THE ROLE OF ASTROCYTE-DEPENDENT, NRF2-ARE INDUCTION IN FLAVONOID-MEDIATED PROTECTION FOR PRIMARY MOUSE CORTICAL CULTURES

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ABBREVIATIONS

$^1O_2$	Singlet oxygen
8-OHG	8-hydroxyguanine
AD	Alzheimer's disease
Ad-DN-Nrf2	Dominant-negative Nrf2 adenovirus
Ad-EGFP	Enhanced green fluorescent protein adenovirus
AhR	Arylhydrocarbon receptor
ALS	Amyotrophic lateral sclerosis
ARE	Antioxidant response element
ATP	Adenosine triphosphate
Aβ	Amyloid beta
bZIP	Basic region leucine zipper
CH2	Methylene
CNC	Cap n' collar
CNS	Central nervous system
CO_2	Carbon dioxide
CoQ_{10}	Coenzyme Q_{10}
Cu	Copper
CuZn-SOD	Copper-zinc superoxide dismutase (SOD1)
DCPIP	Dichlorophenolindophenol
DHAA	Dehydroascorbic acid
DIV	Day-in-vitro
DMEM/F12	Dulbecco's modified eagle's medium/F12
DMSO	Dimethylsulfoxide
EGCG	Epigallocatechin gallate
EGFP	Enhance green fluorescent protein
ER	Endoplasmic reticulum
ETC	Electron transport chain
FBS	Fetal bovine serum
Fe	Iron
GFAP	Glial fibrillary acidic protein
GLT1/EAAT2	Glutamate transporter 1/ excitatory amino acid transporter 2
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HEK293	Human embryonic kidney 293
HNE	4-hydroxynonenal
HO-1	Heme oxygenase 1
Keap1	Kelch-like erythroid CNC homologue [ECH]-associated protein 1
MAP2	Microtubule-associated protein 2
MDA	Malondialdehyde

Mn-SOD	Manganese superoxide dismutase (SOD 2)
MOI	Multiplicity of infection
MPP+	1-methyl 4-phenylpyridine
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
MTT	Thiazolyl blue tetrazolium bromide
nDNA	Nuclear DNA
NFT	Neurofibrillary tangles
$NO^{\cdot}$	Nitric oxide
NO_2	Nitrogen dioxide
NQO1	reduced-nicotinamide adenine dinucleotide phosphate [NAD(P)H] quinone oxidoreductase
Nrf2	Nuclear factor erythroid 2 [NF-E2]-related factor 2
$O_2^{\cdot-}$	Superoxide anion
OCl^-	Hypochlorite ion
OH^-	Hydroxyl ion
$OH^{\cdot}$	Hydroxyl radical
$OONO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-0.1% Tween20
PD	Parkinson's disease
PKB	Protein kinase B
PKC	Protein kinase C
PUFA	Poly-unsaturated fatty acids
Q3G	Quercetin-3-glucoside
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
tBHQ	tert-butylhydroquinone
TR	Thioredoxin reductase
UGT	UDP glucuronosyltransferase
ZnSOD	Zinc superoxide dismutase
γ-GCS	γ-glutamylcysteine synthetase
γ-GluCys	γ-glutamylcysteine

1.0 ABSTRACT

Oxidative stress is proposed to have an early role in the development and progression of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Activation of the nuclear factor E2-related factor 2/antioxidant response element (Nrf2/ARE) pathway leads to increased expression of many protective antioxidant and phase II enzymes, including reduced-nicotinamide adenine dinucleotide phosphate quinone oxidoreductase (NQO1). Numerous compounds, including some phenolic antioxidants, are ARE inducers that convey neuroprotection against oxidative stress. Here, we report that kaempferol, quercetin-3-glucoside (Q3G) and quercetin significantly increased NQO1-ARE gene transcription in an ARE promoter-directed luciferase assay. In addition, kaempferol and Q3G significantly increased NQO1 enzyme activity, at 5 μM and 10 μM respectively. Pretreatment with Q3G or kaempferol provided significant protection to primary cortical cultures against a toxic concentration of H_2O_2 (25 μM), at 7.5-30 μM and 20 μM respectively. Overexpression of a dominant-negative version of Nrf2 (Ad-DN-Nrf2) in C57Bl6 cortical cultures abolished kaempferol-mediated protection, while an enhanced green fluorescent protein adenovirus control (Ad-EGFP) did not. Adenovirus infection occurs only in astrocytes; thus the Nrf2/ARE pathway in astrocytes contributes to kaempferol-mediated protection against oxidative stress-induced cortical culture death. Q3G-mediated protection at 7.5-10 μM is abolished by preventing ARE gene expression with dominant negative Nrf2. However, a significant loss in Q3G-mediated protection after Ad-DN-Nrf2

infection is only observed at 20 μM. This suggests that the Nrf2/ARE pathway contributes to the antioxidant efficiency of some flavonoids and confers protection to primary cortical cell cultures against oxidative stress.

2.0 GENERAL INTRODUCTION

2.1 Oxidative stress and the aging human population

It is generally accepted that while oxidative stress is not the direct cause, it does have an important role in the early pathology of many neurodegenerative diseases [1-3] and contributes to cell death in later stages. The common thread linking these diseases is the increased risk of onset with age and the correlated increase of oxidative stress in the brain. Post-mortem studies reveal elevated amounts of oxidative damage in disease-specific areas of the brain in Alzheimer's disease (AD) [2, 4], Parkinson's disease (PD) [5, 6] and amyotrophic lateral sclerosis (ALS) [7, 8] patients, in comparison to age-matched controls. Almost 8% of Canadian's over the age of 65 will be affected by AD or other dementia [9], correlating to approximately 450,000 people in 2007 (extrapolated data) and an annual expense of $5.5 billion (in 2000) [10]. Parkinson's disease affects 1% of the Canadian population over 65 years of age [11] and 2-3 Canadians die each day from ALS [12]. Globally, the World Health Organization predicts the incidence of AD and PD to rise 36.6% in the next 25 years as the world population over 60 years of age doubles. Similarly, a 37.9% rise is predicted in AD- and PD-related disability adjusted life years, which is a measure of the amount of healthy life lost [13]. The risk of developing a neurodegenerative disease dramatically increases over the age of 70. With an aging population, Canada's health care system will be strained to find the necessary human and monetary resources required to properly care for neurodegenerative disease

patients, making the search for preventatives and therapeutics a necessity in the near future.

2.2 Oxidative stress, oxidative species and free radicals

Oxygen is essential for cell signaling and energy production, but does occasionally leak from these pathways and is utilized in destructive reduction/oxidation (redox) reactions. Oxidative stress is a cellular condition resulting from higher levels of pro-oxidants than antioxidants. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the hydroxyl ion (OH^-), singlet oxygen ($^1O_2$) and the hypochlorite ion (OCl^-), are pro-oxidants containing reactive oxygen atoms. ROS participate in reduction reactions to create free radicals and diffuse to more distant sites to act. Reactive nitrogen species (RNS) consist of nitric oxide ($NO^{\cdot}$) and its derivatives, peroxynitrite ($OONO^-$) and nitrogen dioxide (NO_2). Many ROS/RNS also constitute examples of free radicals, which are molecules with atoms containing an unpaired electron(s) in their outermost shell making them unstable and reactive. Free radicals such as the hydroxyl radical ($OH^{\cdot}$), the superoxide anion ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$) are particularly destructive because they initiate a chain of oxidation events when they remove a hydrogen and its electron from a nearby molecule in attempt to return to their stable ground state. This effectively creates another free radical which participates in a similar reaction until terminated by one of the body's antioxidant defense mechanisms [14, 15].

ROS formation and reduction occurs through a number of well-characterized reactions. The superoxide anion ($O_2^{\cdot-}$) is detoxified by copper-zinc superoxide dismutase (CuZn-SOD) in the cytosol or manganese superoxide dismutase (MnSOD) in the mitochondria, (Eq. 1). The resulting product of hydrogen peroxide (H_2O_2) is converted to water (H_2O) by the enzyme catalase (Eq. 2). Neutrophils catalyze H_2O_2 with myeloperoxidases to produce hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-) for antimicrobial duties (Eq. 3). Being highly reactive and effecting permanent damage on lipid, DNA and proteins makes the hydroxyl radical ($OH^{\cdot}$) possibly the most dangerous ROS. A significant amount of $OH^{\cdot}$ production in the brain occurs due to the relatively high concentration of metals, such as iron (Fe) and copper (Cu), used in ion-mediated ROS generation (Eq. 4-7) [14]. The Fenton Reaction, involving oxidation of iron by hydrogen peroxide, is a well known oxidizing reaction that produces $OH^{\cdot}$ [16].

(Eq. 1) $2O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2 + O_2$

(Eq. 2) $2H_2O_2 \rightarrow 2H_2O + O_2$

(Eq. 3) $H_2O_2 + Cl^- \rightarrow HOCl + OH^-$

$HOCl \rightarrow H^+ + OCl^-$

(Eq. 4) $HOCl + Fe^{2+} \rightarrow OH^{\cdot} + Cl^- + Fe^{3+}$

(Eq. 5) $HOCl + Cu^+ \rightarrow OH^{\cdot} + Cl^- + Cu^{2+}$

(Eq. 6) $H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$

(Eq. 7) $H_2O_2 + Cu^+ \rightarrow OH^{\cdot} + OH^- + Cu^{2+}$

Nitric oxide ($NO^{\cdot}$) has important roles as an intracellular messenger and, even at low concentrations, as a free radical scavenger; however, increased $NO^{\cdot}$ levels during aging contribute to the production of damaging RNS. $NO^{\cdot}$ reacts with superoxide ($O_2^{\cdot -}$) to produce peroxynitrite ($OONO^-$) (Eq. 8) when SOD levels are insufficient to reduce superoxide. Under acidic conditions produced by cellular CO_2 (carbon dioxide), peroxynitrite can undergo homolysis to produce the hydroxyl radical and nitrogen dioxide (NO_2) (Eq. 9). Nitrogen dioxide oxidizes lipids, contributing to tissue damage. In general, the half-lives of RNS are longer than ROS, allowing them greater time to effect damage to DNA, lipids and proteins [14].

(Eq. 8) $NO^{\cdot} + O_2^{\cdot -} \rightarrow OONO^-$

(Eq. 9) $OONO^- + H^+ \rightarrow OH^{\cdot} + NO_2$

2.3 RNS/ROS production

All RNS are ultimately derived from nitric oxide, which is synthesized by one of three isoforms of NO synthase: neuronal, inducible and endothelial [14]. ROS form as a result of exposure to exogenous ultraviolet radiation, chemicals or drug therapy, or endogenously as a byproduct of multiple cellular processes. Systems that produce free radicals include the immune system [17, 18], the cytochrome p450 system, oxidative enzymes [19], peroxisomes, eicosanoid metabolism and the mitochondrial electron transport chain [20].

Mitochondria produce 90% of cells' ROS through leakage of 0.2% of electrons from the electron transport chain (ETC) [21]. The flow of primarily single electrons through the ETC creates a proton gradient necessary to produce energy in the form of adenosine triphosphate (ATP). Flavoproteins and iron-sulfur (FeS) clusters within ETC complexes, and free ubisemiquinone, pass single electrons to oxygen to create ROS [21]. Complexes I, II and IV of the ETC release superoxide into the mitochondrial matrix, while Complex III releases superoxide into the matrix and the intermembrane space (Figure 1). Superoxide in the intermembrane space can then be transported to the cytoplasm via voltage-dependent anion channels [22]. Monoamine oxidase also produces hydrogen peroxide that can diffuse into the cytoplasm [23]. Nitric oxide freely diffuses into the mitochondria to inhibit the ETC at various points, increasing superoxide production [22]. Approximately 85% of the superoxide is reduced to hydrogen peroxide, while the remaining 15% reacts with nitric oxide to produce peroxynitrite [24]. In the brain, Complex I is responsible for most of the superoxide production at an FeS cluster site [25].

2.4 Essential ROS/RNS

A steady-state level of ROS/RNS generation is essential for the metabolic function of eukaryotic cells. $NO^{\cdot}$ has roles in adenylyl cyclase regulation [26], neurotransmitter release [27], hormone release, vasodilation [28, 29] and inhibition of infection or reactivation of various viruses including Herpes Simplex

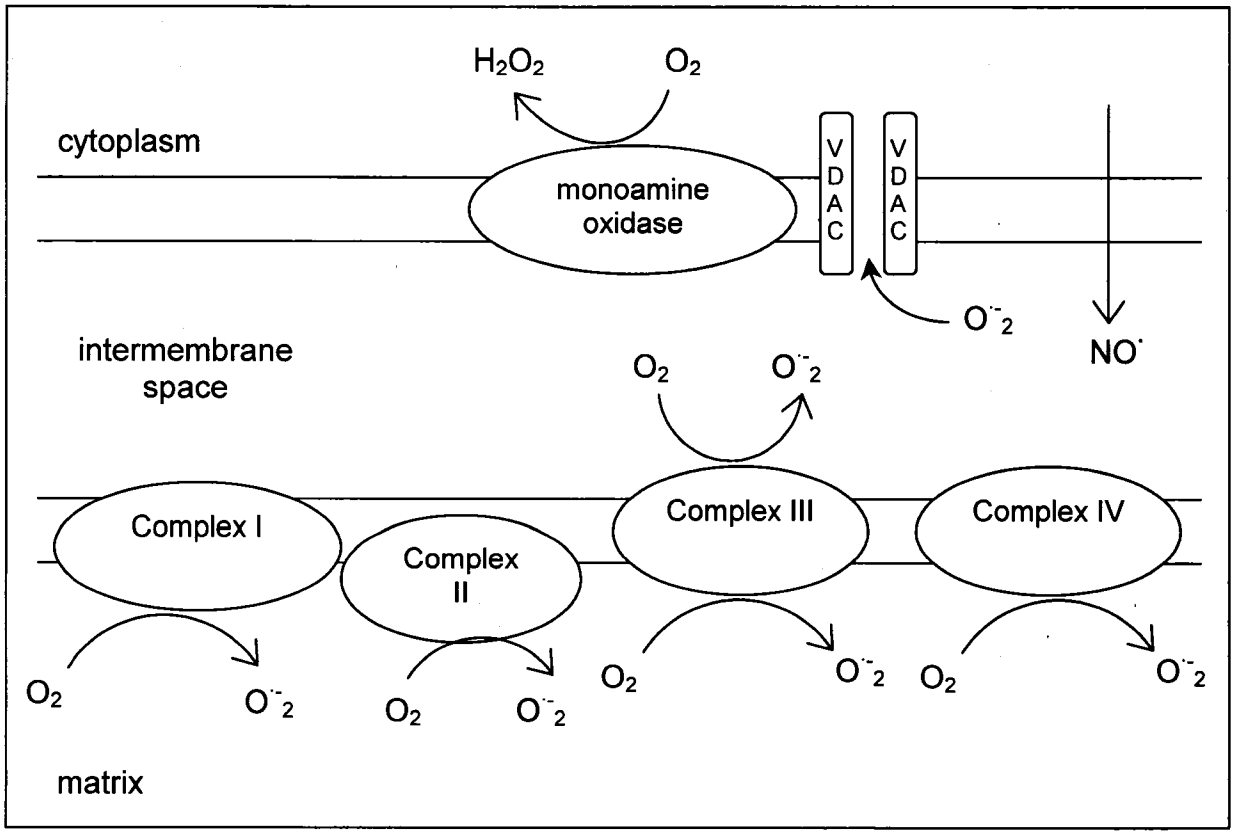

Figure 1. ROS production from the mitochondrial electron transport chain.
O_2 – oxygen, H_2O_2 – hydrogen peroxide, $O_2^{\cdot-}$ – superoxide anion, $NO^{\cdot}$ – nitric oxide, VDAC – voltage dependent anion channel (also known as mitochondrial porin) [23]

virus and Epstein Barr virus [30]. The essential mechanism for cellular energy, ATP production through the electron transport chain, is a tightly controlled series of redox reactions that depend upon ROS for electrons to move down the chain. The direct and indirect activation of the G-protein Ras by $NO^{\cdot}$ and H_2O_2, respectively, can lead to cell proliferation, differentiation, and apoptosis. Activation of numerous protein tyrosine kinases and receptor tyrosine kinases, including the insulin receptor, are stimulated by H_2O_2. H_2O_2 also inhibits protein tyrosine phosphatases via oxidation, leading to an overall increase in tyrosine phosphorylation. A number of signaling pathways, including epidermal growth factor and platelet-derived growth factor stimulation via receptor phosphorylation, involve H_2O_2. H_2O_2 activates mitogen-activated protein kinase and c-Jun N-terminal kinase, and activates transcription factors, as well as playing roles in lipid metabolism and calcium signaling. ROS act as second messengers in transforming growth factor β and nuclear factor kappa B pathways [31, 32].

2.5 Oxidative stress-induced cellular damage

DNA damage plays an important role in aging, with damage to post-mitotic neurons being particularly important; neurogenesis in the human brain is regionally limited and evidence of neuromigration in mammals is just emerging [33, 34]; thus post-mitotic neurons should be protected from ROS-mediated damage because a complete neuron replacement system does not exist. Reactive oxygen species, particularly the hydroxyl radical, preferentially react with electron-rich areas of DNA bases such as the methyl groups of thymine and

cytosine. A form of radical, called an adduct radical, is produced when ROS extract a hydrogen and add a bond to DNA bases [35]. These radicals have reducing or oxidizing properties and interact with other molecules to produce a variety of products such as 8-hydroxyguanine (8-OHG), commonly measured as 8-hydroxy-2'-deoxyguanosine in the laboratory as a marker for oxidative DNA damage [35]. Nucleotide radicals can be substituted to form improper base pairs (e.g. 8-OHG to adenine) and participate in electron-removal from the sugar rings of DNA, leading to DNA strand breaks. DNA repair mechanisms are not always 100% efficient and their efficiency degrades with age. This can also lead to ROS-induced damage in the form of covalent DNA-protein cross-links [35]. The effects of ROS on DNA, including single and double-strand breaks, adducts, cross-linking, mutations and deletions [36], if left unchecked, can lead to improper transcription, translation and dysfunctional proteins and/or apoptosis [37].

A marked decrease in expression of frontal cortex genes has been identified in aging humans, particularly genes important for mitochondrial function, calcium homeostasis and synaptic plasticity. Oxidative damage to the promoters of genes in the cortex is highly correlated with this decreased gene expression. In addition, an increase in stress response and DNA repair gene expression is a reaction to increased oxidative stress with age [38]. Mitochondrial DNA (mtDNA) suffers significantly more oxidative damage than nuclear DNA (nDNA) in the brains of numerous mammalian species. This has been attributed to mtDNA's proximity to ROS production from the ETC, high lipid density from the additional inner lipid

membrane and a less effective DNA repair mechanism [39]. This increased mtDNA oxidation has been correlated with a decreased maximum life span of eight mammalian species [40]. Increased susceptibility of mtDNA to point mutations and deletions with age [41], leads to premature aging in a mouse model [42]. Repair of oxidative damage to nuclear DNA declines with age and has been linked to mutations in DNA repair mechanisms. Damage to cytoplasmic mRNA by ROS can lead to improper translation and protein aggregation, a hallmark of multiple neurodegenerative diseases [43]. It is becoming clearer that oxidative damage is not only a byproduct of aging, but also an early event that contributes to the aging process in a significant way.

Unsaturated fatty acids are the most sensitive macromolecule to ROS damage [44]. Polyunsaturated fatty acids (PUFA) containing the chemical structure =CH-CH2-CH= are susceptible to peroxidation at the electron-rich methylene position (-CH2-). A free radical extracts a hydrogen atom, leaving a carbon-centered radical to react with oxygen and produce a reactive peroxyl radical to continue ROS production along the PUFA chain [14]. An elevated number of double bonds in PUFA is correlated to susceptibility to lipid peroxidation [44, 45] and a decreased life span in mammals [45]. Lipid peroxidation produces secondary radicals and byproducts including malondialdehyde (MDA), which form DNA adducts and protein adducts, and 4-hydroxynonenal (HNE). HNE is slightly more stable than MDA and diffuses further, and so potentially may cause more tissue damage. HNE produces covalent adducts on proteins and DNA, resulting in

inhibition of cell proliferation, alteration in ATP production, reduced efficiency of glutathione, and deactivation of glutathione peroxidase (GPx) which detoxifies HNE [14]. Alteration of calcium (Ca^{2+}) fluxes and glucose transport are also caused by HNE [14]. As with DNA oxidation, mitochondria are more susceptible to lipid peroxidation than the nucleus. [44].

Attacks on protein backbones by ROS removes electrons, creating carbon-centered free radicals and potentially causing protein cross-linking or peptide-bond cleavage [46]. ROS interaction with protein side-chains produces many by-products ultimately leading to alterations in protein structure and function [46]. Carbonyl groups added to proteins by ROS, HNE or MDA are useful indicators of protein oxidation [14]. Although ROS-induced protein malfunction may only be noticed if the conformation or active site is affected, the overall result of free radical attack on proteins ranges from altered membrane fluidity in cells, changes in ion balance and calcium flow and alterations in enzyme activity [14].

The effect of oxidative stress on DNA, proteins and lipids also increases the permeability of the blood brain barrier [15], another pathological feature of AD and PD [47].

2.6 Neurodegenerative diseases and oxidative stress

The *free radical theory of aging*, first proposed by Harman in 1956 [48], suggested that damage caused by oxidative stress products was the basis for

the aging process. This theory has evolved to its current form, the *mitochondrial theory of aging* [49, 50], which proposes that oxidative damage to mitochondria, particularly mtDNA, plays a significant role in aging. The brain is particularly susceptible to oxidative stress, owing to its high metabolic rate [50], a high concentration of PUFA and metals, lower concentrations of antioxidant enzymes [51] and its use of nitric oxide as a signaling molecule [50]. Oxidative stress in post-mitotic neurons has serious implications, because extensive damage or cell death critically affects the function of the central nervous system (CNS). It has been established that neuronal loss occurs with aging [50], that aging is the primary risk factor for development of neurodegenerative diseases and that oxidative stress is an early event in multiple neurodegenerative diseases [2, 3, 52-55]. There is a direct relationship between oxidative stress and neurodegenerative disorders [51], including AD [56], PD [57, 58], and ALS [59]. The brains of AD [2, 4], PD [5, 6], and ALS [7, 8] patients possess increased levels of lipid peroxidation, compared to age-matched controls. Furthermore, this link is specific to targeted brain areas including the substantia nigra of PD, the spinal fluid of ALS and the cortex and hippocampus of AD patients [60]. There is also evidence of elevated nucleic acid oxidation in PD [58, 61], AD [62-64], and ALS [65] patients and elevated nitrosylated protein levels in the cortex and hippocampus in AD, in motor neurons of ALS and in the Lewy Bodies of PD patients [60].

Depleted levels of the antioxidant glutathione in the substantia nigra of PD patients may explain the increased levels of ROS and MDA in that brain region, [54, 55] and associated increased levels of lipid, DNA and protein oxidation [66]. Increased iron concentrations in brains of PD patients [67] contribute to the observed elevated levels of oxidative stress and disease pathology [67, 68]. Dopaminergic neurons, which are selectively lost in PD, are particularly susceptible to oxidative stress due to dopamine's ability to undergo auto oxidation [68]. Basal GPx levels in the substantia nigra are lower than other brain regions, but are elevated in glial cells as neurodegeneration proceeds, perhaps as a compensatory mechanism [69] for the increased levels of oxidative stress. α-synuclein protofibrils, the proposed toxic form of the protein aggregate in Lewy bodies, are promoted and stabilized by free radicals and oxidized dopamine. The formation of complete fibrils is inhibited in the presence of oxidizers and after adduct formation with α-synuclein, resulting in protofibril accumulation [70], providing another link between PD and oxidative stress. The accidental discovery of MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) in the mid-1970s led to its use as a Parkinsonism model based on the ability of its metabolite, 1-methyl 4-phenylpyridine (MPP+), to inhibit mitochondrial complex I. This inhibition mimics PD pathology and causes increased free radical production, peroxidation and subsequent dopaminergic neuron death [71].

AD patients possess decreased antioxidant plasma levels [72-74], and increased peroxidation in early stages of the disease [74]. In the later stages of AD the activity of the powerful antioxidant enzymes SOD, catalase [75], and GPx [76]

are significantly reduced, contributing to the hypothesis that oxidative stress is an early event in AD and continues throughout the disease. There is evidence to suggest that protein carbonyls, a product of protein oxidation that are elevated in AD patients [77, 78], contribute to the formation of hyperphosphorylated Tau and neurofibrillary tangles (NFT), a hallmark of AD pathophysiology [79]. Tau is a microtubule-associated-protein, primarily found in neurons, which stabilizes and promotes microtubule assembly [80]. Tau is regulated by phosphorylation; however, a 4-8 fold increase in phosphorylated tau in the brains of AD patients precedes the formation of hyperphosphorylated tau filaments and tangles inside neurons [81]. After reacting with HNE, a lipid oxidation product, phosphorylated tau is able to form polymers which comprise the toxic neurofibrillary tangles of AD [82]. Oxidized mRNA levels are significantly increased by 30-70% in the frontal cortices of AD patients [83]. This oxidation is specific and affects a number of proteins implicated in AD pathology, including the antioxidant enzyme CuZn-SOD, also known as SOD1 [84]. Interestingly, it has also been shown, through AD transgenic and manganese superoxide dismutase (MnSOD) crossed knockout mice, that oxidative stress increases levels of amyloid beta (Aβ) and Aβ plaque formation [85]. Not only does oxidative stress appear to be a contributing factor to the disease development, but hallmarks of AD, such as hyperphosphorylated Tau and Aβ, also produce significant amounts of ROS [86, 87]. For a comprehensive review of the complex relationship between AD and oxidative stress, see Chauhan and Chauhan [80].

Perhaps the most evident link between a neurodegenerative disease and oxidative stress is the mutated SOD1 found in approximately 20% of familial ALS patients [88, 89]. Although the mechanistic effect of the mutation in SOD1 is not fully understood, [65], it is well established that mutant SOD1 contribute to increased peroxynitrite in the cerebrospinal fluid [90] and motor neurons [91] of ALS patients. Some mutant SOD1 variants act as a peroxidase catalyzing the regular dismutase reaction in the opposite direction, using hydrogen peroxide to produce superoxide [50, 65]. The oxidative stress created by these mutant enzymes would use valuable antioxidant resources, making the motor neurons more susceptible to oxidation and excitotoxicity. A link between oxidative stress and CNS motor neuron death was established when mutant SODs in familial ALS were found to oxidize and inactivate a glial glutamate transporter, GLT1/EAAT2 (glutamate transporter 1/ excitatory amino acid transporter 2), leading to glutamate-induced excitotoxicity in *Xenopus* oocytes [59]. In addition, patients with sporadic ALS exhibit oxidatively-modified GLT1/EAAT2 [92].

2.7 Endogenous antioxidants and antioxidant enzymes

The brain contains a number of antioxidants and antioxidant enzymes to neutralize free radicals, ROS and RNS, although these defenses are considered insufficient to deal with the increased oxidative stress observed in neurodegenerative disorders. A more detailed description of antioxidants and antioxidant enzymes found in the brain follows.

Glutathione is an extremely important brain antioxidant and detoxifier of xenobiotics. It is able to directly reduce free radicals, donate electrons through the GPx-mediated pathway and function as a conjugate to eliminate harmful metabolites [93].

Vitamin E refers to the various tocopherols and tocotrienols that act as endogenous antioxidants [94]. Alpha-tocopherol is the only form of vitamin E found in the brain and is particularly effective in preventing lipid peroxidation as it is the only membrane-bound, lipid soluble antioxidant [94].

Ascorbate is the reduced physiological form of vitamin C (ascorbic acid) and is an effective antioxidant. Due to its low redox potential, it can donate electrons to free radicals, and reduce vitamin E and glutathione [95, 96]. The oxidized form of ascorbate, dehydroascorbic acid (DHAA) is highly toxic to cells. Astrocytes play a vital role in antioxidant defense by reducing DHAA and releasing ascorbate through a glutamate-ascorbate transporter [96]. Ascorbate is one of the first mitochondrial antioxidants to be consumed during a free radical attack, possibly due to its effectiveness as an antioxidant, suggesting that it plays a key role in brain antioxidant defenses [95].

Coenzyme Q_{10}'s (CoQ_{10}) primary role is electron transfer in the mitochondrial respiratory chain. CoQ_{10} also acts as an antioxidant by donating electrons to free radicals, reducing vitamins E and C to their active forms [97], and protecting against lipid peroxidation [98]. Animal studies have shown that supplementation

with CoQ_{10} significantly increases life span, implying that it has a beneficial role in fighting the aging process [97].

Deactivation of ROS scavengers by antioxidant enzymes follows a sequential series of reduction reactions. Superoxide formed from oxygen (O_2) is converted to hydrogen peroxide (H_2O_2) by the metalloproteins, superoxide dismutases (Figure 2). H_2O_2 is degraded to water (H_2O) and oxygen (O_2) by catalase or glutathione peroxidase (GPx). The removal of H_2O_2 by GPx requires the oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG). Another enzyme, glutathione reductase (GR), reduces GSSG to GSH, restoring GSH to its antioxidant form. GPx appears to play a bigger role than catalase in the reduction of peroxides in the brain [93]. Glutathione is an antioxidant that is clearly associated with aging and neurodegenerative diseases. A decline in glutathione levels is a characteristic of human aging [99] and has been shown to lead to neurodegeneration in rodent models [100, 101]. Furthermore, dysfunctioning GSH pathways have been identified in PD and AD [102]. Decreased GSH levels have also been correlated with the degree of PD severity [99].

Astrocytes play a key role in glutathione synthesis by providing neurons with all three glutathione precursors, glycine, glutamine and the rate-limiting cysteine [103]. Astrocytes have the highest amount of antioxidant defenses in the brain [95, 104, 105] and protect against neuronal death by neutralizing ROS and RNS [106].

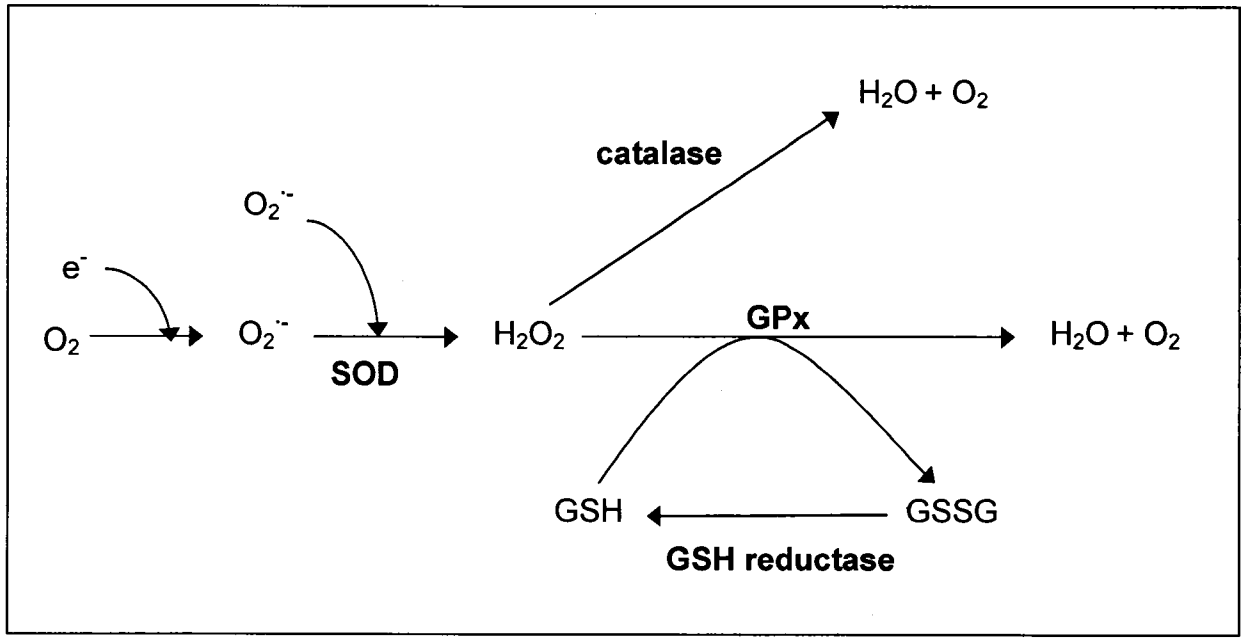

Figure 2. ROS detoxification by antioxidant enzymes.
O_2 – oxygen, H_2O_2 – hydrogen peroxide, $O_2^{\cdot-}$ – superoxide anion, H_2O – water, e^- – electron, GSH – reduced glutathione, GSSG – oxidized glutathione, SOD – superoxide dismutase, GPx – glutathione peroxidase

2.8 Antioxidant therapy for neurodegenerative diseases

A clinical trial with the antioxidant CoQ_{10}, which is significantly lower in PD patients than age-matched controls [97], has been effective in slowing PD progression in early stages (1200 mg/day) [107], relieving PD symptoms (360 mg/day) [108]and a high-dose trial (3000 mg/day) did not reveal any side-effects [50, 109]. In PD models, the substantia nigra of vitamin E-deficient animals shows increased susceptibility to lipid peroxidation [69], and vitamin E supplementation provides protection to dopaminergic neurons against MPP+ toxicity [51]. Although there is no significant difference in vitamin E concentrations between PD patients and age-matched controls, it has been demonstrated that vitamin E deficiency in rats and in patients with abetalipoproteinemia contributes to loss of nigrostriatal nerve terminals [69]. There is no conclusive epidemiological evidence that vitamin E supplementation affects the incidence of PD, however, it has been shown that a Vitamin E-rich diet reduces the risk of development, suggesting that other nutritionally-derived constituents contribute to this reduced risk [51]. Vitamin E is derived solely from plants and their fruit, so perhaps other plant compounds such as flavonoids are responsible for the reduced-risk effect.

AD patients have low plasma levels of vitamins C and E, compared to age-matched controls [51], which are restored with vitamin E (400 IU/day) and C (1000mg/day) supplementation, consequently reducing lipoprotein oxidation in plasma and cerebrospinal fluid [110]. Although research demonstrates the ability of vitamin E to reduce oxidative stress, Aβ, and plaque formation in young mice

of an AD model [51, 111], there is much disagreement regarding its therapeutic uses for AD. The results of large epidemiological studies indicate that vitamin E (360 IU/day) [112, 113] intake from food reduces the incidence of AD. Furthermore, treatment with vitamin E (2000 IU/day) slowed the progression of AD in a clinical trial with 341 patients [114]. However, later data analysis revealed that the levels of Vitamin E used in these trials increases levels of mortality due to non-AD causes [115]. A conflicting study with 50 patients has shown that vitamin E and/or C supplementation after the age of 65 has no effect on AD or dementia development [116]. This study did not classify vitamin intake in mg/day, instead classifying dosage as low (multi-vitamin supplement) or high (vitamin E or C alone).

Although treatment with vitamin E in a clinical trial did not impact the incidence of ALS, it did reduce the severity of the disease. Other trials indicate vitamin E has a slightly beneficial impact in treating ALS, with no significant side-effects [65]. After a small-scale phase I trial showed promise for CoQ_{10} treatment for ALS [117], a subsequent clinical trial of high-dose CoQ_{10} treatment revealed no significant side effects [109]. A further phase II clinical trial has been designed [118], but has not yet been undertaken.

Although many of the clinical trials involving antioxidants use vitamin E, it is important to note that vitamin E does not easily pass through the blood-brain barrier, and high concentrations are required *in vitro* to ameliorate oxidative stress [65]. These high concentrations have been shown to increase the mortality

rate in patients suffering from non-neurodegenerative diseases such as heart disease. A small clinical trial has recently shown that induction of SOD and catalase in the blood, by once-daily dietary supplementation with 675 mg/day Protandim eliminated an age-dependent increase in lipid peroxidation. Protandim is comprised of plant extracts, including milk thistle, green tea and turmeric, suggesting that this may be alternative treatment to vitamin antioxidant supplementation [119]. Thus, there is evidence supporting antioxidant therapy being protective against neurodegenerative disease development and progression. In addition to dietary or vitamin supplementation of antioxidants, other antioxidant pathways are being explored for their potential role in protecting against neurodegenerative diseases.

2.9 Antioxidant response element

The antioxidant response element (ARE) is a *cis*-acting enhancer, with a conserved nucleotide sequence (5'-TGA(C)nnnGC-3') [120], and is located upstream of numerous protective antioxidant enzyme and phase II enzyme genes [121]. Activation of the ARE is dependent upon a cascade of events.

Kelch-like erythroid cap n' collar homologue [ECH]-associated protein 1 (Keap1) is a zinc metalloprotein [122], bound to the cytoplasmic actin skeleton [123] and as a binding partner for nuclear factor erythroid 2 [NF-E2]-related factor 2 (Nrf2). Nrf2 is a basic region leucine zipper domain (bZIP) transcription factor; the basic region is responsible for DNA binding, while the leucine zipper mediates heterodimerization. The basic domain is highly conserved and responsible for

ARE binding. Nrf2 is often referred to as a cap n' collar-like (CNC) transcription factor due to its close homology to the *Drosophila* CNC transcription factor [124].

It is generally agreed that Keap1 plays a role in repressing Nrf2 accumulation in the nucleus where it binds the ARE; however, the mechanism is still debated. As a substrate adaptor for the Cul3-RbX1 E3 ubiquitin ligase complex, Keap1 also binds Nrf2 at the C-terminal kelch domain [125], bringing Nrf2 in proximity to the ubiquitin ligase, enabling the ligase to conjugate ubiquitin to Nrf2 and target it for destruction by the 26 S proteosome (Figure 3A) [125]. Recent research supports the hypothesis that after this ubiquitination cascade is blocked, due to changes in the oxidation state of Keap1 cysteine residues, Nrf2 is synthesized *de novo*, able to bypass the Keap1-degradation system, and translocate to the nucleus (Figure 3B) [126, 127]. The oxidative changes in the Keap1 cysteine residues are not a factor in Nrf2 dissociation from Keap1 [127], supporting *de novo* synthesized Nrf2 as the primary candidate for ARE binding. Quinone-induced oxidative stress increases the rate of Keap1 ubiquitination, while concurrently decreasing the rate of Nrf2 ubiquitination, suggesting that a switch in normal ubiquitination activity contributes to Nrf2 accumulation in the nucleus [128]. Electrophile adduction of Keap1 in the central linker domain appears to be essential in elevating and reducing ubiquitination of Keap1 and Nrf2, respectively, allowing Nrf2 nuclear translocation [126].

Direct ubiquitination of Nrf2 without complexing with Keap1 also occurs, explaining why Nrf2 degradation is still observed under oxidative stress

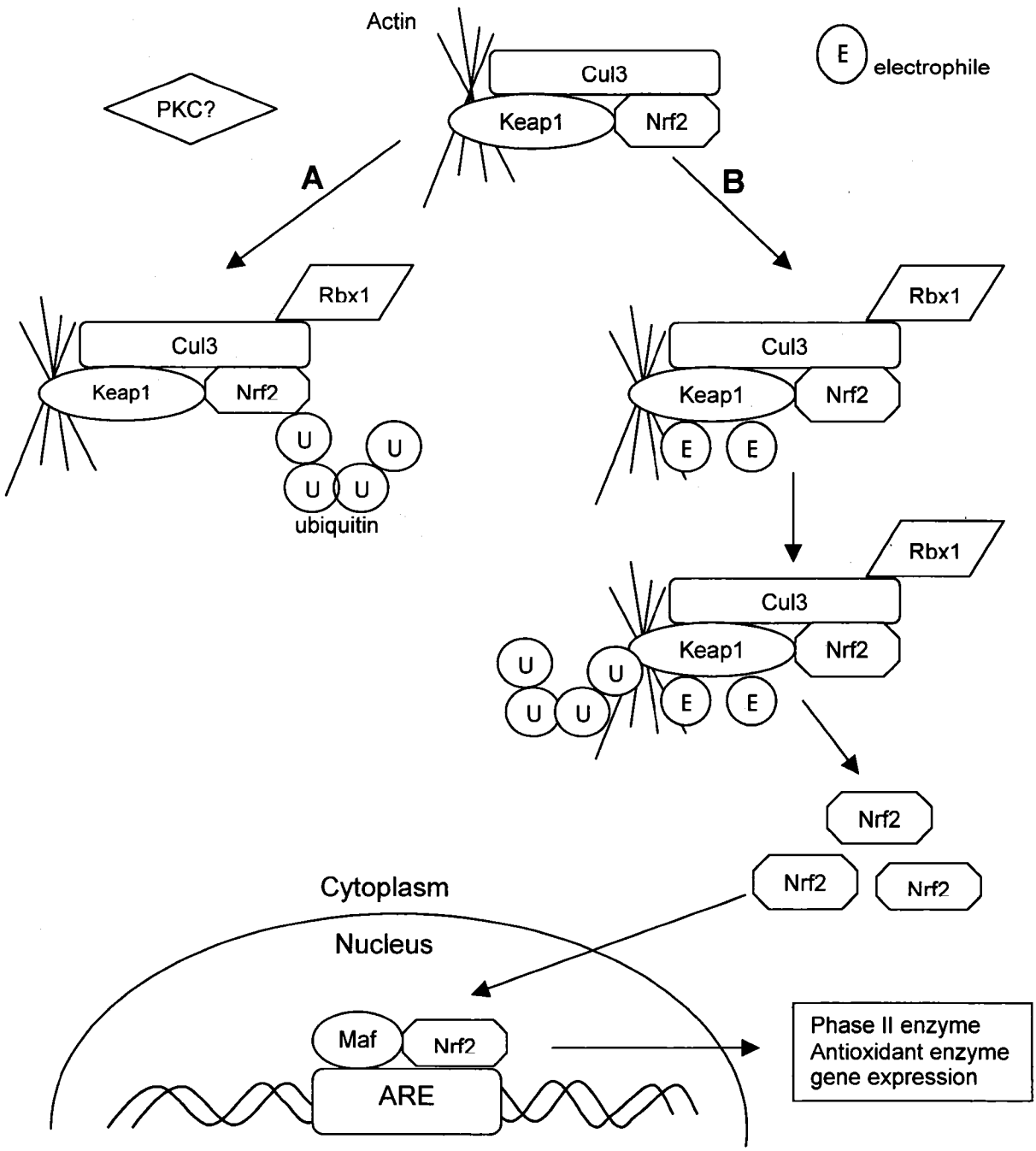

Figure 3. Nrf2/ARE pathway.
A Keap1 binds Nrf2 in the cytoplasm, bringing it in close proximity to Cul3-Rbx1 ubiquitin ligase, enabling ubiquitination of Nrf2. This prevents Nrf2 accumulation. **B** Electrophiles target redox-sensitive residues on Keap1, resulting in Keap1 ubiquitination and a decrease in Nrf2 ubiquitination. *de novo* synthesis of Nrf2 leads to an accumulation in the cytoplasm. Nrf2 translocates to the nucleus where it heterodimerizes with a small Maf protein, then binds to, and activates the ARE, resulting in transcription of Phase II and antioxidant enzymes. ARE-antioxidant response element, Cul3 – cullin 3, Keap1- Kelch-like erythroid CNC homologue [ECH]-associated protein 1, Nrf2 - Nuclear factor erythroid 2 [NF-E2]-related factor 2, E - electrophile, Rbx1 – ring-box 1, U – ubiquitin, PKC – protein kinase C. [126]

conditions where Keap1 is down regulated [129]. Degradation of Nrf2 is maintained in Keap1 null mice and in Nrf2 lacking the Keap1 binding domain, effecting an increase in half-life to 30 minutes [130], mirroring the same half-life extension seen during electrophile exposure [131, 132].

Nrf2 contains both a nuclear localization signal and a nuclear export signal, which play a role in Nrf2 engaging the ARE in the nucleus, and Nrf2 degradation in the cytoplasm [133]. Ngyuen *et al.* contend that Nrf2 is primarily a nuclear protein, constitutively effecting transcription of protective genes. They have shown *tert*-butylhydroquinone (tBHQ)-independent nuclear translocation of Keap1 and subsequent Nrf2 ubiquitination [134]. tBHQ, an aromatic phenol, is an antioxidant and known ARE-inducer [135]. Keap1 contains a nuclear export signal that when inactivated, possibly by oxidative stress, allows both Keap1 and Nrf2 nuclear translocation [136].

Protein kinase C (PKC) phosphorylation has a role in Nrf2 nuclear localization, as PKC inhibitors block translocation [137] and direct PKC phosphorylation of Nrf2 disrupts its binding with Keap1 and increases nuclear accumulation [137, 138]. In other proteins, phosphorylation near or within the degrons, the amino acid sequence that directs ubiquitin to the start site of degradation, decreases the rate of ubiquitination [139], therefore phosphorylation of Nrf2 degrons could reduce its rate of ubiquitination. As methodology and antibodies are improved, a

consensus may be reached on the upstream pathways leading to Nrf2 nuclear localization.

Once in the nucleus Nrf2 must heterodimerize before it is able to bind the ARE. Small Maf proteins (MafF, MafG, MafK), in addition to C-Jun and ATF-4, have been identified as Nrf2 binding partners [124, 140]. Maf homodimers/heterodimers and Bach1-Maf heterodimers bind the ARE to act as negative regulators of transcription [141].

In addition to Nrf2, there are also Nrf1 and Nrf3 CNC transcription factors. Nrf1 is a membrane-bound cytoplasmic protein, localized primarily in the endoplasmic reticulum (ER), and unassociated with Keap1 [142]. Nrf1 has significantly less affinity for the ARE than Nrf2 [124] and may have a primary role in ER stress response [142]. Nrf3 binds with small Maf proteins to competitively inhibit Nrf2 binding to ARE [143].

Nrf2-heterodimer binding initiates the ARE to interact with a promoter, initiating transcription of genes encoding phase II and antioxidant enzymes involved in antioxidant and xenobiotic stress defenses. Phase II enzymes detoxify radicals and reactive metabolites, enabling elimination [144], while antioxidant enzymes reduce radicals to a less reactive state. These genes include glutathione-S-transferases (GST), heme oxygenase 1 (HO-1), γ-GCS (γ-glutamylcysteine synthetase) and NAD(P)H-quinone oxidoreductase (NQO1).

2.10 Phase II enzymes and antioxidant enzymes regulated by Nrf2/ARE

There are a number of antioxidant enzymes and phase II enzymes that require activation of an ARE for transcription to occur. Detailed below are some of the enzymes that increase transcriptionally following Nrf2 overexpression [145].

Inducible mRNA and protein levels of SOD1 and catalase are decreased in Nrf2-null mice [146, 147]. Although it is apparent that Nrf2 affects SOD1 and catalase expression, the exact role is unclear because neither enzyme has an identified ARE to date.

Peroxiredoxin 1, a member of the recently discovered peroxiredoxin family of antioxidant enzymes, is induced through the Nrf2-pathway as evidenced by decreased transcription in the astrocytes of Nrf2-null mice [147]. Peroxiredoxin is ubiquitous, inducible by oxidative stress and has roles in detoxifying peroxides and preventing DNA oxidation [148].

Heme oxygenase 1 (HO-1) has an ARE upstream of its gene [147, 149, 150],and catalyzes the rate limiting step of the pro-oxidant, heme, into biliverdin. Biliverdin is subsequently reduced to bilirubin, an effective neutralizer of peroxyl radicals. Not surprisingly, HO-1 null mice are more susceptible to oxidative stress due to their reduced antioxidant capacity. [151].

NADPH quinone oxidoreductase (NQO1), previously known as DT-diaphorase, is one of the most studied genes containing an ARE [147, 152]. NQO1's primary function is catalyzing a 2-electron reduction of quinones, bypassing the reactive semiquinone state produced by a 1-electron reduction of quinones. NQO1 produces less reactive, more hydrophilic hydroquinones, which are conjugated by glucuronide or sulfate and eliminated. However, less commonly, hydroquinones undergo auto-oxidation or alkylation to produce reactive species [153]. Semiquinones form adducts with DNA or undergo redox cycling to produce ROS that participate in reactions to oxidize lipids, proteins and DNA [154]. Another harmful side-effect of semiquinones is the depletion of cellular antioxidants used in the detoxifying process [155]. NQO1 appears to function as both a phase II enzyme and an antioxidant enzyme: NQO1 has the ability to reduce oxidized vitamin E (alpha-tocopherolquinone) to vitamin E hydroquinone (alpha-tocopherolhydroquinone) [156], an antioxidant protein effective in preventing lipid peroxidation and maintaining other antioxidants in a reduced state [157]. NQO1 also maintains another very important antioxidant, CoQ_{10}, in its reduced form of ubiquinol [158]. NQO1 is localized primarily in astrocytes [159], supporting the hypothesis that astrocytes are the primary line of antioxidant defense for neurons in the brain.

NQO1 protein levels in AD are significantly increased in NFT, the cytoplasm of hippocampal neurons [160, 161], and in astrocytes and neurites surrounding plaques of the frontal cortex [162]. Increased NQO1 activity significantly protects

a neuronal cell line against glutamate-induced oxidative stress and cell death [163]. Taken together, this implies that increased NQO1 expression is part of the body's defense response to elevated oxidative stress in neurodegenerative diseases and enhancing this response is beneficial to neuronal survival.

NQO2 is also regulated by an ARE [164], but has a lesser role in antioxidant defense than NQO1. Unlike NQO1, NQO2 contains a metal-binding site and may have a key role in xenobiotic defense because NQO2 null animals have increased sensitivity to carcinogens [165].

Ferritin is a holoenzyme capable of storing up to 4500 iron atoms [166] and preventing intracellular iron from participating in Fenton reactions to produce scavenging free radicals. Nrf2/ARE control of ferritin expression has been well established [147, 152, 166].

UDP glucuronosyltransferase (UGT) has an important role in the glucuronic acid-conjugation of harmful metabolites and xenobiotics to facilitate their elimination [167]. Although UGT has multiple enhancer and receptor motifs, it is clear through its downregulation in Nrf2-null mice that the Nrf2/ARE pathway plays a role in its expression [168].

Metallothionein-1 production is regulated by an ARE and is upregulated after ischemia-reperfusion injury [169] and is protective in animal models [151].

Metallothioneins are cysteine-rich proteins, localized primarily in astrocytes in the brain, that bind metals and regulate zinc and copper levels [170]. Their quenching abilities prevent metals from participating in deleterious reactions with ROS/RNS, thereby providing protection to the metal-enriched brain.

Thioredoxin reductase (TR) maintains thioredoxin, a potent cellular antioxidant, in its active reducing form. This enables thioredoxin to donate two electrons to scavenging free radicals [151]. Both thioredoxin and TR are regulated through induction of an ARE [151, 171, 172].

Glutathione is the most abundant thiol antioxidant in mammals, directly reducing free radicals or as a substrate for a number of antioxidant enzymes. Astrocytes play an essential role in glutathione synthesis by providing neurons with precursors for GSH synthesis [173]. Astrocytes also harbour a larger concentration of GSH than neurons [145], providing protection against stresses primarily through peroxide stabilization [93, 105]. Increases in astrocytic GSH levels were observed after upregulation of the Nrf2-ARE pathway by the inducer tBHQ [174].

GSH synthesis consists of 2 steps: γ-glutamylcysteine synthetase (γ-GCS), also known as γ-glutamylcysteine ligase forms γ-glutamylcysteine (γ-GluCys) from glutamate and cysteine in the rate limiting step [93], followed by glutathione synthetase (GS)-catalyzed addition of glycine to form the tripeptide GSH [93].

Feedback inhibition of GSH on γ-GCS mediates this cycle [93]. An ARE is found upstream of genes for both the γ-GCS catalytic and modulatory subunit [175]. A 50% reduction in both γ-GCS and Nrf2 levels is responsible for depleted glutathione levels in the liver of aged rats, and GSH levels are significantly improved by Nrf2 induction [176]. Gene expression of both GR and GPx, essential enzymes for GSH-dependent peroxidation inhibition, is dependent upon ARE induction [151, 152].

Glutathione is also conjugated onto toxic hydrophobic electrophiles and ROS by GSTs, facilitating elimination [174]. The gamma and alpha subunits (Gsta1, Gsta2, Gstm1, Gstm2, Gstm3, Gstm4) are particularly induced through Nrf2 activity; a downregulation in Nrf2 null mice results in increased susceptibility to xenobiotics and oxidative stress [177].

With the current knowledge that astrocytes have a large role in protecting neurons against oxidative stress [145], it is not surprising that ARE activation occurs preferentially in astrocytes to confer neuroprotection against oxidative stress [178]. For example, tBHQ, a known ARE inducer, stimulates upregulation of TR protein expression and activity and glutathione protein levels in cortical astrocytes, but not neurons [179].

2.11 ARE induction and neuroprotection against oxidative stress

It has been well documented that *in vitro* activation of the Nrf2/ARE pathway provides neuroprotection against oxidative stress. tBHQ induction of ARE genes, including NQO1 and the modulatory subunit of γ-GCS, protects neuroblastoma and human neural stem cells against H_2O_2-mediated necrotic cell death [180]. NO-induced upregulation of ARE genes, including NQO1, HO-1, TR, γ-GCS, and GST subunits, defends against stress-induced apoptosis in neuroblastoma cells [181]. An increase of glutathione and NQO1 through ARE induction in neuroblastoma cells confers protection against oxidative stress-induced cell death [182]. Finally, tBHQ-mediated ARE activation limits oxidative glutamate toxicity in primary neuronal cultures [145].

The Nrf2/ARE pathway's neuroprotective ability is further evidenced *in vivo* by Nrf2 null mice and their increased susceptibility to oxidative stress [144]. In addition, oxidative stress and resultant brain lesions induced by mitochondrial complex II inhibition are attenuated by overexpression of Nrf2 and its inducible genes [183, 184]. Activation of Nrf2 by tBHQ has also been shown to reduce cortical damage and sensorimotor deficit in rats after ischemic-reperfusion injury [135].

The significance of the Nrf2 pathway has also been studied in animal models of neurodegenerative diseases. SOD1 ALS mutants have downregulated Nrf2 and ARE induction [185]. Induction of Nrf2, and subsequent ARE gene expression, in astrocytes of another ALS mouse model provided significant protection against

motor neuron death [186]. Neurodegeneration in a *Drosophila* PD model was attenuated by overexpression of GSTA1 [187], a gene controlled by the Nrf2-ARE pathway [177, 188].

ARE inducers are generally electrophiles with the ability to undergo redox reactions or alkylation with sulfhydryl group [144]. Included in this group are tBHQ, sulphoraphane and phorbol esters [121]. Other inducers include hydrogen peroxide, oxidized low-density lipoprotein (LDL), phenolic antioxidants, aromatic hydrocarbons, reactive quinones and peroxynitrite [151].

2.12 Flavonoids as ARE inducers

Flavonoids are plant-derived phenolic compounds. Foods high in flavonoids have been used for centuries in China as herbal remedies, with the most well known probably being the components of green tea [189]. North American interest in flavonoids has been piqued in the last decade as the potential for bioactive compounds as therapeutics and preventatives is explored.

The basic structure of a flavonoid consists of two benzene rings joined by a three-carbon bridge (Figure 4A). Hydroxyl groups (-OH) attached to the phenolic rings can donate electrons to reduce scavenging free radicals. Flavonoids are classed into groups based upon their chemical structure: flavonols, flavones, anthocyanidins, flavanones, catechins and proanthocyanidins. Kaempferol, quercetin and quercetin-3-glucoside (Figure 4B, 4C, 4D) are examples of flavonols, which are used in this study.

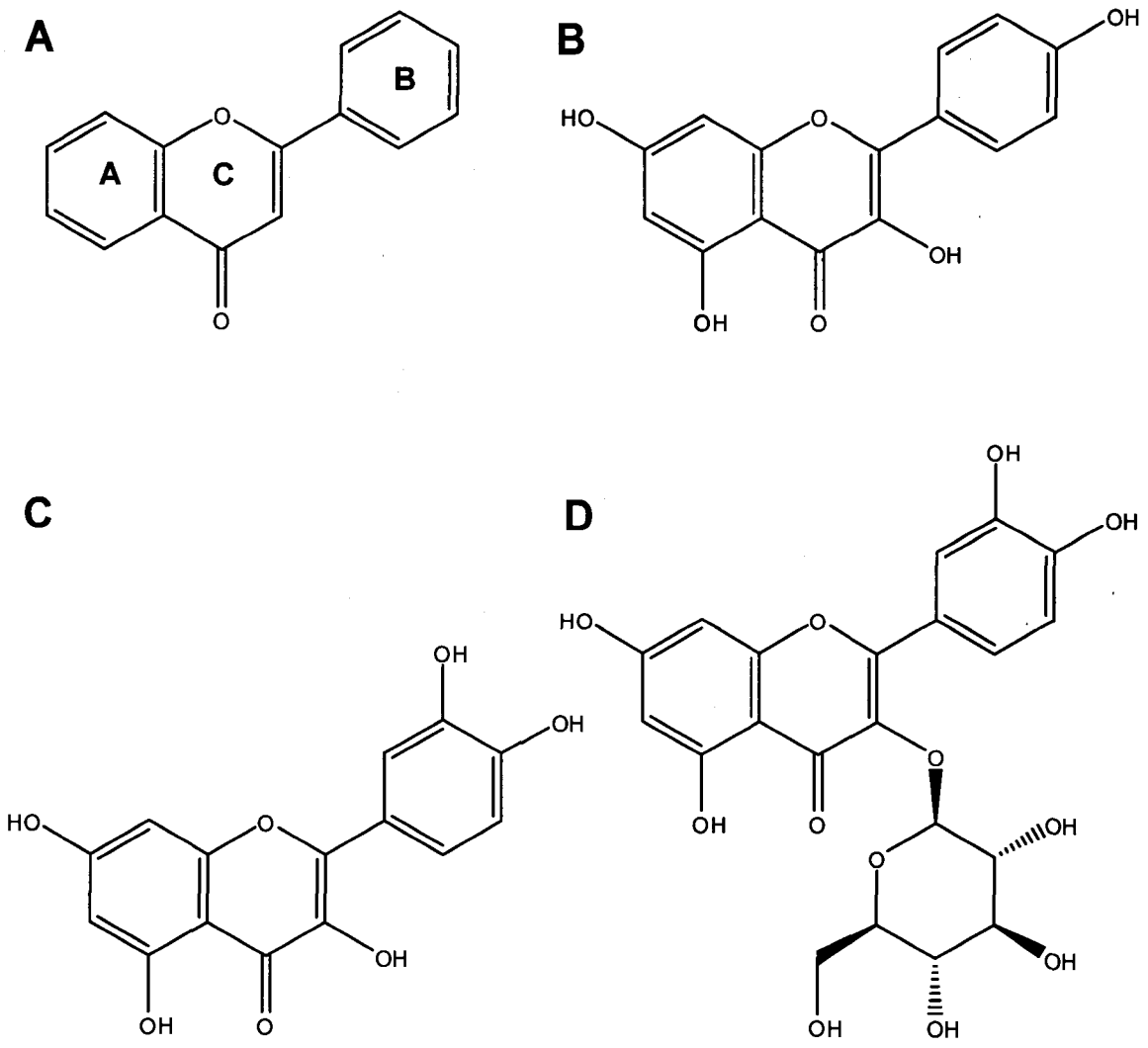

Figure 4. **Flavonoid structure**. **A** Flavonol, **B** Kaempferol, **C** Quercetin, **D** Quercetin-3-glucoside.

Some flavonoids act as bifunctional inducers [190] by activating the arylhydrocarbon receptor (AhR) to induce phase I enzymes, and the resultant intermediates induce phase II enzyme transcription. They also act as monofunctional inducers by inducing phase II enzyme transcription directly through the ARE [191]. Quercetin, kaempferol, genistein and quercetin-4-glucoside all act as monofunctional inducers to increase NQO1 activity in cells lacking the AhR [191], implying that they may act through the Nrf2/ARE pathway. Quercetin and its glycoside significantly activate the ARE and increase intracellular glutathione in rat hepatocytes [192]. Through microarray experiments, epigallocatechin gallate (EGCG) has been shown to induce ARE genes [193], including HO-1, to provide significant protection against H_2O_2-induced cell death in endothelial cells [150]. Quercetin [194] and fisetin [195] induce the human NQO1-ARE in transfected cell lines.

The ability of some of these flavonoids to activate the ARE has been discovered since our research was undertaken, giving further validation for the need to explore the full potential of these compounds as therapeutics and preventatives. The fact that the synthetic flavonoid beta-naphthoflavone is a potent ARE inducer [132] lends credence to the search for additional flavonoids that induce transcription of genes to combat oxidative stress. Astrocytes may provide the best model for examining Nrf2/ARE induction by flavonoids because the ARE is more highly expressed in astrocytes than neurons [178].

2.13 Flavonoids as anti-carcinogens

Many of the phase II enzymes, which are dependent on induction of the Nrf2/ARE pathway, are important in cellular defense against cancer [196]. Flavonoids' chemopreventative properties may be linked to the phase II enzymes and antioxidant enzymes produced when they induce the ARE. The observation has been made that flavonoids inhibit or decrease cancerous growths in various organs in mouse models [197, 198]. This has led to a number of clinical trials and epidemiological studies regarding the effect of flavonoids on cancer development in humans. Treatment with EGCG, a proanthocyanidin, is associated with a reduced risk of breast cancer redevelopment [199] and flavone or flavonol intake significantly reduced the risk of breast cancer in a large Italian study [200]. Green tea catechins significantly reduce development of prostate cancer [201] and a clinical trial is currently underway investigating the efficacy of EGCG in treating B-cell chronic lymphoid leukemia [202]. Other flavonoids in clinical trials include silymarin from milk thistle for treating lymphoblastic leukemia [203, 204] and the over-the-counter flavonoid supplement Isotonix OPC-3 for improving vasodilation.

2.14 Flavonoids and neuroprotection against oxidative stress

Some flavonoids act directly as antioxidants by donating electrons [205] and have the capability to regenerate the antioxidant, vitamin E [206], scavenge free radicals and chelate metal ions [207]. *In vitro* [208] and *in vivo* [209] evidence suggests that flavonoids are capable of crossing the blood brain barrier.

There is *in vitro* evidence of the antioxidant capacity of some flavonoids and their neuroprotective properties. Luteolin and quercetin confer protection to rat oligodendrocytes against H_2O_2-induced cell death [210]. A number of flavonoids, including luteolin, quercetin and fisetin, provide protection against H_2O_2-induced cell death in the HT-22 mouse hippocampal cell line by indirectly elevating intracellular GSH, direct scavenging of free radicals, and decreasing intracellular Ca^{2+} influx [211]. Quercetin and a number of catechin derivatives significantly protect striatal neurons against oxidized- LDL-induced cell death [212]. Quercetin and naringenin protect rat dopaminergic neurons against a variety of oxidative stressors including H_2O_2, 6-hydroxydopamine and MPP+ [213], suggesting that they have potential as therapeutics in PD. Naringenin pretreatment of rat PC12 cells provided significant protection against $A\beta_{25-35}$-induced oxidative stress, lactate dehydrogenase-release, and cell death [214] providing promise for flavonoids as therapeutics in AD. Extract EGb761, from the *Ginkgo biloba* tree, significantly protected primary cortical and hippocampal neurons against $A\beta_{1-42}$-induced toxicity [215, 216] and its ability to protect hippocampal cultures against NO-induced cell death has been attributed to its flavonoid components [217]. Quercetin and kaempferol constitute two of the three major flavonoid constituents of EGb761 [218].

In vivo flavonoid studies have produced some promising results in the search for bioactives. A number of flavonoids have been shown to significantly reduce

hamster LDL-peroxidation and superoxide formation [219]. In an AD mouse model, those fed *Ginkgo biloba* exhibited spatial memory and learning similar to wildtype controls, although soluble Aβ and Aβ plaque burden was unchanged between treatment groups [220]. Treatment of mouse models for AD with an apigenin-glucoside or quercetin significantly reversed poor cognitive performance in aged and lipopolysaccharide-treated mice [221].

2.15 Flavonoid intake and risk of neurodegenerative disease

Ginkgo biloba intake for 2 years (526 patients, age 75) significantly decreased plasma $A\beta_{1-42}$ levels by 32% in non-demented subjects. Although $A\beta_{1-42}$ is not a useful tool for diagnosing AD, it is significantly increased in late-onset AD patients and is considered to be a risk factor in AD development [222].

Flavonoid intake has been inversely correlated with risk of dementia development in a study of French patients over a 5-year trial [223]. This trial was continued for another five years, and at the end of the 10-year trial, flavonoid intake greater than 13.6 mg/day was associated with a decreased risk of cognitive decline [224]. Although no significant effect was found in data obtained from the earlier Zutphen Elderly study [225], there were differences in the clinical trial designs. The Zutphen study had only male subjects, and because almost twice as many women as men have AD [10], it is questionable whether the same inferences from the results can be made for women. The Zutphen study also had a lower number of participants (342) in comparison to the French study (1367).

The flavonoid intake in the diets' of both studies was defined by five flavonoids: the flavonols quercetin, myricetin, and kaempferol, and the flavones luteolin and apigenin. Therefore, there is evidence to support a role for flavonoids in reducing the incidence of neurodegenerative disease.

2.16 Rationale for study

Treatment to increase antioxidants in the body and reduce oxidative stress, the common factor linking many neurodegenerative diseases, may be beneficial in preventing or slowing progression of these diseases [50, 51, 65, 110, 111, 113, 114]. The ARE is a potential target for therapeutics and preventatives because its induction is required for production of multiple protective antioxidant enzymes and phase II enzymes. Furthermore, ARE induction has provided neuroprotection in a number of animal models [135, 144, 183, 184, 186]. For these reasons, we chose to examine inducers of the Nrf2/ARE pathway and its protective effects in cortical cell cultures. Flavonoids were chosen due to previous research implicating them as ARE inducers in other models [191-195]. The ARE is preferentially activated in astrocytes, leading to the hypothesis that "Flavonoids increase phase II enzyme and antioxidant enzymes in astrocytes, which provides indirect protection to primary cortical cell cultures". The aims of this research were to determine the potency and efficacy of flavonoid compounds that augment phase 2 and antioxidant enzymes in astrocytes and to determine the level of protection flavonoids afford to primary cortical cultures against oxidative stress.

3.0 METHODS & MATERIALS

3.1 Materials

Promega's Cell Culture Lysis Buffer and Luciferase 1000 Assay system were obtained from Fisher Scientific (Nepean, ON). All cell culture media and supplements were purchased from Hyclone (Logan, UT), except Neurobasal medium, B27 supplement and gentamicin (Gibco, Burlington, ON). Restriction enzymes and DNA ligase were purchased from Fermentas (Burlington, ON). Quercetin-3-glucoside was obtained from ChromaDex (Santa Ana, CA). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON). The pHTS-MCS plasmid was obtained from Biomyx (San Diego, CA).

3.2 Cell culture

As the most published, commercially available human astrocytoma cell line, the U87-MG cell line was chosen for transfection and enzyme activity experiments. U87-MG cells (American Type Culture Collection, Manassas, VA) were cultured in Minimal Essential Medium/Earle's Balanced Salt Solution supplemented with 1X non-essential amino acids, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 units/ml penicillin, 100ug/ml streptomycin and 10% fetal bovine serum (FBS). Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (1:1) supplemented as above. Cells were maintained at 37°C with 5% CO_2.

All animal experiments were carried out in accordance with the Canadian Council on Animal Care guidelines, and protocols were approved by the University of Prince Edward Island and National Research Council Animal Care Committees. Timed pregnant C57Bl6 mice were anesthetized at embryonic day 16 by intraperitoneal injections of 50 mg/kg pentobarbital, followed by decapitation. Cells from the cerebral cortex were chosen to assess flavonoid toxicity and protection against oxidative stress because they, along with the hippocampus, are the primary site of neuronal loss in Alzheimer's disease. Cerebral cortices were removed from fetal embryos, maintained in Hanks' balanced salt solution for 15 min, dissociated by trituration and seeded on poly-D-lysine coated, polystyrene 96-well plates at a density of 9.8×10^4 cells/cm^2, or on 12 mm glass coverslips at a density of 8.8×10^5 cells/cm^2. Neurobasal medium supplemented with 1 mM L-glutamine, 5 mM HEPES, B27, 25 μg/ml gentamicin and 10% FBS was replaced after 24 h (1 day-*in-vitro* DIV) with supplemented, FBS-free Neurobasal medium. Cells were incubated at 37°C with 5% CO_2 for four additional days before experiments were performed.

3.3 pHTS-ARE plasmid design and construction

To create stable cell lines containing an ARE-driven luciferase reporter, the pHTS-ARE plasmid was designed. The pGL3-NQO1-ARE plasmid [182] was kindly provided by Dr. George Robertson (Dalhousie University, Canada). PCR primers were designed for the ARE sequence in the pGL3-NQO1-ARE plasmid using Invitrogen's OligoPerfect Designer and Vector NTI v6: sense primer 5'-

AGTCAAGTCAGATCTCGGCCGCAATAAAATATCTTT-3' and antisense primer 5'-ATACAATCTCTCGAGGATTCTGCTGAGTCACTGTGAC-3' (Invitrogen, Burlington, ON).

ARE amplification from pGL3-NQO1-ARE was performed with a Mycyler thermal cycler (Bio-Rad, Montreal, QC) and Platinum PCR SuperMix High Fidelity (Invitrogen). The approximately 220 base pair (bp) primer product was isolated on a 5% DNA PAGE gel, excised and purified with the MinElute Gel Extraction Kit (Qiagen, Mississauga, ON).

The plasmid pHTS-MCS and purified ARE were digested with BglII and XhoI at 37°C for 2 h and purified with MinElute Reaction Cleanup Kit (Qiagen). The digested pHTS-MCS and ARE were ligated with T4 DNA ligase at 16°C for 16 h, followed by inactivation at 65°C for 10 min. The ligation mixture was transformed into XL1-blue cells with TransformAid Bacterial Transformation Kit (Fermentas), and selected on 100 μg/ml ampicillin-agar plates for 16 h, 37°C. DNA was extracted from bacterial colonies and purified from 0.7% agarose gels by phenol-chloroform extraction [226]. To ensure that the ligated pHTS-ARE plasmid contained the ARE in the correct orientation, the mini-prepped DNA was sequenced with primers supplied with the pHTS-MCS plasmid, by the University of Calgary's Core DNA Services (Calgary, AB).

3.4 U87-MG transfections and luciferase assays

U87-MG cells were seeded at a density of 1 x 10^5 cells/cm^2 in a 6-well plate, in FBS/antibiotic-free medium (Day 0). Cells were transiently transfected with 2 μg of pHTS-ARE, pHTS-MCS or the control pHyg-EGFP (Clontech, Mountain View, CA) using Lipofectamine 1000 (Invitrogen) (Day 1). To ensure an even distribution of transfected cells, cells were scraped and plated at a density of 7.9 x 10^4 cells/cm^2 in a 96-well plate 24 h post-transfection (Day 2). Uniform transfection of cells was confirmed by a separate transfection with the pHygEGFP plasmid. On Day 2, treatments, including flavonoids and controls (DMSO vehicle and the positive ARE inducer tBHQ) were added to transfected cells. On Day 4, cells were washed with phosphate buffered saline (PBS) and lysed for 5 min at RT with 20 μL Cell Culture Lysis Buffer (Promega). Luminescence was measured on a Luminoskan Ascent microplate luminometer (Thermo Electron, Milford, MA) using a Luminescence Assay Kit (Promega). The 48 h timepoint was chosen for the study of flavonoids based on previous findings that tBHQ significantly induced ARE after this incubation time [178].

3.5 Western blotting

Whole cell lysates of U87 MG cells were assayed for protein concentration, by the Lowry method [227], for the NQO1 activity assay (DC Protein Assay kit, Bio-Rad, Montreal, QC). Twenty μg of protein was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels for 40 min, equilibrated in transfer buffer for 30 min, and semi-dry transferred with a Trans-Blot SD transfer cell

(Bio-Rad) to polyvinylidene fluoride. Blots were blocked in 5% goat serum overnight at 4°C, incubated with 1:1000 anti-mouse NQO1 antibody (Santa Cruz, Santa Cruz, CA) for 2 h at room temperature and washed three times for 5 min in PBST (phosphate buffered saline-0.1% Tween20). Blots were incubated in 1:10,000 peroxidase-conjugated AffiniPure goat anti-rabbit IgG for 1 h at room temperature and washed three times in PBST. Bands were detected with BM Chemiluminescence blotting substrate kit (Roche) and a Chemidoc XRS system (Bio-Rad). Images were captured with Quantity One 4.6.1 software (Bio-Rad).

3.6 NQO1 Activity Assay

An NQO1 activity assay was chosen as an additional indication of ARE induction because the ARE of this commonly studied Phase II enzyme was also used in the luciferase assays. U87-MG cells were seeded at a density of $3x10^5$ in 12-well plates. After 24 h incubation, cells received a 48 h flavonoid treatment. Cells were rinsed with PBS, pelleted at 12,200 g (Eppendorf mini-spin, Mississauga, ON) and once more rinsed with PBS. Pellets were lysed in 200 μL of ice-cold cell lysis buffer (100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 0.6 M NaCl, 4% Igepal, 20% sucrose) for 10 min and centrifuged at 4300 g, 5 min, 4°C.

NQO1 enzyme activity was measured as a colorimetric dicumarol-sensitive reduction of dichlorophenolindophenol (DCPIP) [228]. Briefly, 40 μg protein was incubated with 24 mM Tris-Cl (ph 7.4), 0.7 mg/ml bovine serum albumin, 5 μM flavin adenine dinucleotide and 0.2 mM nicotinamide adenine dinucleotide, with

and without the NQO1-specific inhibitor, 20 μM dicumarol, for 10 min. Absorbance at 540 nm was measured for 3 min after the addition of 0.4 mM DCPIP (Molecular Devices Spectramax M2, Sunnyvale, CA). The specific activity of NQO1 was expressed as nmol of DCPIP reduced per minute per mg of protein, with an extinction coefficient of 21 mM^{-1} cm^{-1}.

3.7 Flavonoid protection of primary cortical cultures

To determine if flavonoids provide protection to primary cortical cultures against oxidative stress, at 5DIV the flavonoids quercetin-3-glucoside (Q3G), quercetin, or kaempferol were administered to C57Bl6 cortical cultures. Flavonoid treatments were in triplicate wells of neurons, to a final concentration of 0.01-0.20% dimethylsulfoxide (DMSO). After 48 h the medium was replaced with Neurobasal medium, minus antioxidants, containing 25 μM H_2O_2. After 24 h, mitochondrial activity was measured, as an indication of cell viability, by thiazolyl blue tetrazolium bromide (MTT) colorimetric reduction at 570 nm. All future cell viabilities were measured by MTT assay. Removing the flavonoids, before the H_2O_2 was added, prevented them from acting directly as antioxidants to reduce the H_2O_2 extracellularly. This better enabled examination of the effect of the flavonoids on gene expression.

3.8 Adenovirus infection

The adenoviruses Ad-EGFP and Ad-DN-Nrf2, constructed by the Adenovirus Core Facility (Canadian Stroke Network Core Facility, University of Ottawa,

Ottawa, ON), were generous gifts from Dr. Timothy Murphy (University of British Columbia, Canada). The dominant negative Nrf2 (DN-Nrf2) was constructed using the Cre-Lox system [229], with Nrf2 cDNA excised from pEF-Nrf2 [149]. The DN-Nrf2 was cloned into the pTrack-CMV vector, which contains an enhanced green fluorescent protein (EGFP) gene with a separate promoter, used to determine transfection efficiency. The replication-deficient adenoviruses were amplified and titered in HEK293 cells. Multiplicity of infection is the average number of virus particles that infect a host cell. Optimal multiplicity of infection (MOI) was determined by toxicity assays on C57Bl6 cortical cultures and confirmed by detection of infection via EGFP fluorescence with an Axiovert 40 CFL immunofluorescent microscope (Zeiss, Toronto, ON).

To evaluate the efficiency of the DN-Nrf2, 5DIV C57Bl6 cortical cultures were transfected in 96-well plates with 0.25 μg pHTS-ARE plasmid per 0.25 μl Gencarrier-2 reagent (Epoch Biolabs, Houston, TX). After 5 h incubation, cells were infected with adenovirus for 1 h. Medium was then replaced, supplemented with 20% FBS and 50 μg/ml gentamicin, and treated with tBHQ or DMSO vehicle for 48 h. Cells were washed with PBS, lysed for 5 min at RT with 20 μL Cell Culture Lysis Buffer (Promega) and luminescence measured. In parallel, cell cultures with identical treatments were transfected with the pHygEGFP plasmid and fluorescent cells were counted to ensure there was no statistical difference in transfection efficiency between treatments (2 fields of vision at 200X magnification, n=7 performed in triplicate wells). Transfection efficiency of

primary cortical cultures was relatively low at 3.8%; however, the signal was within the luciferase standard curve detectable by the luminometer.

To determine the role of the Nrf2-ARE pathway in flavonoid-mediated protection of cortical cultures, conditioned medium was removed from 5DIV C57Bl6 cortical cultures and they were infected at 30 MOI with Ad-EGFP adenovirus control or Ad-DN-Nrf2 for 1 h. The conditioned medium was then replaced and flavonoids were added. After 48 h, cultures infected with Ad-DN-Nrf2 or Ad-EGFP were stressed with 25 μM H_2O_2, and 30 μM H_2O_2, respectively for 24 h. Cell viability was measured by MTT assay.

3.9 Immunofluorescent Assay (IFA)

To determine the primary cortical cell type in which adenovirus infection occurs, 5DIV cortical cells were infected with Ad-DN-Nrf2 or the control Ad-EGFP at 30 MOI. After 72 h, cells were fixed with 4% paraformaldehyde for 10 min and permeablized with HEPES-Triton buffer (0.02M HEPES pH 7.4, 0.3 M sucrose, 0.05 M NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 5 min. Cells were incubated with 1:500 anti-glial fibrillary acidic protein (GFAP) (DAKO, Mississauga, ON) or 1:200 anti-microtubule-associated protein 2 (MAP2) (Chemicon, Temecula, CA) overnight at 4°C, followed by a 1 h incubation with 1:200 or 1:800 Alexa Fluor 546 secondary antibodies, respectively. Cells were then incubated with 10 μg/mL Hoechst nuclear stain for 30 sec followed by mounting with FluorSave reagent (Calbiochem, San Diego, CA). IFA slides were viewed on an Axioskop 2 Plus

fluorescent microscope (Zeiss). Images were captured at 200X magnification, with an AxioCam MRm camera and AxioVision V4.2 software (Zeiss). 10 fields of view were counted from 2 separate experiments.

3.10 Data analysis

Cell survival in MTT assays was expressed as a percentage of the untreated absorbance control.

A comparison of Ad-EGFP and Ad-DN-Nrf2 infected cultures, treated with flavonoids, was made after correcting the flavonoid data with the DMSO vehicle controls.

2-way ANOVA with Bonferroni's post-test was used to determine the statistical difference between treatments and their vehicle controls, using Graphpad Prism V4 software (San Diego, CA).

4.0 RESULTS

4.1 Flavonoid induction of NQO1-ARE-luciferase in U87-MG cells

A previous study has shown that the flavonol quercetin increases ARE induction 2-fold in a human breast carcinoma cell line [194]. We hypothesized that flavonoids would increase ARE induction in astrocytes. ARE induction in U87-MG cells was determined by luciferase assay 48 hours after pHTS-ARE transfection. The positive control tBHQ significantly induced NQO1-ARE-luciferase in a concentration-dependent manner (Figure 5).

A 48 h treatment, post-transfection, with quercetin, quercetin-3-glucoside (Q3G) or kaempferol significantly induced NQO1-ARE-luciferase in U87-MG astrocytoma cells (Figure 6). Quercetin was omitted from further testing because its neuroprotective properties have been established in a similar *in vitro* model [178].

4.2 Flavonoids increase NQO1 activity in U87-MG cells

Previous studies have shown that flavonoids significantly increase NQO1 enzyme activity in murine hepatoma cell lines [191, 230]. To determine whether flavonoid-mediated increases in ARE-induced gene expression resulted in functional proteins, NQO1 enzyme activity was examined after flavonoid treatment. We chose the human U87-MG astrocytoma cell line as our model and first established the presence and inducibility of the NQO1 protein, by Western blot, after 48 h treatment with the positive control tBHQ (Figure 7). All three

flavonoids increased enzyme activity significantly ($p<0.05$), however, 10 μM quercetin-3-glucoside was responsible for the largest increase, greater than 25% (Figure 8).

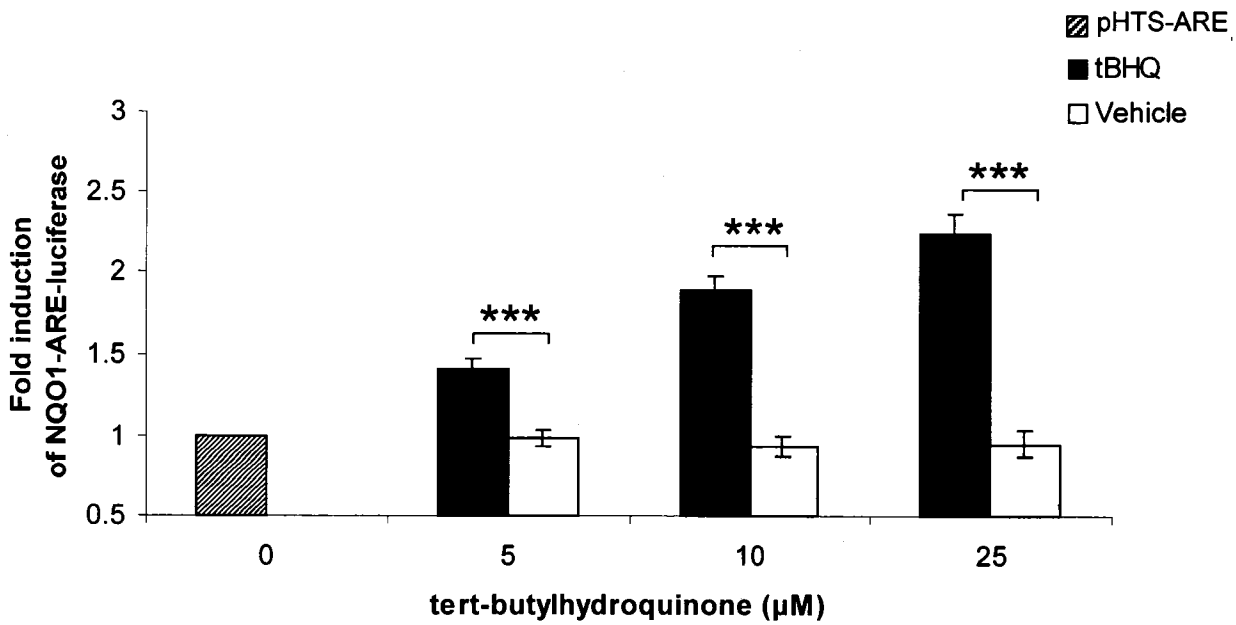

Figure 5. tert-butylhydroquinone (tBHQ) induction of NQO1-ARE-luciferase in U87-MG cells. U87-MG cells were transiently transfected with pHTS-ARE luciferase reporter plasmid, treated with tBHQ or DMSO vehicle and assayed for luciferase activity 48 h later. Values are the mean fold induction of untreated controls ± SEM (n=8, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. ***p<0.001

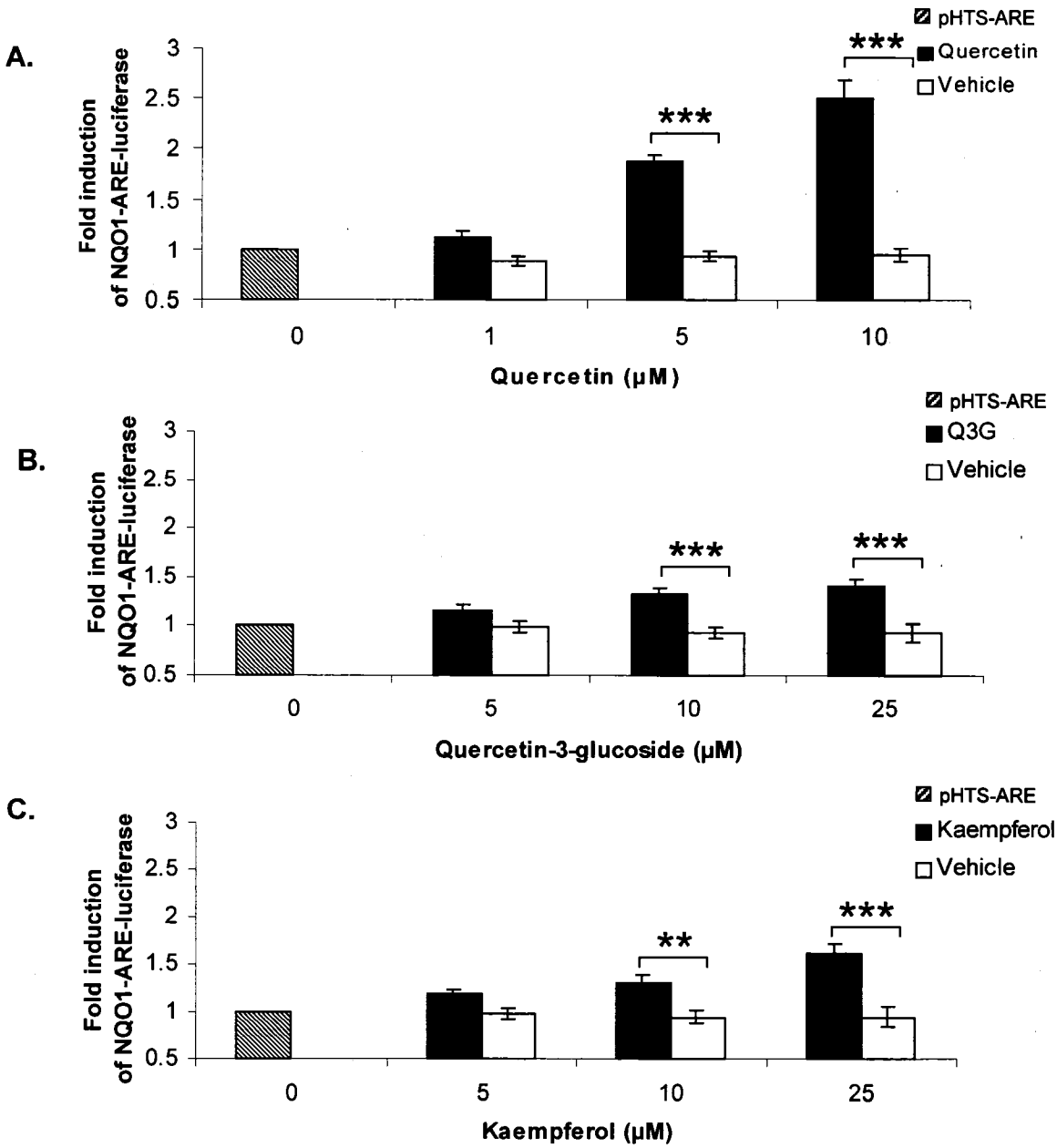

Figure 6. Flavonoid induction of NQO1-ARE-luciferase in U87-MG cells. U87-MG cells were transiently transfected with pHTS-ARE luciferase reporter plasmid, then treated with **A.** quercetin **B.** quercetin-3-glucoside **C.** kaempferol or DMSO vehicle and assayed for luciferase activity 48 h later. Values are the mean fold induction of untreated controls ± SEM (n=7, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. **p<0.01, ***p<0.001

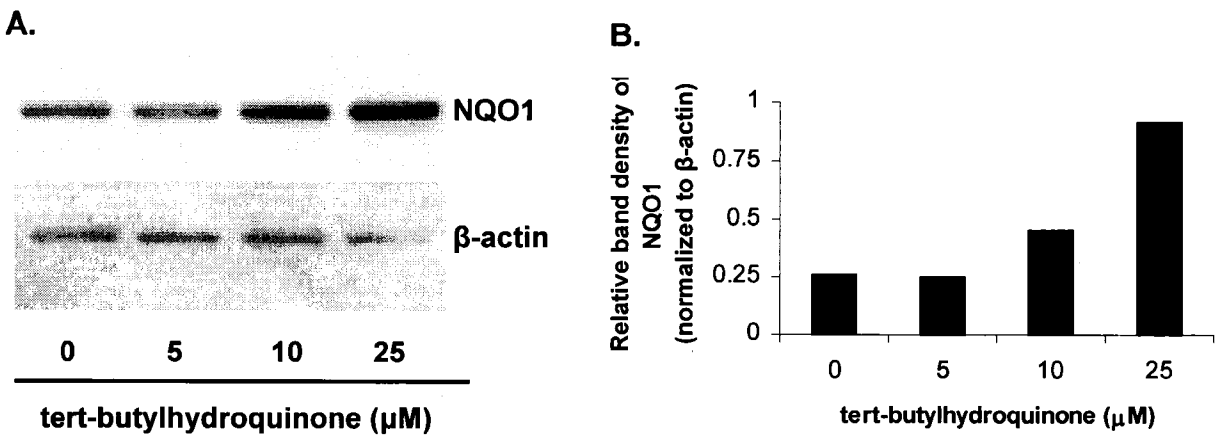

Figure 7. NQO1 protein is induced in U87-MG cells upon treatment with tBHQ. A. This is a representative Western blot showing the presence of the NQO1 protein in U87-MG astrocytoma cells and its increase by tert-butylhydroquinone (tBHQ) after 48 h treatment. **B.** A densitometry analysis of the Western blot in **7A**, normalized to β-actin. This is representative of 4 independent experiments.

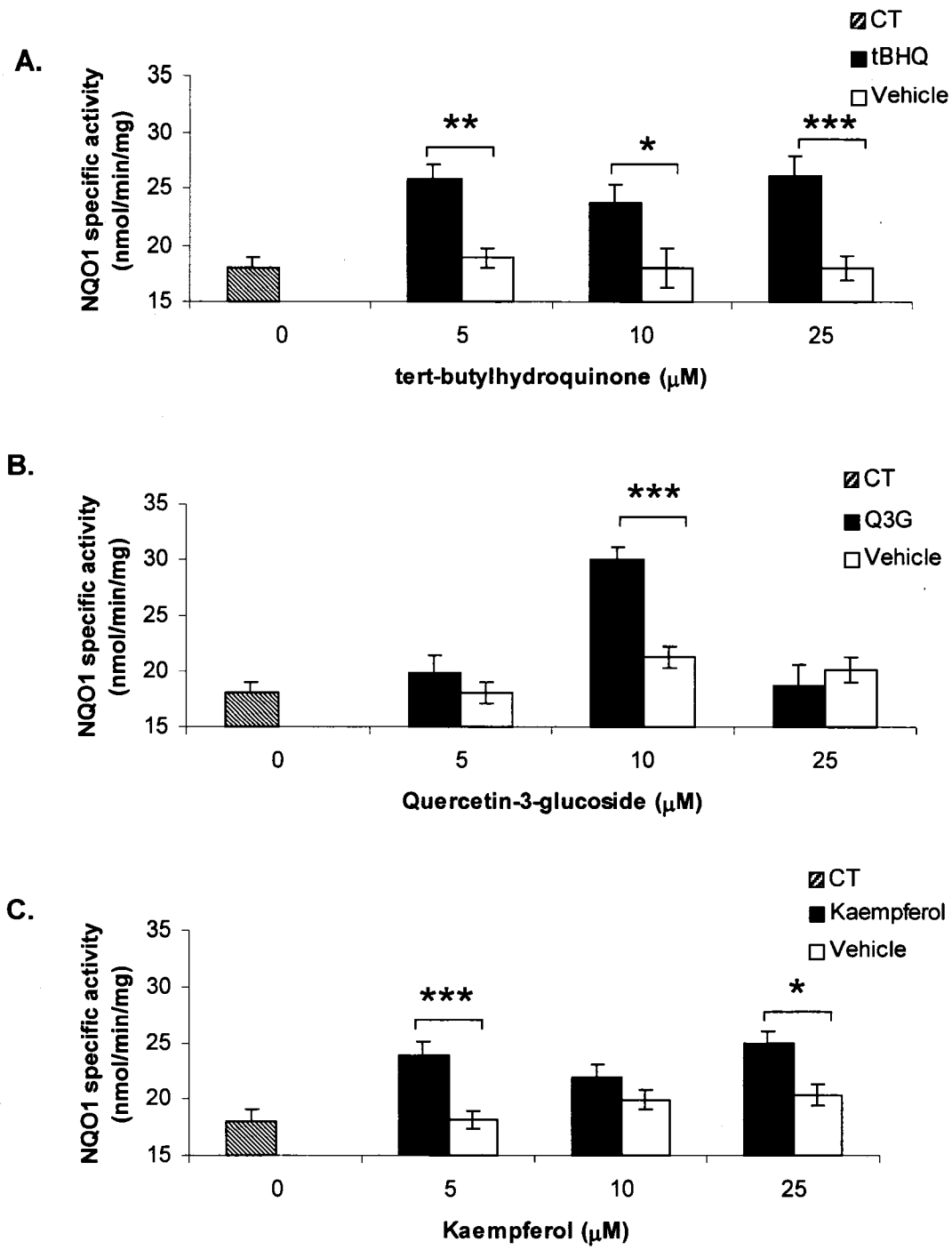

Figure 8. Flavonoids increase NQO1 enzyme activity in U87-MG cells. U87-MG cells were pretreated for 48 h with **A.** tert-butylhydroquinone **B.** quercetin-3-glucoside **C.** kaempferol or DMSO vehicle and assayed for NQO1 enzyme activity. CT is untreated control. Values are the mean specific enzyme activity ± SEM (n≥5, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. *p<0.05, **p<0.01, ***p<0.001

4.3 Flavonoid-mediated toxicity in C57Bl6 cortical cell cultures

Flavonoids found to increase ARE induction were tested for toxicity in a C57Bl6 cortical cell culture model by MTT assay. The ARE is induced by stress response; therefore before their neuroprotective properties could be studied, it was important to determine that the flavonoids were not toxic to cortical cultures. After 48 h incubation quercetin-3-glucoside was non-toxic ($p>0.05$) at concentrations 25 μM or less ($p<0.001$), kaempferol displayed no significant toxicity within the dose range tested ($p>0.05$), and tBHQ was highly toxic above 10 μM ($p<0.05$) (Figure 9). Quercetin displayed dose-dependent toxicity beginning at 15 μM ($p<0.05$).

4.4 Flavonoids provide protection to primary cortical cell cultures in a H_2O_2 oxidative-stress model

Q3G and kaempferol were tested, at non-toxic concentrations, for their neuroprotective properties in a C57Bl6 oxidative stress model. H_2O_2 was chosen as a stressor because its oxidative stress products are targets of ARE-regulated enzymes. Q3G provided significant protection ($p<0.05$) against H_2O_2 at 7.5-30 μM (Figure 10). Significant protection was observed with kaempferol at 20 μM ($p<0.05$).

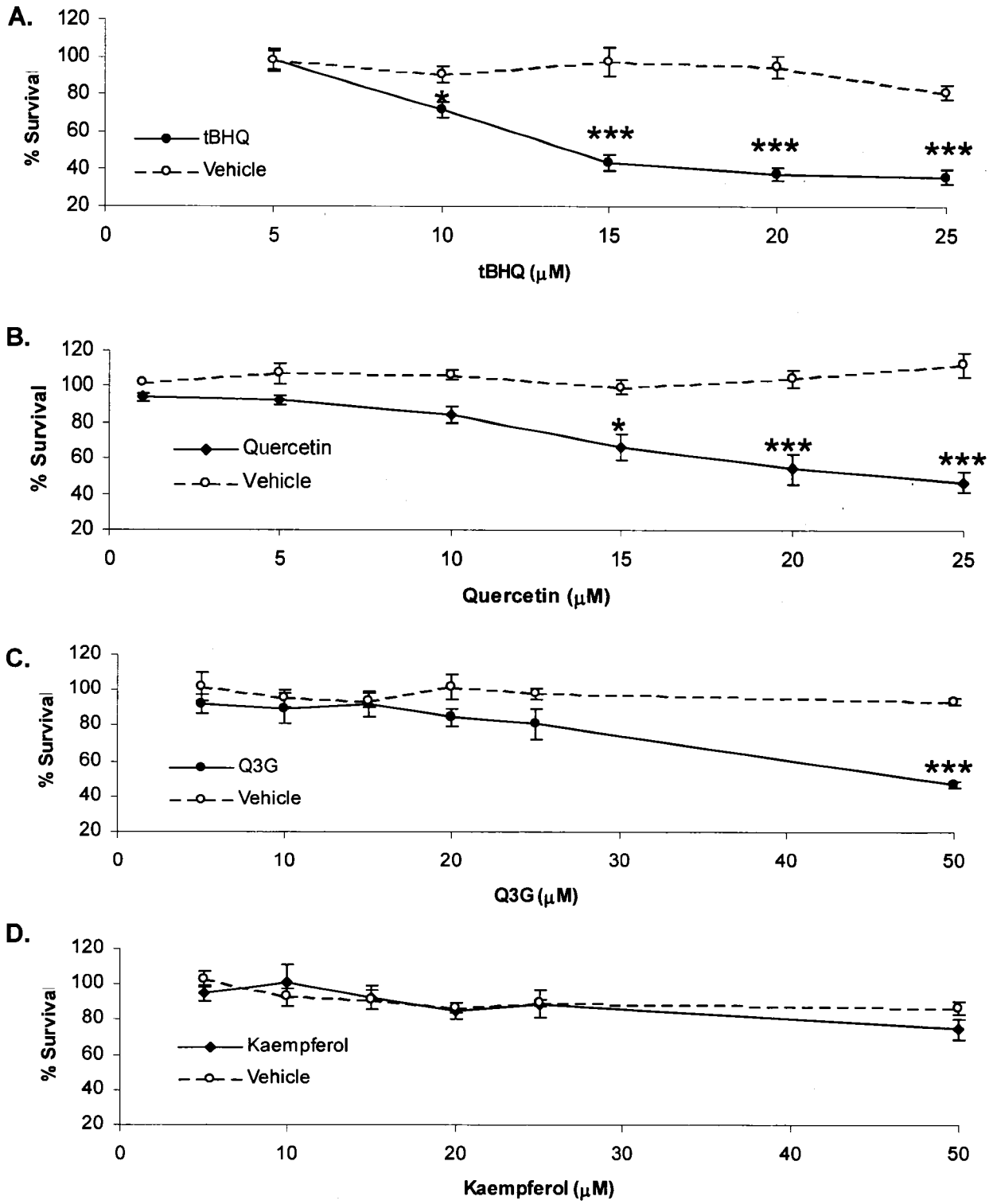

Figure 9. Flavonoid-mediated toxicity on C57Bl6 cortical cell cultures. C57Bl6 cortical cultures were incubated with **A.** tert-butylhydroquinone **B.** quercetin **C.** quercetin-3-glucoside (Q3G) **D.** kaempferol or DMSO vehicle for 48 h and assessed for cell viability by MTT assay. Values are expressed as percent of the untreated control ± SEM (n=3, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. *p<0.05, ***p<0.001

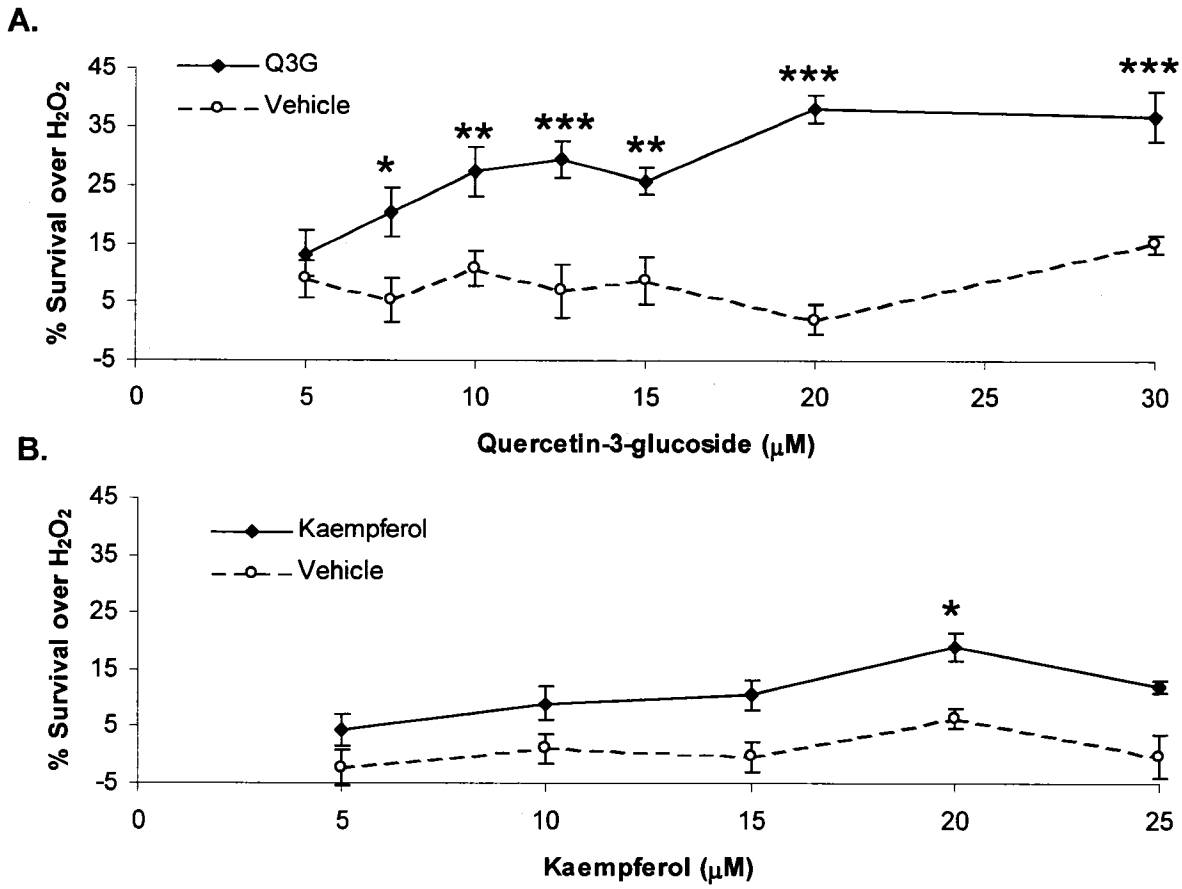

Figure 10. Flavonoids protection against oxidative stress in primary cortical cell cultures. C57Bl6 cortical cultures were incubated with **A.** quercetin-3-glucoside (Q3G) **B.** kaempferol or DMSO vehicle for 48 h, stressed with 25 μM H_2O_2 for 24 h and assessed for cell viability by MTT assay. Values are expressed as percent of the untreated control ± SEM (n=5, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. *p<0.05, **p<0.01, ***p<0.001

4.5 Adenovirus preferentially infects astrocytes in primary cortical cell cultures

It is known that some flavonoids directly reduce oxidative species, but further examination is needed on the role that the Nrf2/ARE pathway has in flavonoids' ability to protect primary cortical cultures from oxidative stress. To identify the cell types in which Nrf2/ARE induction could be inhibited, C57Bl6 cultures were infected with Ad-DN-Nrf2 or Ad-EGFP and stained for neuronal or glial markers, MAP2 and GFAP, respectively. There was no significant difference in staining between Ad-DN-Nrf2 and Ad-EGFP. Thirty MOI resulted in an 88.5% infection level in astrocytes, while no infected neurons were identified (Figure 11). This is in accord with previous findings where neurons were rarely infected at a high (75) MOI [178].

4.6 Ad-DN-Nrf2 inhibits induction of NQO1-ARE-luciferase

To confirm the efficacy of Ad-DN-Nrf2, C57Bl6 cultures were transfected with pHTS-ARE and infected with either Ad-DN-Nrf2 or the control Ad-EGFP. After 48 tBHQ treatment, luminescence was measured as an indicator of ARE induction.

No significant induction of the ARE was observed after infection with Ad-DN-Nrf2, while the control infection with Ad-EGFP produced significant luciferase activity with 5 μM and 10 μM tBHQ (Figure 12).

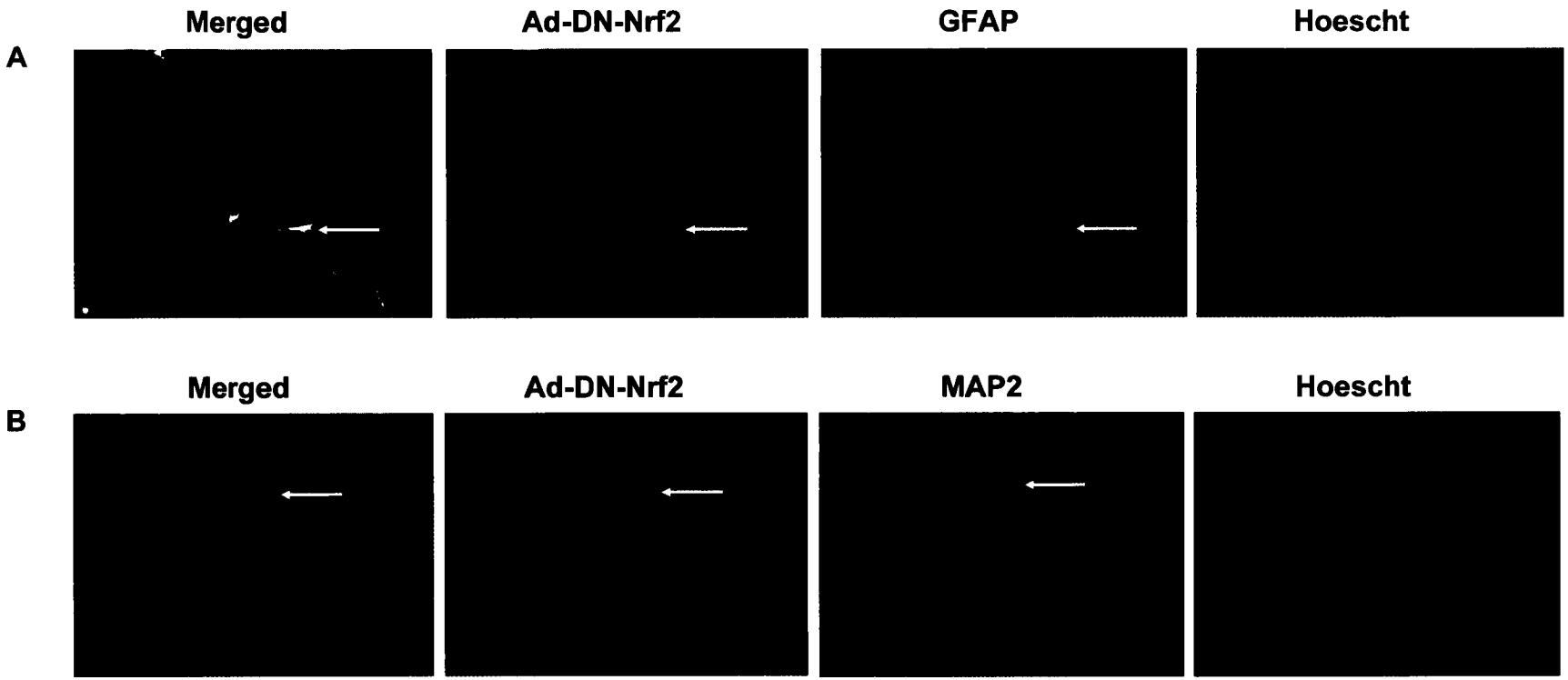

Figure 11. Adenovirus preferentially infects astrocytes. **A.** is a representative immunofluorescent assay of primary cortical cultures 48 h post-Ad-DN-Nrf2 infection. Cells are stained for astrocytic marker GFAP, demonstrating 88.5% adenovirus infection efficiency of astrocytes at 30 MOI. Arrows indicate adenovirus-infected cells. Hoechst is a nuclear stain. **B.** is a representative immunofluorescent assay of primary cortical cultures 48 h post-Ad-DN-Nrf2 infection stained for neuronal marker MAP2, demonstrating 0% adenovirus infection of neurons at 30 MOI

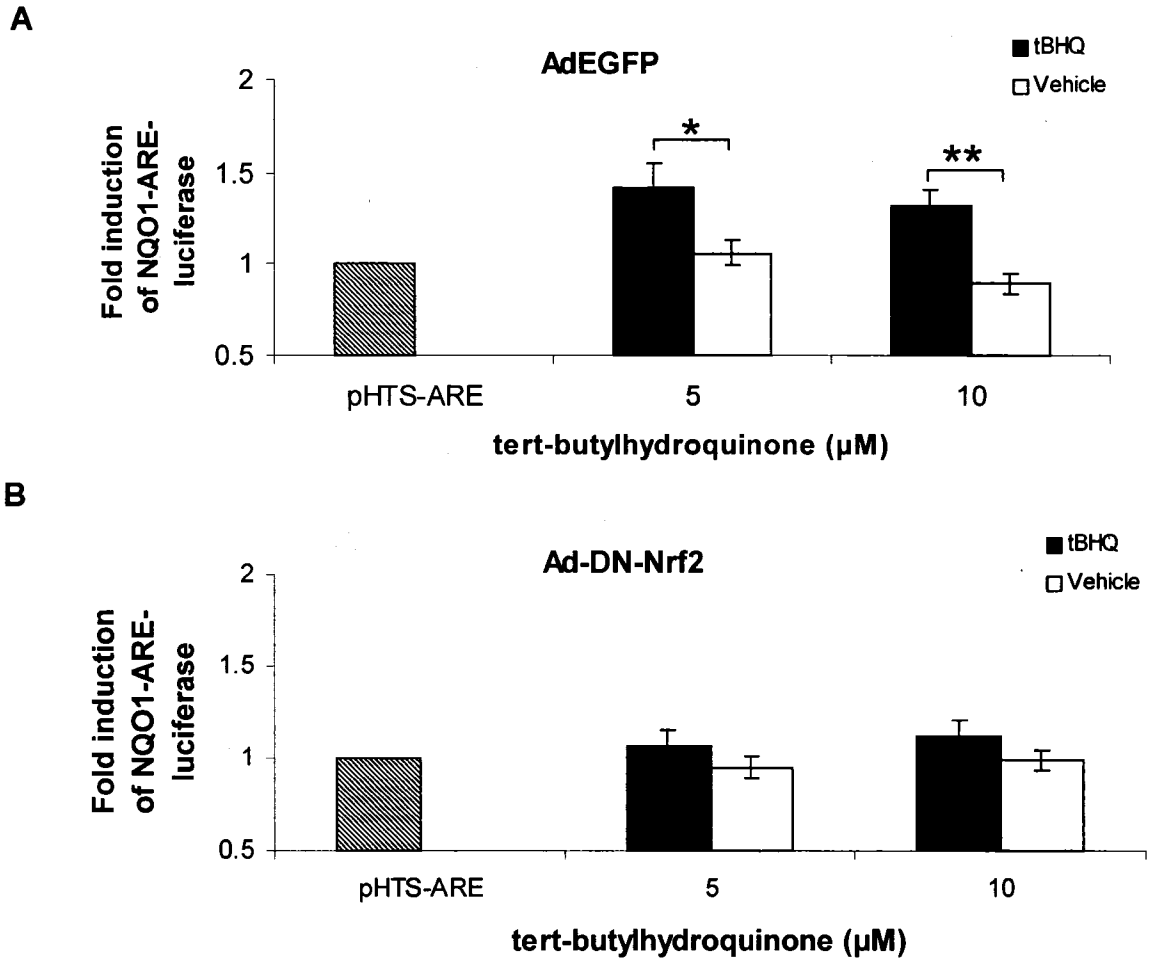

Figure 12. Ad-DN-Nrf2 inhibits NQO1-ARE-luciferase induction. Primary cortical cell cultures were transfected with pHTS-ARE and infected with **A** 30 MOI Ad-EGFP control **B** 30 MOI Ad-DN-Nrf2. Values are the mean fold induction of untreated controls ± SEM (n=7, in triplicate). 2-way ANOVA of treatments vs. DMSO controls, Bonferroni's post-hoc. *$p<0.05$, **$p<0.01$

4.7 Kaempferol-mediated protection of primary cortical cell cultures against oxidative stress involves Nrf2

Kaempferol significantly increased NQO1-ARE-mediated luciferase activity and NQO1 enzyme activity. To determine the role of the Nrf2-ARE pathway in kaempferol-mediated protection against oxidative stress, primary cortical cell cultures were infected with 30 MOI Ad-DN-Nrf2 or the Ad-EGFP control. After 48 h kaempferol treatment, cultures were stressed with H_2O_2 for 24 h and assayed for cell viability. The significant protection afforded by 15 μM kaempferol following infection with Ad-EGFP (Figure 13A) was not observed in Ad-DN-Nrf2-infected cultures (Figure 13B), suggesting that the Nrf2-ARE pathway plays a role in kaempferol-dependent protection against oxidative stress. Inhibiting the Nrf2/ARE pathway significantly reduced kaempferol-mediated protection against oxidative stress in cortical cell cultures (Figure 13C).

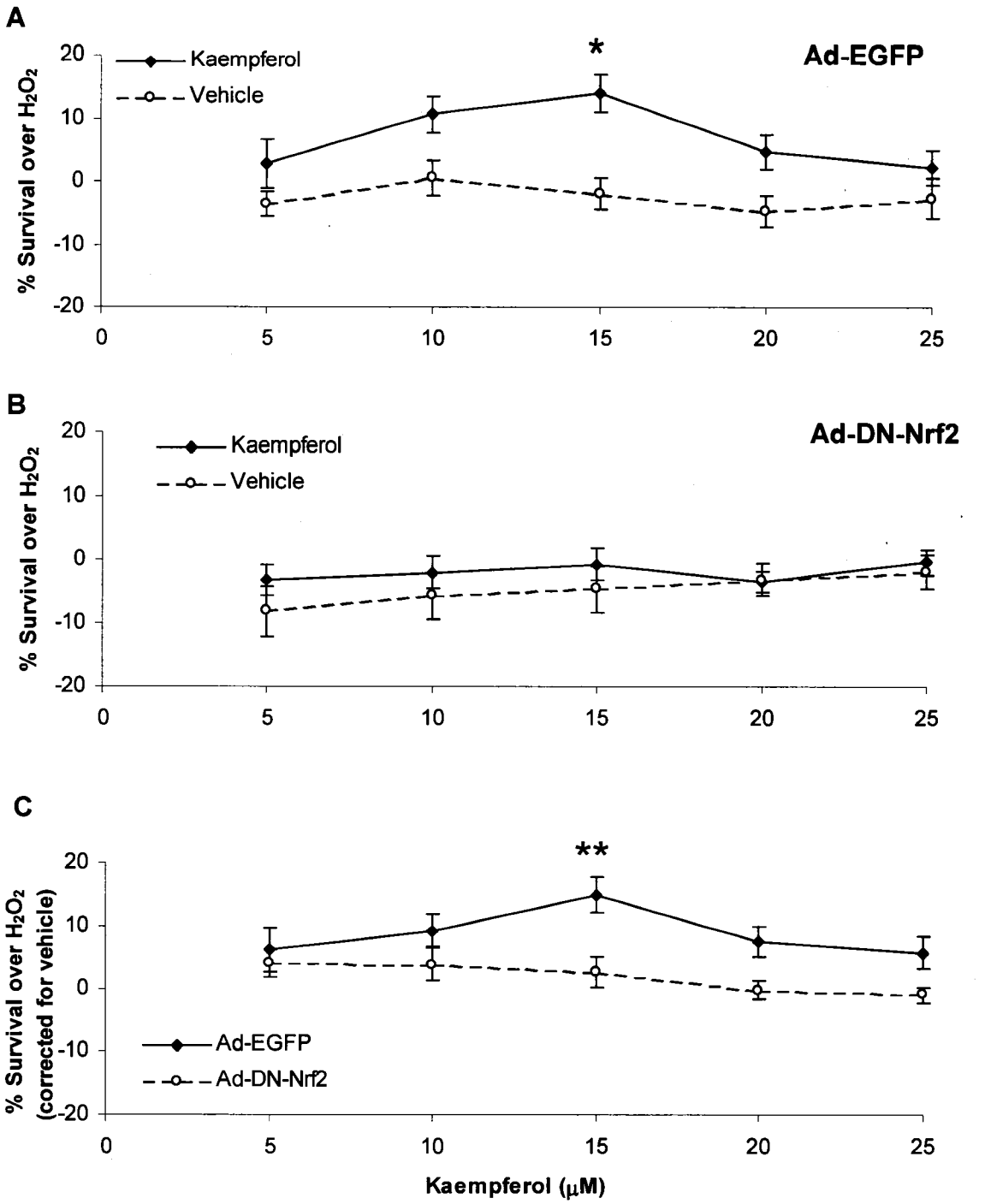

Figure 13. Kaempferol primary cortical cell culture protection against oxidative stress is Nrf2-dependent. **A** Primary cortical cultures were infected with Ad-EGFP, incubated with kaempferol for 48 h, then stressed with 30 μM H_2O_2 for 24 h. Survival was measured by MTT assay. **B** Cultures were infected with Ad-DN-Nrf2, incubated with kaempferol for 48 h, then stressed with 25 μM H_2O_2 for 24 h. **C** Difference in cortical culture survival after infection with Ad-EGFP or Ad-DN-Nrf2, followed by 48 h kaempferol and 24 h H_2O_2. Values are the mean and S.E. compared to DMSO vehicle (*$p<0.05$, **$p<0.01$ 2-way ANOVA, Bonferroni's post-hoc). n=5 experiments performed in triplicate.

4.8 Quercetin-3-glucoside primary cortical cell culture protection and adenovirus infection

After determining that Q3G provides protection to primary cortical cultures against oxidative stress, an attempt was made to establish mechanism of protection. Primary cortical cell cultures were infected with Ad-DN-Nrf2 or the Ad-EGFP control, treated with Q3G for 48 h, and stressed with H_2O_2 for 24 h. The significant protection ($p<0.05$) observed with 7.5 μM to 20 μM Q3G in uninfected cultures (Figure 10A) was maintained in the control Ad-EGFP-infected cultures (Figure 14A) as anticipated. The significant protection ($p<0.05$) observed at 7.5 μM and 10 μM Q3G was lost in Ad-DN-Nrf2-infected cortical cell cultures (Figure 14B). However, after correcting for the DMSO vehicles, statistical analysis revealed that inhibition of the NRf2/ARE pathway with adenovirus only resulted in a significant difference ($p<0.001$) in protection of cultures incubated with 20 μM Q3G (Figure 14C).

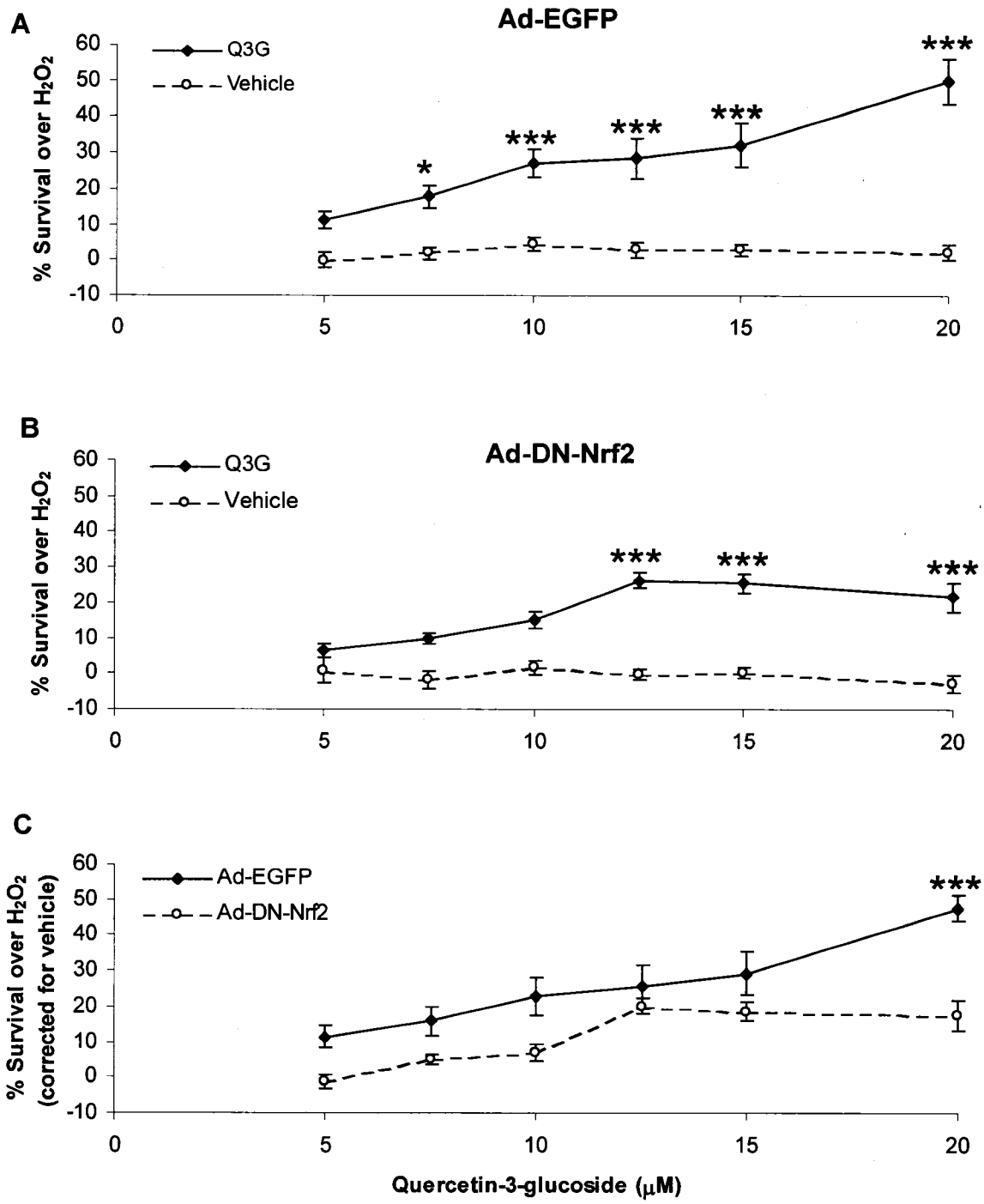

Figure 14. Quercetin3-glucoside protection against oxidative stress after adenovirus infection. **A** Primary cortical cultures were infected with Ad-EGFP followed with 48 h Q3G incubation. Protection against 30 μM H_2O_2 (24 h) stress was then measured by MTT assay. **B** Protection against 25 μM H_2O_2 (24 h) was measured in Ad-DN-Nrf2-infected cells, which were treated and stressed as in 14A. **C** Difference in cortical culture survival after infection with Ad-EGFP or Ad-DN-Nrf2, followed by 48 h Q3G and 24 h H_2O_2. Values are the mean and S.E. compared to DMSO vehicle (*p<0.05, ***p<0.001, 2-way ANOVA, Bonferroni's post-hoc). n=7 experiments performed in triplicate.

5.0 DISCUSSION

The current study provides evidence that the flavonoids, kaempferol and quercetin-3-glucoside provide significant protection to cortical cell cultures against oxidative stress. Flavonoid activation of the Nrf2-ARE pathway appears to have a role in the flavonoid-mediated cortical culture protection that was observed.

It is well documented that transcription of genes with an ARE enhancer is elevated in neurodegenerative disease and is localized to pathologically relevant areas of the brain [144, 231-234]. One such gene product is the enzyme NQO1, the expression of which is elevated early in AD [160]. In addition, relatively little NQO1 staining is observed near the end-stage of PD [233], suggesting that its protective role is in early to mid stages of the disease. The upregulation of ARE enzymes may be a compensatory response for the increased oxidative stress observed in the brains of patients with neurodegenerative disease [56, 57, 235]. However, upregulation of multiple ARE-dependent genes is probably necessary for conferring neuroprotection, as upregulation of one enzyme does not provide *in vitro* protection [236]. Our findings that flavonoids induce the ARE, applies not only to the NQO1-ARE, but most likely to the AREs of other genes, as the ARE contains a conserved sequence across genes. Identification of drugs or natural products that enhance ARE activity may be a successful strategy to prevent or treat neurodegenerative diseases.

The United States Department of Agriculture has compiled a summary of the flavonoid content of selected foods, based on scientific. Total flavonoid levels are high in green tea, leafy vegetables and fruit. In particular, a variety of berries, green tea and onions contain high levels of quercetin. Kaempferol is often found in the same foods as quercetin, but at lower concentrations except in kale, broccoli, brussel sprouts and a number of other leafy greens [237]. While information is not available on the Q3G-content of these foods, it has been shown that Q3G is also present in foods rich in quercetin, including whole onions [238] and apple peels [239]. A study by Commenges and colleagues found a significant decrease in the incidence of dementia correlated with a diet high in flavonoids, including kaempferol and quercetin [223]. It is expected that Q3G would also be present in foods containing quercetin. Thus, there is evidence to suggest that kaempferol, and quercetin and its glucosides reduce the risk of developing dementia. Drinking two cups of tea per day, which contains high levels of quercetin and kaempferol in addition to other flavonoids, has been associated with a decreased risk of developing PD [240].

The structures of quercetin and kaempferol are very similar, differing only in an additional hydroxyl group on the B ring of quercetin (Figure 4). The structure of Q3G is the same as quercetin, with a glucoside added to the 3-position of the C ring. Quercetin has the most antioxidant activity of the three flavonoids, measured by ferric reducing activity power and antiradical power assays, [241] because of its two hydroxyl groups on the B ring, which donate their hydrogen

atoms to scavenging free radicals [242]. Q3G and kaempferol have similar antioxidant activities, approximately half that of quercetin [241]. Of the three compounds, kaempferol is the most lipophilic because it lacks the second hydroxyl group on the B ring and the sugar moiety of Q3G. The higher lipophilicity of kaempferol and quercetin make it likely that higher concentrations of these flavonoids pass through cellular membranes, compared to Q3G.

Although kaempferol's neuroprotective properties are well documented for various oxidative stress conditions [243-246], we have demonstrated for the first time kaempferol's antioxidant effects through the Nrf2-ARE pathway in primary cortical cultures. Furthermore, we have shown that kaempferol's activation of this pathway in astrocytes contributes to its protection of cortical cell cultures. Our findings of kaempferol's Nrf2/ARE-dependent protection in primary cortical neurons supports previous studies illustrating the neuroprotective effects of ARE induction [145, 183, 184, 186]. The difference in concentrations of kaempferol required to afford protection to cortical neurons, 15 μM in adenovirus-infected cultures and 20 μM in uninfected cultures, may be due to effects of adenovirus infection in cell cultures. Kaempferol's shallow dose-response curve initially makes it appear an unlikely candidate for therapy because it may be difficult to attain a therapeutic concentration *in vivo* for protection. The maximum plasma concentrations of flavonoids after eating flavonoid-rich foods are between 0.06-7.6 μM [247], well below the 20 μM kaempferol-mediated protection of cortical cell cultures. Further complicating our interpretations is the uncertainty on how

much flavonoid crosses the blood brain barrier. A study of cultured rat cortical neuronal cultures has shown that 2 h incubation of neurons with 35 μM kaempferol results in an intra-neuronal kaempferol concentration of 175 μM [248]. Therefore, it is possible to achieve high concentrations of kaempferol in neurons, if a method was employed to bypass metabolism of kaempferol when ingested orally. The non-significant increase in NQO1 activity observed with 10 μM kaempferol may be due to a confounding effect of the DMSO vehicle, which increased NQO1 activity at higher concentrations. It is possible that under our experimental conditions maximal NQO1 activity is achieved with 5 μM kaempferol, explaining the lack of a dose-response. Kaempferol-mediated protection at higher doses of 20 μM are still possible, perhaps due to the conserved nature of the ARE across genes; increased expression of multiple ARE-dependent genes is likely necessary to confer protection.

Astrocytes play a key role in antioxidant defense, with the majority of Nrf2/ARE induction occurring in these neuronal support cells [145, 178]. Our findings show that astrocytes play an important role in kaempferol-dependent protection of neuronal cultures. Glutathione is one of the most important antioxidants in the brain and astrocytes play an important role in supplying glutathione precursors to neurons [249]. Thus, ARE-dependent production of multiple proteins responsible for glutathione synthesis, use and transport [145, 250] may contribute to kaempferol-mediated protection of cortical cultures.

A goal of this study was to identify non-toxic, plant-derived compounds that would activate the ARE in astrocytes and induce transcription of protective genes. The pHTS-ARE luciferase assay developed in this research is a useful tool for identifying potential therapeutic compounds. However, many known ARE inducers, including ethoxyquin, heavy metals, phorbol-12-myristate and tBHQ, are harmful [121], possibly because they are recognized as xenobiotics by cellular systems. Our findings support the conclusion that strong ARE-inducers are not necessarily protective and can be toxic, as exemplified by tBHQ and quercetin, making toxicity screening necessary. A potent induction of the ARE by tBHQ would allow an increase in glutathione-reduction and glutathione-conjugation, due to γ-GCS being ARE-dependent and the rate-limiting enzyme in glutathione synthesis [251]. Glutathione-conjugated tBHQ has greater than 3-fold increases in redox potential [252], meaning it has a much greater ability to oxidize lipids, proteins and nucleic acids. This cycle of toxic by-products may explain tBHQ's toxicity to neuronal cultures, which are predisposed to be more susceptible to oxidative stress. In experiments with tBHQ we did not see a protective effect against H_2O_2 at 10 μM, unlike Kraft and colleagues [178]. At this concentration we observed a dose-dependent trend to increased cell death. This may be due to a difference in treatment paradigm between our studies, where Kraft *et al.* [178] used a shorter 2 h H_2O_2 treatment, catalase treatment and 5 h recovery period before assessing cell viability. It is possible that relatively long-term (24 h) H_2O_2 treatment, as in our model, is a harsher insult than Kraft *et al.*'s and from which it may be more difficult for cortical cultures to recover.

The quercetin dose-response survival curve (Figure 9B) matches previous findings [253]. Quercetin toxicity to cortical cell cultures may result from inhibition of Akt/PKB (protein kinase B) phosphorylation, while decreasing BAD phosphorylation and increasing caspase-3 activity [253]. Phosphorylation of the cell survival factor, PKB, is critical for its activation. PKB phosphorylation of the pro-apoptotic factor BAD results in inactivation and sequestration of BAD in the cytoplasm, preventing apoptosis [254].

Kaempferol, but not quercetin, has been shown to reduce amyloid-β-induced rat cortical neuron death, while both quercetin and kaempferol still significantly reduced ROS [255]. Others have also shown that quercetin reduces ROS in rat cerebellar neurons [256]. Although quercetin has been demonstrated to protect against oxidative stress, the cell type must be taken into account. For instance, 100 μM quercetin protected kidney tubule epithelial cells against H_2O_2 induced cell death [255], but this high concentration of quercetin causes extensive neuronal death. Also, quercetin failed to provide protection against 6-hydroxydopamine in a PD animal model, having no effect on lesion size [257]. Almost all studies supporting quercetin neuroprotection [255, 258-260] have been carried out in immortalized cell lines. In general, neuroblastoma immortalized cells are less susceptible to death [261] at concentrations we found toxic to primary neurons.

Kaempferol and quercetin differ in structure by only one hydroxyl group, yet kaempferol provided significant protection to cortical cell cultures at concentrations at which quercetin was toxic. Surprisingly, this may be related to quercetin's antioxidant activity, which is more than double kaempferol's. The extra hydroxyl group on quercetin's B ring, which accounts for its elevated antioxidant activity compared to Q3G and kaempferol, may also be responsible for the toxicity and lack of protection offered by quercetin. The number of hydroxyl groups on the B ring is proportional to a flavonoid's prooxidant activity [242]. Furthermore, there is evidence that after exerting its antioxidant effect, the resultant oxidized quercetin can arylate thiols in the absence of GSH [262]. As quercetin has the highest antioxidant activity of these flavonoids, at high concentrations it may produce an excess of oxidized quercetin in cells, utilizing much of the cellular GSH for conjugation. In addition to draining the cells of essential antioxidant GSH, the remaining unconjugated oxidized-quercetin would be free to arylate the thiols of important proteins and cause toxicity. There is also *in vivo* evidence to support quercetin toxicity; 0.1% quercetin diet supplementation in mice significantly reduced their life span [263].

Previously, quercetin-4-glucoside has been shown to activate the ARE in a murine hepatoma cell line [264], but no evidence existed on quercetin-3-glucoside's capabilities for ARE induction or neuroprotection. Q3G provided protection to cortical neurons from oxidative stress induced by hydrogen peroxide, leaving the mechanism for this protection in question. Q3G increased

induction of ARE-luciferase and NQO1 enzyme activity. However, Q3G-mediated protection to cortical cell cultures was only significantly inhibited at 20 μM (Figure 14C) after the Nrf2/ARE pathway was repressed by dominant negative-Nrf2. With this and the non-significant trend in decreased cell survival at concentrations lower than 20 μM, compared to the Ad-EGFP control, we infer that a portion of Q3G-mediated protection is Nrf2/ARE-dependent.

While we have shown that quercetin and Q3G activate the ARE and similar properties have been demonstrated in other quercetin-glycosides [192], this appears insufficient to account for Q3G's neuroprotective properties. It is possible that the glucoside moiety in the C3 position of quercetin plays a role in Q3G-mediated protection, because quercetin lacks this structural element and is very toxic at concentrations that are not toxic with Q3G. A study of flavonoid uptake in rat primary cultured cortical neurons revealed that quercetin aglycone was not detected in neurons after 8h incubation. However, the concentration of quercetin in the medium had decreased and another compound found in the cells led researchers to infer that quercetin is taken up by neurons and quickly metabolized to a different form [248]. It is not known how Q3G uptake differs from quercetin.

While we have demonstrated, by inhibiting ARE activation, that kaempferol and Q3G activities are partially dependent on the Nrf2-ARE pathway, we have not specifically shown that these flavonoids induce phase II and antioxidant enzymes

beyond NQO1. Both of these flavonoids increase ARE-luciferase activity;. however, this does not provide direct evidence of resulting functional proteins. Lee *et al.* have shown that Nrf2 -/- astrocytes have lower mRNA levels of many antioxidant and phase II enzymes [147]. Thus we may infer that inhibiting Nrf2 with dominant-negative adenovirus would also lower mRNA levels of these enzymes. It has been suggested that the dominant-negative Nrf2 adenovirus competitively inhibits ARE activation by binding to heterodimerization partners or the ARE itself [178]. Further investigations to confirm the ability of these flavonoids to induce antioxidant and phase II enzymes would involve determining protein levels and activity of these enzymes pre- and post- flavonoid incubation.

The *in vitro* results of cortical culture protection by flavonoids are preliminary and are limited to looking at one end point (cell death). The primary cortical cultures contained both neurons and astrocytes, and the MTT assay does not distinguish between survival of cell types. Thus, although Q3G and kaempferol may provide protection to neurons, we have not specifically shown this. Future experiments will determine the extent of protection these flavonoids provide to neurons. Experiments will be performed on glass coverslips, followed by fixation and staining for the neuronal marker MAP2. Scripts written for the imaging program Image-J will enable measurement of MAP2 staining as an indicator of neuronal viability.

Further exploring the effects of the flavonoids on protecting neuronal functions, through a neurodegenerative diseased mouse feeding trial, may reveal other benefits in addition to antioxidant protection. Recently, the flavonoid fisetin was found to increase long-term potentiation and memory in a mouse model [265]. Both of these neuronal functions are negatively affected in animal models of neurodegenerative diseases [266, 267].

Other considerations in selecting compounds for therapeutic development include flavonoid bioavailability and the antioxidant properties of metabolized flavonoids. Quercetin glucosides are more readily absorbed in the small intestine after oral administration in the rat when compared to quercetin; however, Q3G is not detectable in rat plasma, possibly due to conjugation or hydrolysis [268, 269]. In a study of ileostomy patients, the addition of a glucose moiety to quercetin increased its bioavailability by 28% [270]. These pharmacokinetic factors are important in determining the value of a bioactive compound, in addition to its protective properties. Further exploration in methods of transporting unmetabolized flavonoids to the brain, where they may reduce oxidative stress and neuronal death, would be beneficial. Possibilities include direct injection of flavonoids into the brain and modification of the flavonoid structure to decrease their metabolism and conjugation when taken orally.

In summary, both Q3G and kaempferol possess antioxidant and protective capabilities for cortical cell cultures. Kaempferol requires the Nrf2/ARE pathway

to confer significant protection to cortical cell cultures. Q3G protection is non-significantly decreased at lower concentrations (5-15 μM) when the Nrf2-ARE is blocked, but is significantly decreased at 20 μM. These two compounds are closely enough related in chemical structure to be classified as flavonols, but the slight differences in structure, mainly an extra hydroxyl group and a glucoside, may account for Q3G-mediated protection in the presence of dominant-negative Nrf2.

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