxicity and caspase activation-induced apoptosis, respectively. Furthermore, both compounds showed an increased survival in different models of neurodegenerative diseases. Rosmarinic acid caused a noticeable decrease in motor neuron death in a mouse model of ALS. Also, carnosic acid extended the survival of an Alzheimer's disease mouse model. We believe that more investigation with other models would prove valuable to study. Since both compounds protected CGNs through different pathways, further

studies that look at the synergistic properties of the combined compounds may be a promising therapy for neurodegenerative disorders.

PQQ protected CGNs from oxidative stress induced by two insults, hydrogen peroxide and copper chloride, even with very low concentrations of PQQ. These data are promising *in vitro*, and encourage further study into the mechanisms by which PQQ protects CGNs from these two insults. The ability of PQQ to induce mitochondrial biogenesis and also induce the nerve growth factor (NGF) has been demonstrated by several studies. Our future studies will examine the involvement of mitochondria biogenesis on the protective effects of PQQ by inhibiting PGC-1 α , a key transcriptional regulator of mitochondria biogenesis. We also plan to investigate the protection mechanisms, and if they depend on PQQ activity for promoting NGF expression. PQQ showed a significant improvement in pre-clinical disease models of Parkinson diseases. Further pre-clinical studies with another model of neurodegenerative diseases, such as ALS mice, would be appropriate. Table 1 represents the summary for the data.

Insults and stressors	Chlorogenic acid	Caffeic acid	Ferulic acid	Quinic acid	Rosmarinic acid	Carnosic acid	PQQ
SNP	+	+	-	-	+	+	-
H ₂ O ₂	-	+	-	-			+
Glutamate/ Glycine	-	+	+	-	+	-	-
Brefeldin A	-	+	-	-			
PS-341	-	+	-	-			
LPS	-	+	-	-			
CuCl ₂							+
5K	-	+	-	-	-	+	-

Table 1

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APPENDIX A: ABBREVIATIONS

6-OHDA; 6-hydroxydopamine
AChE; acetylcholinesterase
AD; Alzheimer's disease
ALS; amyotrophic lateral sclerosis
Bcl-2; B cell lymphoma protein 2
AMPA; α – Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARE; Antioxidant Response Element
BChE; butyrylcholinesterase
Bcl-2; B-cell lymphoma 2
BME; Basal Modified Eagle's medium
CA; caffeic acid
Ca²⁺; calcium
CAPE; caffeic acid phenethyl ester
CAR; Carnosic acid
CGA; chlorogenic acid
CGN; cerebellar granule neuron
CNS; central nervous system
COX-2; cloxygenase-2
CREB; cyclic AMP response element binding protein
DMSO; dimethylsulfoxide
EAAT2; excitatory amino acid transporter 2
EGCG; epigallocatechin 3-gallate
ER; Endoplasmic reticulum
ERAI; ER stress-activated indicator
FA; ferulic acid
FDA; Food and Drug Administration
FIM; Foundation for Innovation in Medicine
Glu/Gly; glutamate/glycine
GluRdelta2; excitatory amino acid receptor delta2
GPx1; glutathione peroxide 1
GST; glutathione S-transferase
h; hours
H₂O₂; hydrogen peroxide
hiPSC; human induced pluripotent stem cells
HIV; human immunodeficiency virus
HO-1; heme oxygenase-1
hRSV; human respiratory syncytial virus
Hsp70; heat shock protein
IFN- γ ; interferon gamma
IL-1 β ; Interleukin 1 beta
IL-6; Interleukin 6
KCL; potassium chloride

LPS; Lipopolysaccharide
MPTP; methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NMDA; N-Methyl-D-aspartate
nNOS; neuronal NO synthase
NO; nitric oxide
NF- κ B; nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor
NQO1; NADH-quinone oxidoreductase 1
Nrf2; NF-E2-related factor-2
O₂⁻; superoxide
PBS; phosphate buffered saline
PC12; rat pheochromocytoma adrenal medulla cell line
PCD; programmed cell death
PD; Parkinson's disease
PGC-1 α ; peroxisome proliferator-activated receptor- γ coactivator-1 α
PI3K; phosphoinositide 3-kinase
PQQ; Pyrroloquinoline quinone
QA; quinic acid
RNS; reactive nitrogen species
ROS; reactive oxygen species
ROS; Rosmarinic acid
SNP; sodium nitroprusside
SOD1; super oxide dismutase 1
SOD2; super oxidative dismutase 2
TNF α ; Tumor necrosis factor
TRAIL; TNF-related apoptosis-inducing ligand
UPR; unfolded protein response

APPENDIX B: PREFACE

Chapter 2 entitled: “Neuroprotection comparison of chlorogenic acid and its metabolites against mechanistically distinct cell death-inducing agents in cultured cerebellar granule neurons and BV2 cell line” In press to Brain research.

Chapter 3 entitled: “The Neuroprotective Effects of Rosmarinic and Carnosic Acid on Primary Cerebellar Granule Neurons” In preparation for submission to Journal of Neurochemistry.