SULFORAPHANE AS A POTENTIAL NUTRITIONAL INTERVENTION TO REDUCE NEUROINFLAMMATION ASSOCIATED WITH AGING AND SICKNESS BEHAVIOR

BY

BRIGITTE E. TOWNSEND

DISSERTATION

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Doctoral Committee:

Professor Emerita Elizabeth H. Jeffery, Chair
Professor Rodney W. Johnson, Director of Research
Professor Emeritus John W. Erdman
Assistant Professor Ryan N. Dilger

Abstract

Innate immune cells provide critical protection against pathogens and produce immune factors that drive adaptive sickness behaviors to facilitate recovery from acute illness and injury. Microglia, the resident innate immune cells in brain, recognize pathogens and danger associated molecular patterns, then express proinflammatory signaling molecules such as interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase (iNOS). Aging is associated with chronic neuroinflammation that is represented by behavioral and cognitive impairment, and increased risk of neurodegenerative disease. Aging-associated inflammation is thought to shift microglia into a primed state that contributes to increased neuroinflammation and oxidative stress in the aging brain.

Dietary broccoli and its derived bioactive sulforaphane (SFN) has potential use as a novel nutritional intervention to reduce aging-related neuroinflammation. Sulforaphane activates nuclear factor E2-related factor 2 (Nrf2), which induces antioxidant response element genes that regulate cellular redox balance. In animal models of neurodegeneration and brain trauma, activation of the Nrf2 pathway is protective, but its function in the aging brain has not been investigated.

Microglial activity is reportedly mitigated by SFN, forming the basis for our hypothesis that SFN would attenuate elevated inflammatory markers in unstimulated aged microglia and in microglia treated with lipopolysaccharide (LPS). To test this hypothesis, microglia from adult and aged mice were isolated and treated in vitro with SFN ± LPS in order to investigate the effects of SFN on aged and LPS-stimulated microglia. In support of the hypothesis, SFN decreased IL-1β, IL-6, and iNOS in LPS-simulated microglia from adult and aged mice, and

tended to decrease basal IL-1β in aged microglia. Sulforaphane increased Nrf2 target genes were increased in both adult and aged microglia, and increased Nrf2 activity in the BV2 microglia cell line.

Based on SFN's ability to reduce inflammatory markers in microglia, we hypothesized that acute exposure to SFN may attenuate neuroinflammation and sickness behavior in LPS-challenged mice. Mice were administered SFN for 3 d then challenged with LPS to mimic peripheral infection. Nrf2 target genes and inflammatory mediators in brain and liver mRNA were quantified after 6 h. In hippocampus and liver of mice that were treated with LPS, SFN decreased IL-1β and iNOS, but did not improve sickness behavior. This led us to hypothesize that longer exposure to SFN, such as could be attained through dietary supplement with broccoli, may have a more pronounced impact on sickness behavior. To test this hypothesis, adult and aged mice were fed control diet or diet containing 10% broccoli. After 28 d, mice were injected with saline or LPS, and sacrificed 24 h later. Broccoli diet improved glial reactivity markers in aged mice. However, broccoli diet did not reduce IL-1β in brain or liver of aged or LPS-challenged mice, and had no effect on sickness behavior.

Collectively these data provide novel evidence that SFN supplementation and dietary broccoli may be a beneficial nutritional intervention to reduce inflammation that is associated with aging and with peripheral infection.

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Chapter 1 - General Introduction and Justification

During the past century, the United States population of adults over age 65 years has grown more rapidly than other age groups, and this trend is projected to continue. Between 2014 and 2060, the population 65 years and older is expected to double, expanding from 46 million to 98 million [1]. Additionally, the oldest-old population, representing those 85 years and older, is expected to increase substantially as the baby boomer generation ages [2]. Although the increase in elderly population represents significant health improvements and medical advances that have contributed to extended longevity, a longer lifespan presents additional healthcare challenges. Chronic diseases, frailty, and cognitive decline are encountered more frequently in advanced years, with an estimated 80% of elderly adults having at least one chronic health condition [2]. Still, heterogeneity in the effects of aging on individuals suggests that complex factors underlie the extent to which aging impacts physiological and cognitive function. The majority of the older population exhibits either mild decline in cognition and functional abilities or pathological aging that is exacerbated by chronic disease such as Alzheimer's that may severely impact daily living. In searching for a mechanistic link between aging and decline in cognition and behavioral function, epidemiological studies indicate a correlation between inflammation and chronic diseases that are associated with aging, including neurodegeneration [3,4]. This is particularly informative, because studies using aged animal models indicate that aging is associated with heightened basal and inducible expression of proinflammatory cytokines which are implicated in cognitive impairment, prolonged sickness response, and depressive disorders [5-9]. Furthermore, advances in neuroimmunology reveal that bidirectional inflammatory signaling occurs between the brain and periphery, impacting

cognition and behavior, but that these signaling pathways may become dysregulated during aging [10-12].

As the percent of the older population grows, the attention of aging research is increasingly drawn to interventions that will mitigate chronic health problems. While the determinants of healthy aging are undoubtedly multifaceted, nutrition is one component that has received much research interest as a factor that can be modulated to improve aging outcomes. Nutritional support for long-term brain health is a significant component of aging research, due to the growing body of evidence suggesting that dietary supplementation with antioxidant-rich fruit or vegetable extracts reverses aging-related cognitive decline [13]. Sulforaphane, a broccoli bioactive, has garnered interest as a potent stimulator of endogenous antioxidant pathways, and recent evidence suggests that it conveys anti-inflammatory properties as well. The overall objective of this dissertation was to determine the anti-inflammatory potential of supplemental sulforaphane and dietary broccoli in the context of aging and microglial-mediated neuroinflammation.

1.1 References

[1] Colby SL, Ortman JM. Projections of the size and composition of the U.S. population: 2014 to 2060. In: Commerce USDo, editor. Washington, DC: United States Census Bureau; 2014. p. 25-1143.

[2] He W, Sengupta M, Velkoff VA, DeBarros KA. 65+ in the United States: 2005. In: U.S. Census Bureau CPR, editor. Washington, D.C.: U.S. Government Printing Office; 2005. p. 23-209.

[3] Teunissen CE, van Boxtel MPJ, Bosma H, Bosmans E, Delanghe J, De Bruijn C, et al. Inflammation markers in relation to cognition in a healthy aging population. J Neuroimmunol. 2003;134:142-50.

[4] Dziedzic T. Systemic Inflammatory Markers and Risk of Dementia. Am J Alzheimers Dis. 2006;21:258-62.

[5] Buchanan JB, Sparkman NL, Chen J, Johnson RW. Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice. Psychoneuroendocrino. 2008;33:755-65.

[6] Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behav Immun. 2008;22:301-11.

[7] Godbout JP, Moreau M, Lestage J, Chen J, Sparkman NL, O'Connor J, et al. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. Neuropsychopharmacology. 2008;33:2341-51.

[8] Lee CK, Weindruch R, Prolla TA. Gene-expression profile of the ageing brain in mice. Nat Genet. 2000;25:294-7.

[9] Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia. 2007;55:412-24.

[10] Dantzer R, BluthÉ R-M, LayÉ S, Bret-Dibat J-L, Parnet P, Kelley KW. Cytokines and Sickness Behavior. Ann N Y Acad Sci. 1998;840:586-90.

[11] Dilger RN, Johnson RW. Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. J Leukocyte Biol. 2008;84:932-9.

[12] Joseph JA, Shukitt-Hale B, Casadesus G. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. Am J Clin Nutr. 2005;81:313S-6S.

[13] Lau F, Shukitt-Hale B, Joseph J. Nutritional Intervention in Brain Aging. In: Harris R, Bittman R, Dasgupta D, Engelhardt H, Flohe L, Herrmann H, et al., editors. Inflammation in the Pathogenesis of Chronic Diseases: Springer Netherlands; 2007. p. 299-318.

Chapter 2 - Review of the Literature

2.1 Innate Immunity and the Aging Brain

Inflammation is a physiological response to stimuli that are damaging to a host. The stimulus may be a bacterial or viral pathogen, oxidative stress, cellular damage, exposure to environmental toxins, or other pathogens and danger-associated signals [1-3]. The body responds to acute and chronic immune challenges through a carefully controlled system of immune cells and signaling pathways that allow the host to eliminate the inflammatory stimulus. Acute response to inflammatory stimuli is mediated through the innate immune system. The innate immune system is activated when endogenous or pathogenic ligands bind to pattern recognition receptors that recognize certain molecular patterns, but lack the elaborate specificity of the adaptive immune system. Receptor-mediated signaling cascades alert innate immune cells to eliminate pathogens by phagocytosis or initiate an inflammatory response through increased transcription of proinflammatory cytokines and release of reactive oxygen and nitrogen species [4].

Neuroinflammation, which describes inflammatory processes that occur in the cells of the central nervous system (CNS), is a relatively modern term that was not widely referenced until the later 1990s. The distinction between inflammation and neuroinflammation is derived from the fact that immune mechanisms are strictly regulated in the brain, and to a great extent, set apart from the peripheral immune system by the blood brain barrier and lack of a central lymphatic system [5]. Neuroinflammation is regulated through interactions among neurons and glial cells, and initially was studied only in medical conditions that directly affected the CNS. However, the discovery that proinflammatory cytokine expression increased in the brain during

peripheral infection gradually expanded the understanding of neuroinflammation to comprise a bi-directional signaling network between the nervous system and the peripheral immune system. Several key pathways have been identified through which peripheral-to-brain communication occurs, namely the humoral and neural signaling pathways [6]. Because cytokines are generally too large to diffuse across the blood brain barrier, a humoral signaling pathway for circulating cytokines was considered plausible only at the circumventricular regions which lack a blood brain barrier, or through specialized cytokine transporters. Another hypothesis suggested that circulating cytokines outside the blood brain barrier induced synthesis of small molecule intermediates, such as prostaglandins, which can diffuse readily through the blood brain barrier and act as secondary messengers in the brain [7,8]. Although more recent evidence suggests that minimal cytokine leakage occurs through circumventricular organs, cytokine receptors and nerves at these sites facilitate the relay of peripheral immune signals to the brain [9]. Transporters for the proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNFα) have also been identified in epithelial cells of the blood brain barrier, allowing tightly regulated signaling to occur through this barrier. Cytokines generated in the periphery also relay signals to the brain through the vagal nerve pathway that provides extensive neural communication from the gastrointestinal tract, liver, heart, lungs, and other organs [6,10]. Identification of IL-1 and prostaglandin receptors on vagal nerve endings supported the early hypothesis that peripheral cytokine signals could rapidly be transmitted to the brain through a neural pathway [11,12].

Inflammatory status in both the brain and periphery is an important indicator of health and longevity. Evidence from clinical and pre-clinical research consistently points to age-related

alteration of inflammatory mechanisms that can result in chronic low-grade inflammation and onset of aging-related diseases [13-16]. Vulnerability to infection increases with age, and infection in elderly populations is often accompanied by mild cognitive impairment which leads to difficulty in self-care and prolonged recovery time [16-18]. Advanced age is also the primary risk factor for dementia and neurodegenerative disease, implying that the brain is highly sensitive to immunological changes that occur with aging [19,20]. Increased oxidative damage to proteins and DNA has been identified in brains of healthy aged patients as well as patients with Alzheimer's disease, indicating that molecular changes are not restricted to presence of neurodegenerative pathology. [21-24].

Experimental studies in lipopolysaccharide (LPS)-stimulated aged mice have demonstrated that both prolonged sickness behavior and cognitive impairment occurs in conjunction with increased proinflammatory markers in the brain, particularly in microglia, suggesting that this cell type may be an important mediator of aging-related neuroinflammation [25-28]. Changes in microglial morphology and function in the aging human brain further indicate that microglia may play an important role in brain aging [29].

2.2 Microglia and Neuroinflammation

Microglial involvement in brain pathology was mentioned as early as the 19th century when psychiatrist Franz Nissl described glial "stäbchenzellen" or rod-shaped cells that were motile, phagocytic, and present in neurodegenerative pathology [30]. Nearly twenty years after Nissl's discovery, Pio del Rio-Hortega distinguished microglia from oligodendrocytes, neurons, and astrocytes, and further characterized their activation states [30]. Microglia are now often

referred to as the resident innate immune cells of the brain because of their rapid, multifaceted response to disruptions in homeostasis [31]. In a non-activated state, microglia are ramified cells with thin protruding processes that facilitate surveillance of their surroundings. In contrast, activated microglia adopt an amoeboid morphology. In addition to morphological variation, microglia display an amazing degree of functional plasticity. Older literature referred to quiescent, or resting microglia, that, when stimulated, transitioned into neurotoxic, activated microglia. However, the term “resting” is somewhat deceptive, because ramified microglia exhibit dynamic restructuring of their processes, providing a constant state of surveillance. It is thought that the microglial network is able to perform complete scans of the brain every several hours [32,33]. Like their peripheral counterpart, the macrophage, microglia are currently classified according to whether their activation state is polarized towards a proinflammatory (M1) or anti-inflammatory (M2) phenotype. Classical activation, which indicates the M1 phenotype, is stimulated *in vitro* by LPS or interferon-γ and is characterized by expression of proinflammatory cytokines such as IL-1β, IL-6, and TNFα [34]. Animal models of traumatic brain injury and stroke also initiate M1 activation [35-37]. Although M1 activation is a critical mechanism to protect neurons from invading pathogens, prolonged M1 activation is neurotoxic. Therefore, to resolve acute neuroinflammation, healthy microglia transition from the M1 phenotype to an M2, or alternative activation state. The M2 phenotype is associated with anti-inflammatory cytokines IL-13 and IL-4 and secretion of growth factors that support neuronal and tissue repair. The phenotypic reprogramming that occurs through M1 and M2 polarization diversifies microglial response, equipping them for distinct functions [38,39].

Pattern recognition receptors and antigen presenting molecules facilitate the critical surveillance function of microglia. Like other peripheral immune cells, microglia express membrane-spanning toll-like receptors (TLRs). TLRs bind ligands that correspond to pathogen-associated molecular patterns and signal microglia to stimulate an innate immune response. A variety of TLRs have been identified, with each TLR corresponding to different category of ligand pattern recognition. For example, TLR4 recognizes gram-negative bacterial LPS, leading to the popular use of LPS as a microglial immune stimulus. Activated TLR4 initiates a signaling cascade that results in proinflammatory signaling involving activation of TNFα and the proinflammatory transcription factor, NFκB [40]. In addition to TLRs, complement type-3 receptor (CR3; CD11b/CD18) is a constitutively expressed pattern recognition receptor that comprises a subunit of the larger macrophage antigen complex-1 (MAC1). Complement type-3 receptor is essential to initiation of phagocytosis that eliminates pathogens during acute inflammation. Additionally, the CD11b subunit is commonly used as an identifying microglia marker [41,42]. Another surface protein, major histocompatibility complex class II (MHC-II), is a membrane-bound, antigen-presenting protein that is expressed on microglia [43,44]. Expression of MHC-II and CD11b increase in response to immune stimuli and, interestingly, there is some evidence that both proteins are more highly expressed in aged animals compared to adults [45,46].

Activated microglia are involved in nearly all described pathologies of the CNS, indicating that this cell type is highly sensitive to disruptions in homeostasis [47]. Some research suggests that microglial dysregulation occurs during aging, in which the microglia are resistant to the M2

phenotype. Therefore, microglia are often studied in context of aging, with particular attention given to mechanisms that regulate the M1 and M2 phenotypes.

Aging leads to M1 priming in which mildly activated microglia are chronically sensitized to inflammation and produce an exaggerated response when challenged by a second immune stimulus [48-50]. Several studies have demonstrated increased basal levels of cytokines in microglia from aged mice, suggesting that primed microglia contribute to aging-related neuroinflammation [50,51]. In addition to the inflammatory milieu, aberrant microglial activation in aged mice is tied to impairment of social behavior and cognition [25,47,50,52-54]. Pharmacological and nutritional therapies that reduce microglial activation are therefore highly applicative to aging research [54,55].

2.3 Cytokine-mediated Sickness Behavior

Malaise, lethargy, fever, and anhedonia are classic sickness symptoms. Collectively, these symptoms represent a behavioral component of the acute phase response that immediately follows an immune challenge. Sickness behaviors that accompany common human illnesses, including colds and influenza, are associated with increased healthcare costs, absenteeism, and reduced ability to function normally in a professional or academic environment. Cognitive impairment and reduced psychomotor skills during sickness in older patients is accentuated compared to younger adults [56]. In older adults, depression and medical comorbidity is not uncommon, and may be reflective of a maladaptive sickness response to elevated cytokines [57].

The biological basis for sickness behavior was not identified until after the discovery of cytokine IL-1, when researchers noticed similarities between the side effects of systemic IL-1β administration and symptoms of peripheral infection [58]. This recognition was influential in understanding cytokine-mediated interconnections between behavioral patterns and immunology [59]. Once it became apparent that adaptive sickness behaviors were initiated by cytokines, researchers were better able to target cytokine signaling pathways using inhibitory modulators (i.e. cytokine receptor antagonists) to mitigate sickness responses [60,61].

Cytokines comprise a diverse class of pro- and anti-inflammatory signaling peptides that are secreted by immune cells [62]. Although a number of cytokines contribute to the innate immune response, IL-1β has perhaps been the most widely studied for its role in mediating sickness behavior and cognitive impairment during an inflammatory response. IL-1β receptors are expressed in the brain, with regional variations indicating higher receptor density in areas such as the hippocampus [63]. Experimental models and clinical observations provide evidence of increased IL-1β transcripts in the brain following endotoxin exposure, ischemia, or brain injury, along with sickness behaviors including reduced social exploration and food intake [64,65]. Central administration of IL-1 receptor antagonist attenuates sickness behavior and reduces elevated IL-1β mRNA in the hippocampus of aged mice following a peripheral injection of LPS, confirming that peripheral immune stimulation is communicated to the brain, and indicating that central IL-1β is an important mediator of sickness behavior [66].

The use of LPS injection as a model of peripheral infection has been an important component of studies involving cytokine-induced sickness behavior. In the early 1990s, the first paper was published reporting evidence of IL-1 staining in microglia following peripherally

administered LPS [58]. Since then, it has become common to use LPS to stimulate inflammation in both *in vitro* and *in vivo* models. Stereotypical sickness behaviors in response to subseptic doses of LPS have been defined in mice models to provide measurable parameters of sickness response and recovery. These include anorexia (reductions in food intake and loss of body weight), lethargy (decreased spontaneous locomotor activity), and anhedonia (reduced sucrose preference; decreased social investigation) [67]. As might be expected, animals recover from sickness behavior more rapidly after a low dose of LPS than after a high dose [68]. Additionally, recovery from LPS-induced sickness response is typically longer in aged mice than young adult mice. It is thought that age-related changes in the CNS, including increased microglial activity and exaggerated proinflammatory cytokine response, contribute to prolonged cytokine signaling and delayed recovery [27,49,69].

Nutritional bioactives have been studied for their potential ability to inhibit sickness response or hasten recovery time in aged mice. The dietary antioxidant α-tocopherol, administered by intraperitoneal injection or incorporated into diet, did not affect peak sickness in mice challenged with low-dose LPS, but reduces recovery time [68]. Further examination revealed that α-tocopherol alleviated sickness behavior and was associated with reduced proinflammatory cytokine expression in the brain [70]. Quercetin, another dietary antioxidant that also upregulates antioxidant enzymes, also reduced the LPS-induced sickness response by attenuating proinflammatory cytokines and reactive oxygen species, increasing evidence in support of a link between antioxidant and anti-inflammatory mechanisms [71].

2.4 Oxidative Stress and the Nrf2 Pathway

Oxygen has a long-standing association with aging due to its involvement in metabolic rate and its propensity to form highly reactive free radicals. In the 1920s, Raymond Pearl formulated the rate of living hypothesis, suggesting that high metabolic output decreases longevity. For instance, a rodent with seven times the metabolic rate of a human will age very quickly, comparatively. A quarter of a century later, Denham Harman introduced the free radical theory of aging, proposing that aging is a result of free radical reactions that are damaging to tissues. Although these theories have since been challenged and somewhat reformulated, oxidative stress is still a focal consideration that underlies aging-related deterioration of physiological and biochemical mechanisms [72-75]. Neurodegenerative disease, atherosclerosis, cancer, and a wide variety of other diseases typically associated with aging are linked to an imbalance in antioxidant status and ROS production [76,77]. In fact, as mice age there is a nearly linear increase in oxidative stress and associated cognitive deficits. Several *in vivo* studies have used various antioxidant treatments to investigate whether increased oxidative stress is an inevitable, damaging component of aging or a preventable symptom of aging. Based on the results from these studies, it seems that antioxidants that are chronically administered during the aging process prevent oxidative damage and impaired learning and memory. Others have demonstrated that subchronic administration of antioxidants partially reverses cognitive deficits in aged animals. However, once antioxidant exposure is withdrawn, oxidative stress and cognitive deficits return, suggesting that a chronic intervention is necessary to maintain aging brain health [78-83].

Free radicals, reactive oxygen species, and reactive nitrogen species are produced intrinsically by mitochondria and in phagocytic cells through complex systems such as NADPH oxidase and nitric oxide synthase [84,85]. Strategies to reduce oxidative stress using antioxidant supplements, caloric restriction, and other dietary methods are widely promoted as anti-aging therapy. The American public continues to funnel money into antioxidant supplements despite little evidence indicating that they improve longevity [86,87]. As an alternative to consuming antioxidant supplements to counteract age-related oxidative stress, it may be more effective to utilize pharmaceutical supplements or nutritional tools that activate the body's endogenous antioxidant pathways.

Transcriptional regulation of endogenous antioxidant enzymes through nuclear factor E2-related factor 2 (Nrf2) is at the forefront of oxidative defense mechanisms and is a potential target for pharmacological and nutritional therapeutics. Nrf2 was isolated in 1994, some years after Talalay proposed the existence of a specific enzyme-inducing mechanism that regulated cytoprotective enzymes [88,89]. The newly identified protein was characterized as a transcription factor that forms heterodimers with small Maf proteins and binds to the antioxidant response element (ARE), a regulatory region that was known to promote expression of enzymes that neutralize reactive species or conjugate harmful metabolites to soluble compounds that are rapidly excreted [90]. Prior to the discovery of Nrf2, a diverse group of electrophilic chemical agents had been identified as ARE-inducing compounds, but it was unknown whether these compounds upregulated ARE genes through one dominant pathway or separate pathways [91,92]. Further exploration of the Nrf2 pathway revealed Nrf2-dependent increases in ARE gene expression, providing a unifying mechanism underlying inducible

expression of these genes. The laboratory of Yamamoto was one of the first to use homozygous Nrf2-deficient mice to further characterize Nrf2-dependent cellular protection. Importantly, their research demonstrated that dietary administration of the phenolic antioxidant butylated hydroxyanisole (BHA) resulted in Nrf2-mediated induction of the ARE-containing genes glutathione-S-transferase (GST) and NAD(P)H quinone oxidoreductase (NQO1) [93]. In support of this evidence, McMahon et al. demonstrated that Nrf2 was necessary for gastrointestinal upregulation of GST and NQO1 by a number of dietary compounds including BHA and the phytochemical sulforaphane. The knowledge that dietary compounds could induce ARE genes through Nrf2 provided a novel area for targeted nutritional intervention [94].

Research has gradually uncovered various physiological and pathological states that are affected by Nrf2. Because the ARE promoter sequence is contained in many genes that are involved in prevention of chemical carcinogenesis, initial interest in Nrf2-mediated activation of the ARE genes was driven by the desire to induce anti-carcinogenic enzyme activity [93]. The ARE genes also include endogenous antioxidant enzymes, and many subsequent studies of Nrf2 have focused on areas that are exposed to highly oxidative conditions. In the lung, for example, Nrf2 is a critical factor in resistance to damage induced by environmental toxins and cigarette smoke [95]. In the liver and gastrointestinal tract, Nrf2 protects against oxidative stress, xenobiotics, and carcinogens [96,97]. Over two hundred genes are regulated by Nrf2 in the liver alone, and its ubiquitous expression makes it a versatile therapeutic target for cancer and pathologies that are linked to oxidative stress [98,99].

In 2006, Thimmulappa and colleagues published research that formed the framework for yet another significant aspect of Nrf2 function. This work presented evidence of Nrf2-blunted

peripheral inflammation following an acute LPS dose, demonstrating that Nrf2 modulates the innate immune response. In an experimental model of sepsis, innate immune system factors including signaling regulators, cytokines, and acute phase proteins, were elevated in Nrf2 knockout mice compared to wild-type controls. Wild-type mice were also more resistant to high doses of LPS than the Nrf2 knockout mice [100].

Several Nrf2 target genes have been extensively studied in the context of their cytoprotective properties. The flavoprotein NQO1 is a commonly used enzymatic biomarker for screening antioxidant, anti-carcinogenic compounds, and was a valuable tool in the initial characterization of Nrf2's ARE-inducing function [101]. Ubiquitously expressed NQO1 serves as a defense mechanism against redox-reactive xenobiotics and endogenous reactive species that are generated by the electron transport chain. Two-electron reduction of quinones and reactive quinone metabolites is catalyzed by NQO1, effectively removing their toxicity. Additionally, NQO1 scavenges superoxide radicals and maintains tumor suppressor proteins [84,85,102]. Elevated NQO1 has been observed in hippocampi from Alzheimer's disease subjects, indicating that its expression may be involved in a compensatory mechanism against the increased oxidative stress that is present in neurodegenerative pathology [103].

Heme oxygenase 1 (HMOX1) is the rate-limiting enzyme in the degradation of heme, and is upregulated by transcription factors including Nrf2 [104]. Heme is a strong oxidant, and this reaction effectively neutralizes heme's reactive potential and releases metabolites with strong antioxidant activity. Through the break-down of heme, HMOX1 contributes to normal cellular maintenance. Under oxidative stress conditions, HMOX1 is upregulated, enabling the cell to neutralize additional oxidants [105]. The critical detoxification role this enzyme is especially

apparent when HMOX1 is deficient. Oxidative stress elevates HMOX1 and results in a mild free radical increase in primary fibroblasts. In contrast, primary cells isolated from HMOX1 knockout mice are less resistant to oxidative stress. Mice deficient in HMOX1 also display markers of chronic inflammation [106]. There is some evidence that HMOX1 increases with age in liver which may represent a compensatory mechanism for increased oxidative stress [107,108].

2.5 Nrf2 Pathway and Neuroinflammation

Nearly a decade after Nrf2 was identified, its potential role in brain health emerged as a new research interest, stimulated by the clear relationship between increased oxidative stress and neurodegenerative pathology. Initial investigation using neuroblastoma cell lines and CNS-derived primary cells detected a protective role for Nrf2 against oxidative stress and neurotoxins [109,110]. Basal expression of NQO1 is more reduced in brain and liver of Nrf2 knockout mice than in wild-type controls, suggesting that Nrf2 target genes are involved in antioxidant response in central as well as peripheral tissues. Loss of Nrf2 also exaggerates neuroinflammation and is accompanied by reduced neurological function in animal models of traumatic brain injury and neurotoxicity. Additionally, Nrf2 seems to be essential to basal and inducible expression of ARE genes in the brain [111-113]. Interestingly, the Nrf2-activator sulforaphane (SFN) mitigates damage from traumatic brain injury, presumably by upregulation of ARE genes and reduction of injury-associated oxidative stress [114-116].

Deficiency in Nrf2 increases glial reactivity in a mouse model of experimental Parkinson's disease. In particular, microglia cultured from these mice displayed elevated activation markers and loss of inducible ARE gene expression [117]. Clinical research has gathered some evidence

of low Nrf2 protein levels in patients with neurodegenerative disorders, particularly in brain areas associated with onset of disease symptoms, suggesting the interesting possibility that a dysfunctional Nrf2 system is involved in neurodegenerative pathology [118,119]. Despite these advancements in understanding the functions of the Nrf2 pathway in the brain, little is known about Nrf2 involvement in age-related neuroinflammation.

2.6 Nutrition and Neuroinflammation

Nutrition is a key predictor of overall health and a critical factor in disease prevention [120]. Nutritional research involves development of mechanistic and translational information about dietary components that can be used to equip an individual with the best possible biochemical tools to avoid illness or recover from sickness and injury. Nutrition is necessary for sustaining brain health, from providing macronutrients that maintain brain structure and supplying glucose that supports brain metabolism, to micronutrients such as the B vitamins that are critical to neurotransmission. Increased availability of literature describing integrated nutrition and brain research implies an expanding interest in nutritional links to cognition and behavior. For example, it has long been recognized that dietary intake of caffeine and other psychostimulants have a powerful effect on alertness and cognition, but for a long time, this view was a common sense realization rather than a mechanistic understanding [121]. More recently, researchers have increased their efforts to elucidate biochemical pathways related to nutrition and long-term brain health, while others tested outcomes of dietary patterns and bioactive supplementation on brain function across the life stages. One significant development that has arisen is the realization that nutrition affects the interrelationship between the

immune system and the nervous system. Nutrition is now viewed as a tool to manipulate anti- and proinflammatory pathways that impact the brain. Diets rich in omega-3 fatty acids and phytonutrients from vegetables and fruits supply antioxidants that reduce oxidative stress and inflammation, while diets high in processed foods promote inflammation. Epidemiological studies indicate that healthier diet styles are associated with reduction of depression, lowered risk of neurodegenerative diseases, and improvement in cognitive function [122,123].

Nutrition impacts long-term brain health, and it is well-documented that incorporation of essential nutrients and bioactives into a dietary routine influences physiological and psychological parameters that benefit cognition and behavior [124]. Recent studies indicate that resveratrol, a phytochemical found in red wine, decreases the effects of LPS-induced sickness behavior and increases learning and memory in aged mice [25]. Luteolin, a flavonoid obtained from celery, reduces proinflammatory cytokine production in reactive microglia, which may support healthy brain aging [54]. Phytochemicals from berries also convey neuroprotective support to aged rodent models, by increasing antioxidant activity and reducing inflammation [125]. Collectively, these studies point to a beneficial aspect of nutritional bioactives in mitigating the adverse aging-related effects of neuroinflammation.

2.7 Clinical and Nutritional Relevance of Sulforaphane

Cruciferous vegetables are remarkably suited for health-promoting effects due to their nutrient and bioactive profile, and frequent intake is correlated with reduced risk of cancer. Broccoli, cabbage, kale, Brussels sprouts, and other crucifers store glucosinolates, parent compounds to isothiocyanates which inhibit carcinogen formation and activate enzymes that

facilitate detoxification [55,126,127]. Bioactive SFN is derived from its glucosinolate parent compound, glucoraphanin. Broccoli and broccoli sprouts are especially rich producers of glucoraphanin, which is hydrolyzed to SFN by the enzyme myrosinase. Myrosinase is produced by the plant, compartmentalized separately from glucoraphanin, which prevents SFN formation until cell walls are broken down by crushing, milling, or chewing broccoli. Colonic bacteria also produce enzymes with thioglucosidase activity that can hydrolyze glucoraphanin to SFN [126,128,129].

Analysis of glucosinolate concentrations in broccoli reveals large varietal differences. Storage and processing conditions also affect glucosinolate stability and myrosinase activity, making these significant factors in the amount of SFN that can be hydrolyzed from broccoli. Commercially-produced frozen broccoli lacks active myrosinase and converts little to none of the glucoraphanin to SFN. Because myrosinase is heat-sensitive, processing methods that include blanching, microwaving, or steaming > 60°C renders SFN unavailable. In contrast, dehydration or freeze-drying methods prevent heat-inactivation of myrosinase [130-132]. Appropriate cooking or processing methods for dietary and supplemental broccoli are important in order to prevent loss of SFN bioavailability. Raw broccoli has higher SFN bioavailability and peak plasma concentrations are reached in less time compared to microwaved broccoli [133,134]. Pharmacokinetic studies demonstrate that purified SFN, administered intravenously or orally, is very well absorbed at dietary doses, with peak plasma concentrations reached after an hour and complete clearance within 24 h [135]. SFN is also widely distributed, with metabolites detected in brain and peripheral tissues several hours after oral gavage [136].

The well-characterized ability of SFN to reduce oxidative tissue damage encompasses the liver, pancreas, lung and airway, heart, skeletal muscle, retina, and brain. In addition, SFN has been studied extensively *in vitro* for its ability to suppress tumor development signals through inhibition of carcinogen-promoting Phase I enzymes and regulation of genes controlling differentiation and cell division [137,138]. *In vivo*, SFN increases transcription and activity of Phase II detoxification enzymes in colon, liver and mammary gland [132,139]. While purified, chemically-synthesized SFN is commonly used in cell culture treatments and for non-dietary routes of administration in animal models, several research groups have investigated health-promoting benefits of broccoli or broccoli sprout-supplemented diet. Dietary broccoli conveys protection against environmental toxins, carcinogens, and oxidative damage that could result in cancer, hypertension, and atherosclerosis [140-142]. Increasing evidence suggests that diets high in SFN increase antioxidant enzyme activity and cytoprotective pathways in colon, prostate, kidney, and bladder tissues. Several mechanistic studies provide evidence that changes incurred are dependent on Nrf2 activation, as SFN loses its chemopreventive, antioxidant-inducing effect in small intestine, stomach, and liver of Nrf2 knockout mice [99,143].

Elevation of Nrf2 target genes and reduced oxidative stress in the brain have also been reported following SFN treatment, in some cases up to days after a single dose or short-term repeated administration [144]. Sulforaphane administration prior to ischemia activates Nrf2 in the blood brain barrier and in perivascular astrocytes, effectively protecting the system from stroke-induced oxidative stress [145]. Growing interest in SFN's ability to protect brain health has led to several interesting findings. One research group reported that SFN induced Nrf2 in

striatum and cortex, while another group investigated the ability of SFN to induce HMOX1 expression in neurons and astrocytes [115,146]. Currently there is only limited data demonstrating SFN's ability to improve cognitive function following brain injury [145,147], and SFN's effect on cognitive and behavioral impairment in an aging model has not been investigated. It is, however, becoming more widely established that SFN attenuates inflammation in the CNS, in part by activating the Nrf2 pathway in microglia [144,148]. Thus, this phytochemical may be an effective activator of antioxidant and anti-inflammatory mechanisms that could support healthy brain aging.

2.8 Summary and Objectives

Scientific advances over the past two decades have generated a well-characterized understanding of the relationship between cytokines, neuroinflammation, and sickness behavior [149]. Notably, elevated cytokines and microglial activation in aging have been linked to behavioral and cognitive impairment, which mirrors increased inflammation and mild cognitive decline in otherwise healthy elderly individuals. Bioactives such as SFN are targets for aging therapy due to their ability to attenuate inflammation and activate endogenous antioxidant pathways. To date, the majority of research involving SFN and dietary broccoli has focused on chemoprevention and, more recently, neuroprotection against traumatic brain injury and ischemia. While it has been theorized that SFN supplementation may improve long-term brain health, its impact on the non-diseased aging brain has not been investigated. This dissertation was designed to examine whether SFN could attenuate markers of inflammation and oxidative stress in microglia, and whether these effects were associated with reduced

inflammation in the aging brain. In addition, a broccoli-supplemented diet was investigated as a potential intervention for LPS-induced sickness behavior and cytokine response in aged mice. These experiments provide important insight into the effects of SFN as a nutritionally relevant intervention to target inflammation in the aging brain.

2.9 References

[1] Chung HY, Kim HJ, Kim JW, Yu BP. The inflammation hypothesis of aging: molecular modulation by calorie restriction. Ann NY Acad Sci. 2001;928:327-35.

[2] Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. J Neuroinflamm. 2004;1:14.

[3] Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81:1-5.

[4] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801.

[5] Banks WA. The blood-brain barrier in neuroimmunology: Tales of separation and assimilation. Brain Behav Immun. 2015;44:1-8.

[6] Dantzer R, Konsman J-P, Bluthé R-M, Kelley KW. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton Neurosci. 2000;85:60-5.

[7] Quan N, Banks WA. Brain-immune communication pathways. Brain Behav Immun. 2007;21:727-35.

[8] Buller KM. Circumventricular organs: gateways to the brain role of circumventricular organs in pro-inflammatory cytokine-induced activation of The hypothalamic– pituitary–adrenal axis. Clin Exp Pharmacol P. 2001;28:581-9.

[9] Maness LM, Kastin AJ, Banks WA. Relative contributions of a CVO and the microvascular bed to delivery of blood-borne IL-1α to the brain. Am J Physiol. 1998;275:E207-E12.

[10] Ek M, Kurosawa M, Lundeberg T, Ericsson A. Activation of vagal afferents after intravenous injection of interleukin-1β: Role of endogenous prostaglandins. J Neurosci. 1998;18:9471-9.

[11] D'Mello C, Swain MG. Liver-brain inflammation axis. Am J Physiol Gastrointest Liver Physiol. 2011;301:G749-G61.

[12] Berthoud H-R, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. Auton Neurosci. 2000;85:1-17.

[13] Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: impact on macrophage function. Aging Cell. 2004;3:161-7.

[14] Miller RA. The aging immune system: primer and prospectus. Science. 1996;273:70-4.

[15] Ginaldi L, De Martinis M, D'Ostilio A, Marini L, Loreto MF, Quaglino D. Immunological changes in the elderly. Aging Clin Exp Res. 1999;11:281-6.

[16] Ginaldi L, Loreto MF, Corsi MP, Modesti M, De Martinis M. Immunosenescence and infectious diseases. Microbes Infect. 2001;3:851-7.

[17] Johnston M, Wakeling A, Graham N, Stokes F. Cognitive impairment, emotional disorder and length of stay of elderly patients in a district general hospital. Brit J Med Psychol. 1987;60:133-9.

[18] Wofford JL, Loehr LR, Schwartz E. Acute cognitive impairment in elderly ED patients: Etiologies and outcomes. Am J Emerg Med. 1996;14:649-53.

[19] Akinyemi RO, Mukaetova-Ladinska EB, Attems J, Ihara M, Kalaria RN. Vascular risk factors and neurodegeneration in ageing related dementias: Alzheimer's disease and vascular dementia. Curr Alzheimer Res. 2013;10:642-53.

[20] Ghosh D, Levault KR, Brewer GJ. Dual-energy precursor and nuclear erythroid-related factor 2 activator treatment additively improve redox glutathione levels and neuron survival in aging and Alzheimer mouse neurons upstream of reactive oxygen species. Neurobiol Aging. 2013;35:179-90.

[21] Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, et al. Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. Ann Neurol. 1993;34:609-16.

[22] McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Rev 1995;21:195-218.

[23] Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, et al. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proceedings of the National Academy of Sciences. 1991;88:10540-3.

[24] Jenner P. Oxidative damage in neurodegenerative disease. The Lancet. 1994;344:796-8.

[25] Abraham J, Johnson RW. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuv Res. 2009;12:445-53.

[26] Barrientos RM, Higgins EA, Biedenkapp JC, Sprunger DB, Wright-Hardesty KJ, Watkins LR, et al. Peripheral infection and aging interact to impair hippocampal memory consolidation. Neurobiol Aging. 2006;27:723-32.

[27] Huang Y, Henry CJ, Dantzer R, Johnson RW, Godbout JP. Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. Neurobiol Aging. 2008;29:1744-53.

[28] Wynne AM, Henry CJ, Godbout JP. Immune and behavioral consequences of microglial reactivity in the aged brain. Integr Comp Biol. 2009;49:254-66.

[29] Streit WJ, Sammons NW, Kuhns AJ, Sparks DL. Dystrophic microglia in the aging human brain. Glia. 2004;45:208-12.

[30] Theele DP, Streit WJ. A chronicle of microglial ontogeny. Glia. 1993;7:5-8.

[31] Rivest S. Regulation of innate immune responses in the brain. Nat Rev Immunol. 2009;9:429-39.

[32] Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, et al. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci. 2005;8:752-8.

[33] Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci. 2007;10:1387-94.

[34] Fairweather D, Cihakova D. Alternatively activated macrophages in infection and autoimmunity. J Autoimmun. 2009;33:222-30.

[35] Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, et al. Microglia/Macrophage Polarization Dynamics Reveal Novel Mechanism of Injury Expansion After Focal Cerebral Ischemia. Stroke. 2012;43:3063-70.

[36] Loane DJ, Byrnes KR. Role of microglia in neurotrauma. Neurotherapeutics. 2010;7:366-77.

[37] Wang G, Zhang J, Hu X, Zhang L, Mao L, Jiang X, et al. Microglia/macrophage polarization dynamics in white matter after traumatic brain injury. J Cereb Blood Flow Metab. 2013;33:1864-74.

[38] Chhor V, Le Charpentier T, Lebon S, Oré M-V, Celador IL, Josserand J, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain Behav Immun. 2013;32:70-85.

[39] Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflamm. 2014;11:98.

[40] Lehnardt S. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia. 2010;58:253-63.

[41] Le Cabec V, Carréno S, Moisand A, Bordier C, Maridonneau-Parini I. Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively. J Immunol. 2002;169:2003-9.

[42] Akiyama H, McGeer PL. Brain microglia constitutively express β-2 integrins. J Neuroimmunol. 1990;30:81-93.

[43] Mattiace LA, Davies P, Dickson DW. Detection of HLA-DR on microglia in the human brain is a function of both clinical and technical factors. Am J Pathol. 1990;136:1101-14.

[44] Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996;19:312-8.

[45] Roy A, Fung YK, Liu X, Pahan K. Up-regulation of microglial CD11b expression by nitric oxide. J Biol Chem. 2006;281:14971-80.

[46] Burton MD, Rytych JL, Freund GG, Johnson RW. Central inhibition of interleukin-6 trans-signaling during peripheral infection reduced neuroinflammation and sickness in aged mice. Brain Behav Immun. 2013;30:66-72.

[47] Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996;19:312-8.

[48] Eggen BJ, Raj D, Hanisch UK, Boddeke HW. Microglial phenotype and adaptation. J Neuroimmune Pharmacol. 2013;8:807-23.

[49] Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J. 2005;19:1329-31.

[50] Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain Behav Immun. 2009;23:309-17.

[51] Frank MG, Barrientos RM, Watkins LR, Maier SF. Aging sensitizes rapidly isolated hippocampal microglia to LPS ex vivo. J Neuroimmunol. 2010;226:181-4.

[52] Richwine AF, Johnson RW. Microglial cells and inflammatory cytokines in the aged brain. In: Siegel A, Zalcman SS, editors. The Neuroimmunological Basis of Behavior and Mental Disorders: Springer US; 2009. p. 411-23.

[53] Ye SM, Johnson RW. An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. Neuroimmunomodulat 2001;9:183-92.

[54] Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. J Nutr. 2010;140:1892-8.

[55] Joseph JA, Shukitt-Hale B, Casadesus G, Fisher D. Oxidative stress and inflammation in brain aging: Nutritional considerations. Neurochem Res. 2005;30:927-35.

[56] Smith AP. Twenty-five years of research on the behavioural malaise associated with influenza and the common cold. Psychoneuroendocrino. 2013;38:744-51.

[57] Katz IR. On the inseparability of mental and physical health in aged persons: Lessons from depression and medical comorbidity. Am J Geriat Psychiat. 1996;4:1-16.

[58] van Dam A-M, Brouns M, Louisse S, Berkenbosch F. Appearance of interleukin-1 in macrophages and in ramified microglia in the brain of endotoxin-treated rats: a pathway for the induction of non-specific symptoms of sickness? Brain Res. 1992;588:291-6.

[59] Aubert A. Sickness and behaviour in animals: a motivational perspective. Neurosci Biobehav R. 1999;23:1029-36.

[60] Bluthe´ R-M, Dantzer R, Kelley KW. Effects of interleukin-1 receptor antagonist on the behavioral effects of lipopolysaccharide in rat. Brain Res. 1992;573:318-20.

[61] Burton MD, Sparkman NL, Johnson RW. Inhibition of interleukin-6 trans-signaling in the brain facilitates recovery from lipopolysaccharide-induced sickness behavior. J Neuroinflamm. 2011;8:54.

[62] Turnbull AV, Rivier CL. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. 1999;79:1-71.

[63] Farrar WL, Kilian PL, Ruff MR, Hill JM, Pert CB. Visualization and characterization of interleukin 1 receptors in brain. J Immunol. 1987;139:459-63.

[64] Watkins LR, Hansen MK, Nguyen KT, Lee JE, Maier SF. Dynamic regulation of the proinflammatory cytokine, interleukin-1β: Molecular biology for non-molecular biologists. Life Sci. 1999;65:449-81.

[65] Rothwell NJaL, G. Pharmacology of interleukin-1 actions in the brain. Adv Pharmacol. 1994;25:1-20.

[66] Abraham J, Johnson RW. Central inhibition of interleukin-1beta ameliorates sickness behavior in aged mice. Brain Behav Immun. 2009;23:396-401.

[67] Crawley JN. What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. 2nd ed. Hoboken, NJ: John Wiley and Sons, Inc.; 2007.

[68] Berg BM, Godbout JP, Kelley KW, Johnson RW. Alpha-tocopherol attenuates lipopolysaccharide-induced sickness behavior in mice. Brain Behav Immun. 2004;18:149-57.

[69] Bodles AM, Barger SW. Cytokines and the aging brain – what we don't know might help us. Trends Neurosci. 2004;27:621-6.

[70] Godbout JP, Berg BM, Krzyszton C, Johnson RW. α-Tocopherol attenuates NFκB activation and pro-inflammatory cytokine production in brain and improves recovery from lipopolysaccharide-induced sickness behavior. J Neuroimmunol. 2005;169:97-105.

[71] Sah SP, Tirkey N, Kuhad A, Chopra K. Effect of quercetin on lipopolysaccharide induced-sickness behavior and oxidative stress in rats. Indian J Pharmacol. 2011;43:192-6.

[72] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci. 1993;90:7915-22.

[73] Harman D. Free radicals in aging. Mol Cell Biochem. 1988;84:155-61.

[74] Harman D. Free radical theory of aging. Mutation Research/DNAging. 1992;275:257-66.

[75] Pearl R. The tate of living: Being an account of some experimental studies on the biology of life duration. New York: A.A. Knopf; 1928.

[76] Oldham KM, Bowen PE. Oxidative stress in critical care: is antioxidant supplementation beneficial? J Am Diet Assoc. 1998;98:1001-8.

[77] Coyle J, Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. Science. 1993;262:689-95.

[78] Liu J, Atamna H, Kuratsune H, Ames BN. Delaying brain mitochondrial decay and aging with mitochondrial antioxidants and metabolites. Ann NY Acad Sci. 2002;959:133-66.

[79] Leutner S, Eckert A, Müller WE. ROS generation, lipid peroxidation and antioxidant enzyme activities in the aging brain. J Neural Transm. 2001;108:955-67.

[80] Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, et al. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitrone. Proc Natl Acad Sci. 1991;88:3633-6.

[81] Liu R, Liu IY, Bi X, Thompson RF, Doctrow SR, Malfroy B, et al. Reversal of age-related learning deficits and brain oxidative stress in mice with superoxide dismutase/catalase mimetics. Proc Natl Acad Sci. 2003;100:8526-31.

[82] Fredriksson A, Archer T. Alpha-phenyl-tert-butyl-nitrone (PBN) reverses age-related maze learning performance and motor activity deficits in C57 BL/6 mice. Behav Pharmacol. 1996;7:245-53.

[83] Liu J, Head E, Gharib AM, Yuan W, Ingersoll RT, Hagen TM, et al. Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: Partial reversal by feeding acetyl-l-carnitine and/or R-α-lipoic acid. Proc Natl Acad Sci. 2002;99:2356-61.

[84] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408:239-47.

[85] Halliwell BG, John. Free Radicals in Biology and Medicine. 4 ed. Oxford, NY: Oxford University Press; 2007.

[86] Lipman RD, Bronson RT, Wu D, Smith DE, Prior R, Cao G, et al. Disease incidence and longevity are unaltered by dietary antioxidant supplementation initiated during middle age in C57BL/6 mice. Mech Ageing Dev. 1998;103:269-84.

[87] Bjelakovic G, Nikolova D, Gluud L, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: Systematic review and meta-analysis. J Am Med Assoc. 2007;297:842-57.

[88] Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci. 1994;91:9926-30.

[89] Talalay P. Mechanisms of induction of enzymes that protect against chemical carcinogenesis. Adv Enzyme Regul. 1989;28:237-50.

[90] Rushmore TH, Pickett CB. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. J Biol Chem. 1990;265:14648-53.

[91] Prestera T, Holtzclaw WD, Zhang Y, Talalay P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci. 1993;90:2965-9.

[92] Jaiswal AK. Antioxidant response element. Biochem Pharmacol. 1994;48:439-44.

[93] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997;236:313-22.

[94] McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, et al. The cap 'n' collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. Cancer Res. 2001;61:3299-307.

[95] Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke–induced emphysema in mice. J Clin Invest. 2004;114:1248-59.

[96] Ramos-Gomez M, Kwak M-K, Dolan PM, Itoh K, Yamamoto M, Talalay P, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci. 2001;98:3410-5.

[97] Klaassen CD, Reisman SA. Nrf2 the rescue: Effects of the antioxidative/electrophilic response on the liver. Toxicol Appl Pharm. 2010;244:57-65.

[98] Kwak M-K, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 Pathway: identification of novel gene clusters for cell survival. J Biol Chem. 2003;278:8135-45.

[99] Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. 2002;62:5196-203.

[100] Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. J Clin Invest. 2006;116:984-95.

[101] Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci. 1996;93:14960-5.

[102] Dinkova-Kostova AT, Talalay P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Biochem Biophys. 2010;501:116-23.

[103] Raina AK, Templeton DJ, deak JC, Perry G, Smith MA. Quinone reductase (NQO1), a sensitive redox indicator, is increased in Alzheimer's disease. Redox Report. 1999;4:23-7.

[104] Naito Y, Takagi T, Higashimura Y. Heme oxygenase-1 and anti-inflammatory M2 macrophages. Arch Biochem Biophys. 2014;564:83-8.

[105] Alam J, Cook JL. How many transcription factors does it take to turn on the heme oxygenase-1 gene? Am J Respir Cell Mol Biol. 2007;36:166-74.

[106] Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci. 1997;94:10925-30.

[107] Lavrovsky Y, Song CS, Chatterjee B, Roy AK. Age-dependent increase of heme oxygenase-1 gene expression in the liver mediated by NFkappaB. Mech Ageing Dev. 2000;114:49-60.

[108] Lu DY, Tsao YY, Leung YM, Su KP. Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. Neuropsychopharmacol. 2010;35:2238-48.

[109] Lee JM, Calkins MJ, Chan KM, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J Biol Chem. 2003;278:12029-38.

[110] Lee JM, Shih AY, Murphy TH, Johnson JA. NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. J Biol Chem. 2003;278:37948-56.

[111] Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci. 2005;25:10321-35.

[112] Kraft AD, Lee J-M, Johnson DA, Kan YW, Johnson JA. Neuronal sensitivity to kainic acid is dependent on the Nrf2-mediated actions of the antioxidant response element. J Neurochem. 2006;98:1852-65.

[113] Jin W, Wang H, Yan W, Zhu L, Hu Z, Ding Y, et al. Role of Nrf2 in protection against traumatic brain injury in mice. J Neurotrauma. 2009;26:131-9.

[114] Ping Z, Liu W, Kang Z, Cai J, Wang Q, Cheng N, et al. Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme. Brain Res. 2010;1343:178-85.

[115] Zhao J, Kobori N, Aronowski J, Dash PK. Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. Neurosci Lett. 2006;393:108-12.

[116] Zhao J, Moore AN, Redell JB, Dash PK. Enhancing expression of Nrf2-driven genes protects the blood brain barrier after brain injury. J Neurosci. 2007;27:10240-8.

[117] Rojo AI, Innamorato NG, Martín-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. Glia. 2010;58:588-98.

[118] Ramsey CP, Glass CA, Montgomery MB, Lindl KA, Ritson GP, Chia LA, et al. Expression of Nrf2 in neurodegenerative diseases. J Neuropathol Exp Neurol. 2007;66:75-85.

[119] Sarlette A, Krampfl K, Grothe C, Neuhoff Nv, Dengler R, Petri S. Nuclear erythroid 2-related factor 2-antioxidative response element signaling pathway in motor cortex and spinal cord in amyotrophic lateral sclerosis. J Neuropath Exp Neur. 2008;67:1055-62.

[120] Dwyer J. Starting down the right path: nutrition connections with chronic diseases of later life. Am J Clin Nutr. 2006;83:415S-20S.

[121] Einother SJ, Giesbrecht T. Caffeine as an attention enhancer: reviewing existing assumptions. Psychopharmacology (Berl). 2013;225:251-74.

[122] Kiecolt-Glaser JK. Stress, food, and inflammation: psychoneuroimmunology and nutrition at the cutting edge. Psychosom Med. 2010;72:365-9.

[123] Lopez-Garcia E, Schulze MB, Fung TT, Meigs JB, Rifai N, Manson JE, et al. Major dietary patterns are related to plasma concentrations of markers of inflammation and endothelial dysfunction. Am J Clin Nutr. 2004;80:1029-35.

[124] Lazarov O, Mattson MP, Peterson DA, Pimplikar SW, van Praag H. When neurogenesis encounters aging and disease. Trends Neurosci. 2010;33:569-79.

[125] Joseph J, Cole G, Head E, Ingram D. Nutrition, brain aging, and neurodegeneration. J Neurosci. 2009;29:12795-801.

[126] Clarke JD, Dashwood RH, Ho E. Multi-targeted prevention of cancer by sulforaphane. Cancer Lett. 2008;269:291-304.

[127] Lin HJ, Probst-Hensch NM, Louie AD, Kau IH, Witte JS, Ingles SA, et al. Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas. Cancer Epidemiol Biomarkers Prev. 1998;7:647-52.

[128] Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidem Biomar. 2001;10:501-8.

[129] Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A. 1992;89:2399-403.

[130] Dosz EB, Jeffery EH. Commercially produced frozen broccoli lacks the ability to form sulforaphane. J Funct Foods. 2013;5:987-90.

[131] Dosz EB, Jeffery EH. Modifying the processing and handling of frozen broccoli for increased sulforaphane formation. J Food Sci. 2013;78:H1459-63.

[132] Keck A-S, Qiao Q, Jeffery EH. Food matrix effects on bioactivity of broccoli-derived sulforaphane in liver and colon of F344 rats. J Agr Food Chem. 2003;51:3320-7.

[133] Vermeulen M, Klöpping-Ketelaars IWAA, van den Berg R, Vaes WHJ. Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. J Agr Food Chem. 2008;56:10505-9.

[134] Clarke JD, Hsu A, Riedl K, Bella D, Schwartz SJ, Stevens JF, et al. Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. Pharmacol Res. 2011;64:456-63.

[135] Hanlon N, Coldham N, Gielbert A, Kuhnert N, Sauer MJ, King LJ, et al. Absolute bioavailability and dose-dependent pharmacokinetic behaviour of dietary doses of the chemopreventive isothiocyanate sulforaphane in rat. Br J Nutr. 2008;99:559-64.

[136] Clarke J, Hsu A, Williams D, Dashwood R, Stevens J, Yamamoto M, et al. Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice. Pharm Res. 2011;28:3171-9.

[137] Barcelo S, Gardiner JM, Gescher A, Chipman JK. CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. Carcinogenesis. 1996;17:277-82.

[138] Myzak MC, Karplus PA, Chung F-L, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res. 2004;64:5767-74.

[139] Gerhäuser C, You M, Liu J, Moriarty RM, Hawthorne M, Mehta RG, et al. Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. Cancer Res. 1997;57:272-8.

[140] Ramsdell HS, Eaton DL. Modification of aflatoxin B1 biotransformation in vitro and DNA binding in vivo by dietary broccoli in rats. J Toxicol Env Health. 1988;25:269-84.

[141] Vang O, Mehrota K, Georgellis A, Andersen O. Effects of dietary broccoli on rat testicular xenobiotic metabolizing enzymes. Eur J Drug Metab Ph. 1999;24:353.

[142] Wu L, Noyan Ashraf MH, Facci M, Wang R, Paterson PG, Ferrie A, et al. Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. Proc Natl Acad Sci U S A. 2004;101:7094-9.

[143] Lee J-M, Li J, Johnson DA, Stein TD, Kraft AD, Calkins MJ, et al. Nrf2, a multi-organ protector? FASEB J. 2005;19:1061-6.

[144] Innamorato N, Rojo, AI, Garcia-Yague, AJ, Yamamoto, M, de Ceballos, ML, Cuadrado, A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol. 2008;181:680-9.

[145] Alfieri A, Srivastava S, Siow RCM, Cash D, Modo M, Duchen MR, et al. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood–brain barrier disruption and neurological deficits in stroke. Free Radical Bio Med. 2013;65:1012-22.

[146] Jazwa A, Rojo AI, Innamorato NG, Hesse M, Fernandez-Ruiz J, Cuadrado A. Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism. Antioxid Redox Sign. 2011;14:2347-60.

[147] Dash PK, Zhao J, Orsi SA, Zhang M, Moore AN. Sulforaphane improves cognitive function administered following traumatic brain injury. Neurosci Lett. 2009;460:103-7.

[148] Kraft AD, Johnson DA, Johnson JA. Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. J Neurosci. 2004;24:1101-12.

[149] Dantzer R, Kelley KW. Twenty years of research on cytokine-induced sickness behavior. Brain, Behav Immun. 2007;21:153-60.

Chapter 3 - Sulforaphane induces Nrf2 target genes and attenuates inflammatory gene expression in microglia from brain of young and aged mice

3.1 Abstract

Increased neuroinflammation and oxidative stress resulting from heightened microglial activation is associated with age-related cognitive impairment, neurodegenerative disease, and prolonged sickness response to an immune stimulus. The primary compensatory mechanism for neutralizing oxidative stress is through the nuclear factor E2-related factor 2 (Nrf2) pathway which induces transcription of antioxidant response element (ARE) genes. Here, BV2 microglia and brain $CD11b^+$ microglial cells isolated from young adult and aged mice were used to investigate the effects of sulforaphane (SFN), a potent Nrf2-activating bioactive, on inflammatory gene expression associated with aging and lipopolysaccharide (LPS) stimulation. We hypothesized that SFN would activate the Nrf2/ARE pathway in BV2 microglia and primary microglia and attenuate elevated proinflammatory cytokine expression resulting from aging and LPS stimulation. In support of our hypothesis, SFN increased Nrf2 DNA-binding activity and upregulated ARE genes in BV2 microglia, while reducing LPS-induced interleukin (IL-)1β, IL-6, and inducible nitric oxide synthase (iNOS). In primary microglia from adult and aged mice, SFN increased expression of ARE genes and attenuated IL-1β, IL-6, and iNOS induced by LPS. Taken together these data indicate that SFN is a potential therapeutic supplement that may be beneficial for reducing microglial mediated neuroinflammation and oxidative stress associated with aging.

3.2 Introduction

Microglia are activated in response to inflammatory stimuli and stress. Activated microglia produce proinflammatory cytokines and reactive oxygen and nitrogen species, and express genes associated with the newly defined microglial sensome [1,2]. Another factor that results in microglial activation is aging. Microglia from old but otherwise healthy mice tend to transition from the quiescent M0 phenotype to the inflammatory M1 phenotype [3]. This shift in microglial phenotype results in chronic, low grade neuroinflammation which is considered a contributing factor to some aspects of cognitive aging. Furthermore, microglia from aged mice are hypersensitive to stress and peripheral immune stimuli and produce excessive levels of inflammatory mediators when further provoked. Microglial hypersensitivity leads to prolonged behavioral deficits (i.e. sickness behavior) following peripheral infection [4,5]. This suggests that it is necessary to regulate the proinflammatory status of microglia in the aged brain to promote successful aging.

Nuclear factor E2-related factor 2 (Nrf2) provides a critical compensatory mechanism to counteract oxidative stress through its ability to upregulate genes containing the antioxidant response element (ARE) promoter sequence [6]. Involvement of Nrf2 in regulation of inflammation and microglial activation has also been reported [7,8]. Because the Nrf2/ARE pathway can be activated by pharmacological and dietary sources, it has become a potential therapeutic target for reducing oxidative stress associated with chronic, age-related neuroinflammation [7,9,10].

Sulforaphane (SFN) is a small-molecule Nrf2 activator that can be obtained naturally from cruciferous vegetables, with especially high concentrations derived from broccoli and broccoli

sprouts [11,12]. Clinical and pre-clinical studies have established that SFN from broccoli is readily absorbed [13] and has a low toxicity profile, making it suitable to be provided in supplement form [14,15]. However, in a recent study where a 10% broccoli diet was fed to aged mice, several markers indicative of reduced glial cell activity were altered, but other markers of inflammation were not [16]. Other studies suggest that Nrf2 signaling might be disrupted during aging, leading to decreased endogenous antioxidant response [17,18], but this has not been examined in microglia. Therefore, the aim of this study was to determine if SFN mitigates markers of neuroinflammation in primary microglia from young adult and aged mice. We hypothesized that SFN would activate Nrf2 target genes and reduce production of inflammatory mediators in microglia from adult and aged mice.

3.3 Materials and Methods

BV2 cell culture and treatments

The immortalized murine microglia cell line, BV2, has been used as a model to investigate the neuroimmune system [19,20]. Cells were maintained at 37°C under 5% CO_2 in 75-cm^2 flasks in Dulbecco modified Eagle medium (Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 200 mM glutamine, and streptomycin/penicillin (Invitrogen, Carlsbad, CA). In order to determine time-dependent change in ARE gene expression, cells were treated with vehicle (complete medium) or 2.5 μM SFN (LKT Laboratories, St. Paul, MN) for 3, 6, 9, or 24 h. In subsequent experiments, cells were treated for 1 h with SFN or vehicle, then with PBS ± LPS (100 ng/mL) (Sigma, St. Louis, MO; 0127:B8) for 8 h. Each treatment was replicated 3 times in separate but identical trials.

CD11b^{+} microglia cell isolation and culture

Because microglial cell activity is closely tied to brain health, it is important to study this specific cell type in the context of aging and exposure to an immune stimulus. In order to obtain primary microglia, we used an isolation method slightly modified from a protocol previously described that yields an enriched population of CD11b^{+}/CD45^{low} microglia that retain phenotypic integrity and inflammatory cytokine production in response to LPS [21]. Cells that were positive for CD11b were isolated from brains of young adult (4-5 month old, n = 16) and aged (19-20 month old, n = 16) BALB/c mice. Whole brains were enzymatically digested using a Neural Tissue Dissociation Kit (Miltenyi Biotec, San Diego CA) for 35 min at 37°C. Digested tissue was then passed through a 40 µm strainer to further separate cells and remove debris and then pelleted by centrifugation at 300 x g for 15 min. Myelin removal was facilitated by suspending the pelleted cells in 30% Percoll-Plus (GE Healthcare, Princeton, NJ) and centrifuging for 10 min at 1000 x g. After centrifugation, myelin and percoll were aspirated and remaining cells were washed with PEB solution consisting of sterile PBS, 0.5% BSA and 0.2 mM EDTA. Cells were then pelleted by centrifugation, PEB solution was removed, and cells were incubated with anti-CD11b magnetic microbeads (10 µL beads 90 µL PEB; Miltenyi Biotec, San Diego CA) for 15 min. MS columns were used to magnetically separate CD11b^{+} cells (Miltenyl Biotec, San Diego CA). Cells were collected and suspended in medium (DMEM, 10% FBS) containing 10 ng/mL GM-CSF and plated in 12-well culture plates pre-coated with poly-L-ornithine (Sigma, St. Louis, MO). After 7-8 days in culture, primary cells were treated and harvested. All primary cells were treated with vehicle (medium) ± SFN (2.5 µM) for 1 h followed by PBS ± LPS (10 ng/mL) for 8 h.

Nrf2 DNA-binding assay

The TransAm Nrf2 kit was used to measure Nrf2 nuclear protein binding to the ARE promoter sequence (Active Motif, Carlsbad, CA). BV2 cells were treated with SFN ± LPS as described above, then harvested with 0.25% Trypsin-EDTA and washed once with cold PBS. Cells were pelleted by centrifugation for 5 min at 500 x g. Nuclear proteins were extracted using NE-PER reagent (Pierce, Rockford, IL). Nuclear protein was quantified using the 660 nm Protein Assay Reagent from Pierce (Rockford IL) and 4.5 µg of nuclear protein per sample was used for the assay.

Markers of inflammation and oxidative stress

RNA was isolated from BV2 cells using E.Z.N.A. total RNA (Omega Biotek, Norcross, GA). RNA from primary microglia was isolated using the Tri Reagent protocol (Sigma, St. Louis, MO). Synthesis of cDNA was carried out using a high capacity RT kit (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qPCR) was used to detect changes in mRNA expression of ARE genes NQO1 (Mm.PT.56a.9609207), HMOX1 (Mm.PT.56a.9675808), and GCLM (Mm.PT.56a.11654780), and proinflammatory markers IL-1β (Mm.PT.56a.41616450), IL-6 (Mm.PT.56a.13354106), and iNOS (Mm.PT.56a.43705194) using PrimeTime qPCR Assays (Integrated DNA Technologies, Coralville, IA). All mRNA expression changes were compared to the housekeeping control gene GAPDH (Mm.PT.39.a.1) and the $2^{-\Delta\Delta Ct}$ calculation method as previously described [22]. Data are expressed as fold change relative to the adult vehicle control.

To determine whether SFN attenuated secretion of proinflammatory cytokines, media from BV2 cells and primary microglia were collected 9 h after SFN. IL-6 protein expression was quantified using a commercially available OptEIA ELISA kit (BD Biosciences, San Jose, CA) according to manufacturer's instructions.

Nitrite production in medium from treated BV2 cells was measured using the Promega Griess Reagent System (Madison, WI). The assay was conducted according to the manufacturer's instructions. Absorbance was read at 532 nm.

Statistical analyses

All data were analyzed using Statistical Analysis System (SAS, Cary, NC). Data from BV2 cells were subjected to two-way analysis of variance (ANOVA) to assess main effects of SFN by time, or effects of SFN and LPS. Primary microglia data were analyzed using three-way ANOVA (Age, SFN, LPS). Where ANOVA revealed a significant interaction, *post hoc* Student's t test using Fisher's least significant differences was used to determine means separation. All data are expressed as means ± SEM.

3.4 Results

SFN increased expression of ARE genes in BV2 cells

Because upregulation of antioxidants may contribute to SFN's anti-inflammatory potential, we measured the transcriptional ARE response to SFN in BV2 cells. SFN upregulates ARE genes including NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HMOX1), and the modulatory subunit of glutamate-cysteine ligase, modifier subunit (GCLM) [23]. We assessed

NQO1, HMOX1, and GCLM mRNA at four time points to determine the optimal time of gene induction. As shown in Figure 3.1, NQO1 was increased at 6, 9, and 24 h after SFN ($p < 0.0001$, for each) compared to vehicle controls. HMOX1 was increased at 3, 6, and 9 h after SFN ($p < 0.0001$, for each) while GCLM was increased at 3, 6, 9, and 24 h after SFN ($p < 0.0001$, for each). These data indicate that the BV2 microglia cell line is highly responsive to SFN.

SFN increased Nrf2 DNA-binding activity and decreased proinflammatory markers in BV2 cells

Activation of Nrf2 in response to oxidative stress or chemical inducers results in its translocation to the nucleus where it regulates antioxidant genes by binding to the ARE promoter region [6]. Both SFN ($p < 0.05$) and LPS ($p < 0.05$) increased Nrf2 activity in BV2 cells (Figure 3.2) but the effects were not additive (SFN x LPS, $p = 0.74$).

In order to examine inhibitory effects of SFN on LPS-induced inflammation, the expression of several proinflammatory markers was assessed. As anticipated, LPS increased IL-1β, IL-6, and iNOS mRNA ($p < 0.01$, for each) (Figure 3.3). Importantly, SFN decreased LPS-induced IL-1β ($p < 0.05$), IL-6 ($p < 0.01$), and iNOS ($p < 0.0001$).

To determine if the change in mRNA expression was reflected by a change in protein, IL-6 protein was measured in supernatants. Consistent with its effects on IL-6 mRNA, SFN reduced IL-6 protein secretion by LPS-treated cells ($p < 0.05$) (Figure 3.3). IL-1β protein is not secreted by BV2 cells and was therefore not assayed. Finally, SFN was found to inhibit nitric oxide production by LPS-stimulated BV2 cells, as measured by the Griess assay ($p < 0.0001$).

SFN increased expression of ARE genes in primary microglia from adult and aged mice

Although SFN-induced ARE gene expression has been previously reported in microglia [24,25], it has not been determined whether aging microglia are sensitive to the antioxidant-inducing effects of SFN. This is important because dysfunctional Nrf2 signaling may underlie age-related changes reported for microglia. To address this question, microglia isolated from adult and aged mouse brain were treated with SFN for 9 h. Exposure to SFN increased NQO1, HMOX1, and GCLM mRNA of adult and aged microglia ($p < 0.0001$, for each) (Figure 3.4), and LPS further stimulated HMOX1 expression in SFN-treated microglia (SFN x LPS, $p < 0.01$). Additionally, SFN increased GCLM in aged microglia more than adult microglia (Age x SFN, $p < 0.05$). These data confirm that primary adult and aged microglia had increased ARE gene expression following exposure to SFN similar to that observed in BV2 microglia. Moreover, microglia from aged mice responded to SFN similarly to microglia from adult mice.

SFN decreased LPS-induced proinflammatory markers in primary microglia from adult and aged mice

The aging brain displays persistent microglial activity which is thought to contribute to chronic, low-grade neuroinflammation that negatively impacts long-term brain health [26]. Because the previous study demonstrated that microglia from aged mice were responsive to SFN, we next examined if exposure to SFN would reduce inflammation in LPS-treated primary microglia from aged mice. As shown in Figure 3.5, LPS increased proinflammatory cytokines IL-1β and IL-6 ($p < 0.0001$, for each). Interactions between SFN and LPS ($p < 0.0001$) were evident for IL-1β and IL-6, providing evidence that SFN reduces LPS-induced proinflammatory markers

in aging microglia. Consistent with the changes observed in IL-6 mRNA, SFN also inhibited LPS-induced secretion of IL-6 (SFN x LPS interaction, $p < 0.0001$) in primary microglia. Because iNOS is induced by proinflammatory cytokines such as IL-1β, we examined the possibility that SFN could indirectly reduce oxidative stress by inhibiting iNOS expression [27]. In agreement with the reduction in LPS-induced IL-1β expression in SFN-treated aged microglia, SFN attenuated LPS-induced increase in iNOS mRNA (SFN x LPS interaction, $p < 0.0001$).

3.5. Discussion

Heightened microglial activation contributes to the chronic low-grade neuroinflammation that is evident in the elderly [28,29]. Due to the rapidly expanding aging population, coupled with the numerous detrimental effects of chronic neuroinflammation such as decreased cognition, research investigating nutritional and pharmacological means to reduce neuroinflammation has garnered increasing interest. The data presented here demonstrated that microglia from aged mice respond to SFN with induction of ARE genes, and that SFN is a potent inhibitor of LPS-induced inflammation and oxidative stress. Therefore, the present study suggests that SFN supplementation may be a viable strategy to reduce age-related neuroinflammation.

Microglia are major producers of inflammatory mediators that exacerbate the production of reactive oxygen and nitrogen species in the CNS [30]. To counter oxidative stress, microglia, along with other cells of the CNS, produce endogenous antioxidants that are regulated predominately through the Nrf2/ARE pathway. Our initial experiments utilizing BV2 microglia sought to demonstrate that exposure to SFN is an effective treatment to increase expression of

ARE genes. The data obtained using BV2 microglia confirmed that ARE gene expression is increased in the presence of SFN and this is consistent with a previous study [24] that showed increased Nrf2 protein expression in BV2 cells after 9 h of exposure to SFN. Interestingly, our study revealed increased Nrf2 activity and induction of Nrf2 target genes using a much lower dose of SFN (2.5 μM compared to 50 μM) than used by Konwinski.

The current study was to determine if SFN could upregulate ARE genes in primary microglia from aged mice. Here, we are the first to provide evidence that SFN increased NQO1, HMOX1, and GCLM transcription in aged mouse microglia. These data indicate that the mechanisms responsible for ARE gene induction are responsive to SFN, a potent Nrf2 pathway inducer, in aging microglia. This finding is particularly important because the progression of age-related cognitive decline and neurodegenerative pathologies is closely associated with increased oxidative stress and microglial activation [31-33]. Additionally, some evidence points to loss of Nrf2 activity during aging, which leads to reduced expression of endogenous antioxidants [17,18]. A dysregulated Nr2 pathway could render aged microglia more prone to take on an inflammatory state. However, studies reporting age-related loss of Nrf2 activity have focused on astrocytes and liver tissue [17,18,34,35], suggesting that there may be regional or cell-specific differences in the aging Nrf2 pathway. The present study found no evidence that the Nrf2 pathway was affected in microglia from aged mice. While it is possible that the aged mice (20-22 mo) were not old enough for loss of Nrf2 activity to be detected, this is unlikely as age-related phenotypic changes have been previously demonstrated in microglia from mice younger than those used in this study (18-20 mo) [5]. Nonetheless, an important finding is that the Nrf2/ARE pathway was intact in aged microglia, meaning that SFN may be useful for

decreasing microglial-mediated neuroinflammation and inducing endogenous antioxidant expression through the Nrf2 pathway.

An additional benefit of SFN supplementation may be derived from reduction in proinflammatory mediators following activation of the Nrf2/ARE pathway. SFN has been reported to reduce inflammation in a Nrf2-dependent manner. Sulforaphane inhibits elevated inflammatory cytokines in wild-type macrophages, but has no known anti-inflammatory effect on cells lacking Nrf2 [36]. Upregulation of the Nrf2 pathway in endothelial cells prevented hydrogen peroxide induced oxidative toxicity and reduced inflammation by suppressing activation of the MAPK pathway [37]. Further, Nrf2-mediated inhibition of P38 phosphorylation reduced inflammatory cytokines in a BV2 model of neuroinflammation [38]. Here, we demonstrate that SFN attenuated the LPS-induced expression of cytokines IL-1β and IL-6 in primary microglia from aged mice. These proinflammatory markers are linked to sickness behavior and increased frailty during aging. Additionally, oxidative stress contributes to the neuroinflammatory milieu, both as a result of decreased antioxidant capacity, and increased oxidative damage. This increase in oxidative stress is associated with cognitive decline [31,39]. Within the CNS, upregulation of iNOS is an important oxidative component of the microglial inflammatory response to toxins and pathogenic stimuli, but excessive production of nitric oxide can damage surrounding cells [40,41]. We observed that SFN attenuated upregulation of iNOS in microglia from aged mice, supporting our hypothesis that the antioxidant-enhancing effect of SFN attenuates increased oxidative stress in stimulated aging microglia. Due to the limited microglia that can be isolated from a single mouse brain, we were unable to detect secreted nitrate as an indication of nitric oxide production. However, in BV2 cells, LPS induced

secretion of nitrate was substantially reduced by SFN, supporting the observed reduction of iNOS mRNA.

Aged microglia are characteristically less responsive to anti-inflammatory signals compared to adult microglia, thus activation of alternative signaling pathways and transcriptional regulation using SFN may offer a unique approach for reducing neuroinflammation during aging [42]. Although our studies used *ex vivo* SFN treatment, peripherally administered SFN has been shown to cross the blood brain barrier and reduce microglial-mediated inflammation and oxidative stress in adult mice [7]. Our current data further demonstrate SFN's anti-inflammatory properties, specifically with regard to microglia isolated from aged mice. Previous studies have demonstrated that elevated neuroinflammatory mediators are found in brain tissue of otherwise healthy aged animals, implying a dysregulated neuroimmune status independent of disease [4]. Although our data did not reveal a main effect due to age, basal expression of IL-1β, IL-6, and iNOS were several-fold higher in aged control microglia compared to adult controls, in agreement with previous reports of heightened basal inflammatory markers in microglia [43]. These results indicate that proinflammatory markers in aging microglia are reduced by SFN, highlighting the need for future *in vivo* studies examining SFN induced attenuation of microglial-mediated inflammation.

In summary, the data presented here indicate that SFN attenuates expression of IL-1β, a key proinflammatory cytokine involved in neuroinflammatory signaling. SFN also reduced proinflammatory cytokine IL-6 and oxidative stress marker iNOS. These data support the hypothesis that SFN can be used therapeutically to reduce potentially adverse properties of activated aging microglia. Furthermore, because neuroinflammation is closely associated with

deficits in cognitive abilities and behavioral function [44-46], an intervention such as SFN that reduces microglial-mediated inflammation may be advantageous to preventing age-induced decline in brain health.

3.6 Figures

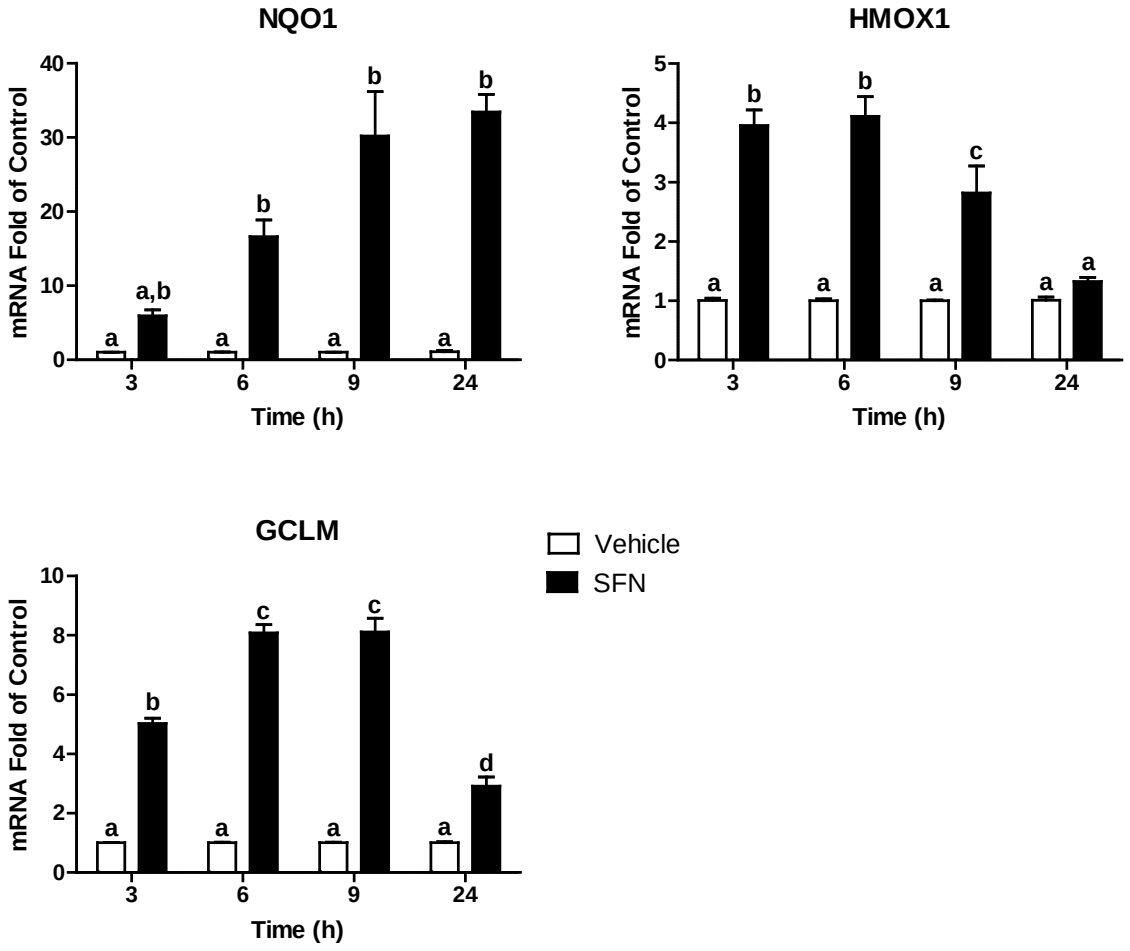

Figure 3.1. *SFN increased expression of ARE genes in BV2 cells*. SFN increased NQO1, HMOX1, and GCLM. Bars represent means ± SEM (n = 3). Means with different letters are significantly different from each other ($p < 0.05$).

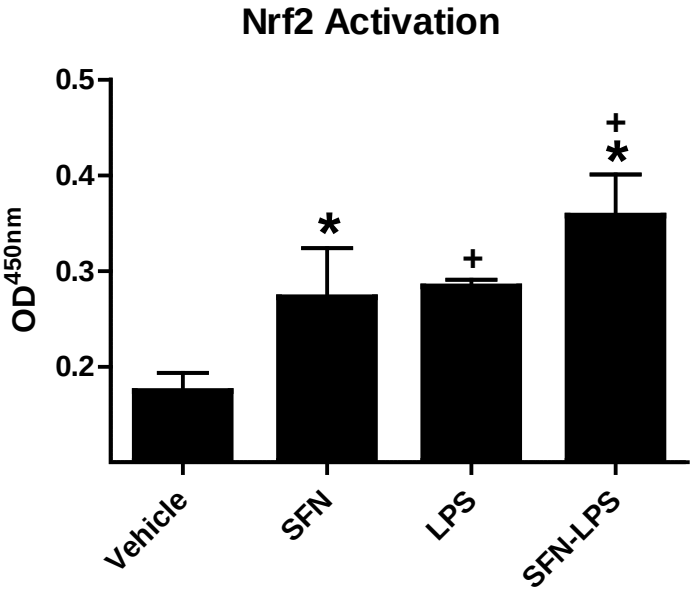

Figure 3.2. *SFN increased Nrf2 activity in BV2 cells*. SFN increased Nrf2 DNA-binding activity. Bars represent means ± SEM (n = 3). * indicates main effect of SFN and + a main effect of LPS ($p < 0.05$).

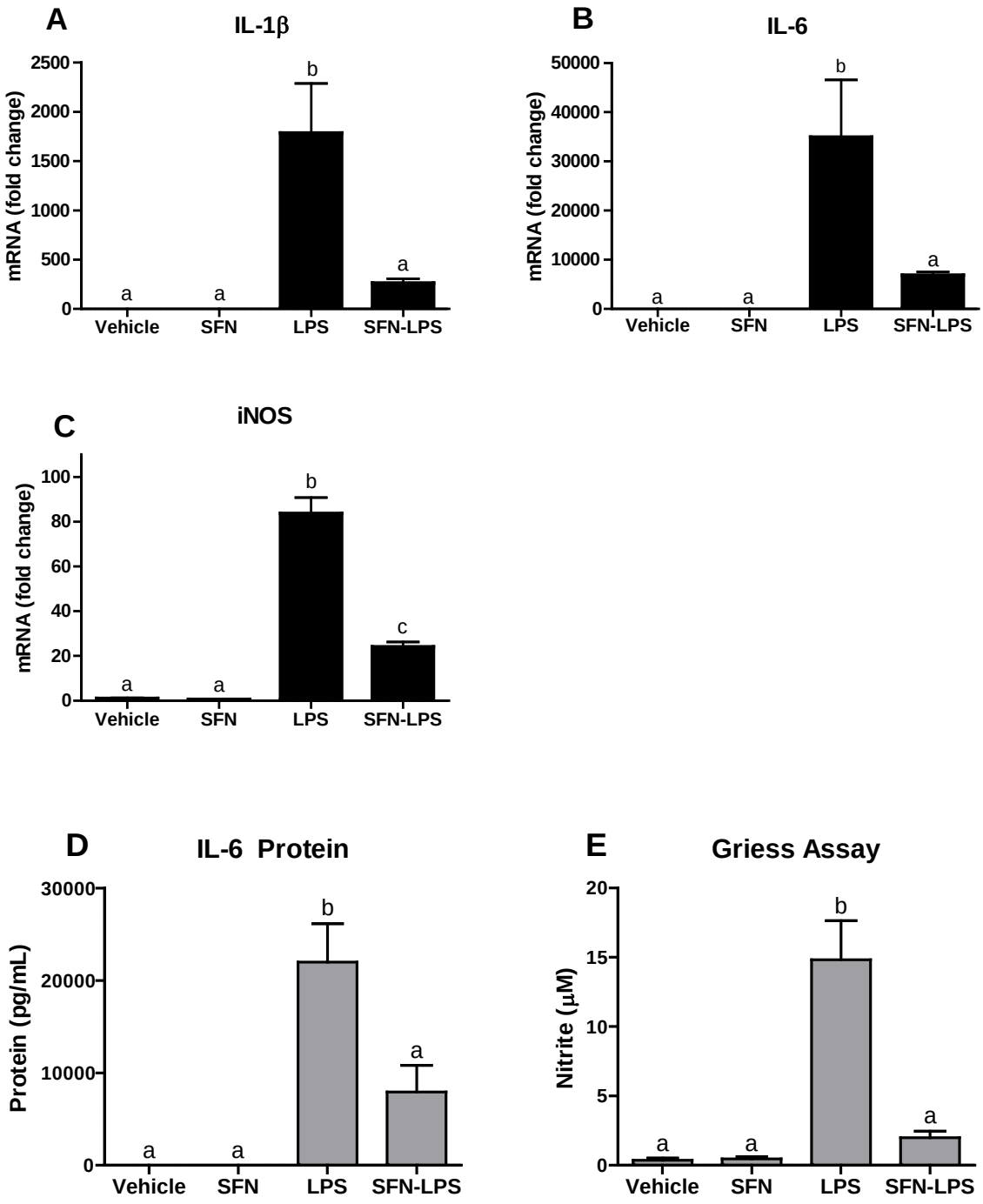

Figure 3.3. *SFN decreased proinflammatory markers in BV2 cells.* (A-C) SFN decreased LPS-induced IL-1β, IL-6, and iNOS mRNA. (D-E) SFN decreased LPS-induced IL-6 protein and nitrite equivalents. Bars represent means ± SEM (n = 3). Means with different letters are significantly different from each other ($p < 0.05$).

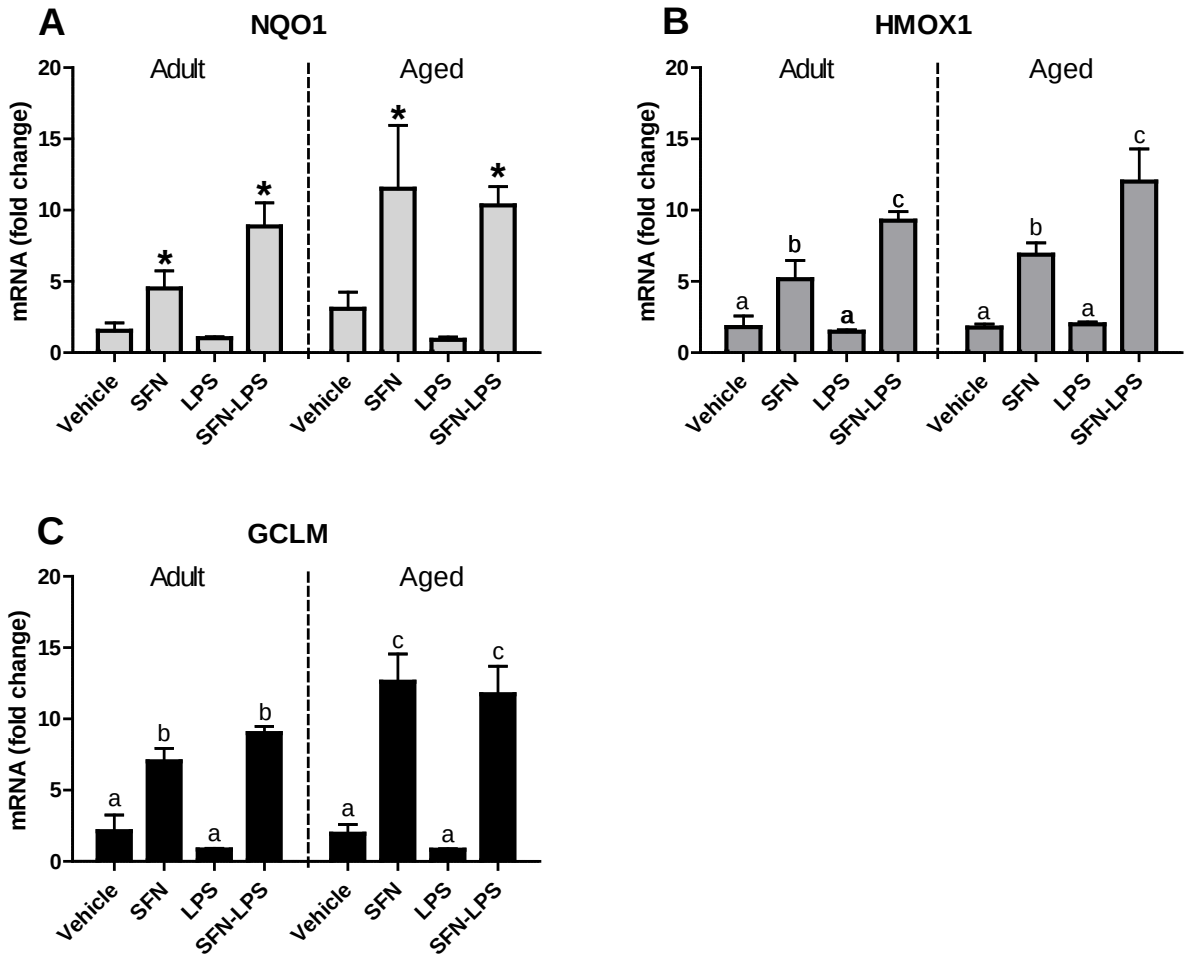

Figure 3.4. *SFN increased expression of ARE genes in adult and aged primary brain microglia.* SFN increased NQO1, HMOX1, and GCLM mRNA. Bars represent means ± SEM (n = 4). Means with an asterisk indicate main effect of SFN ($p < 0.0001$). Means with different letters are significantly different from each other ($p < 0.05$).

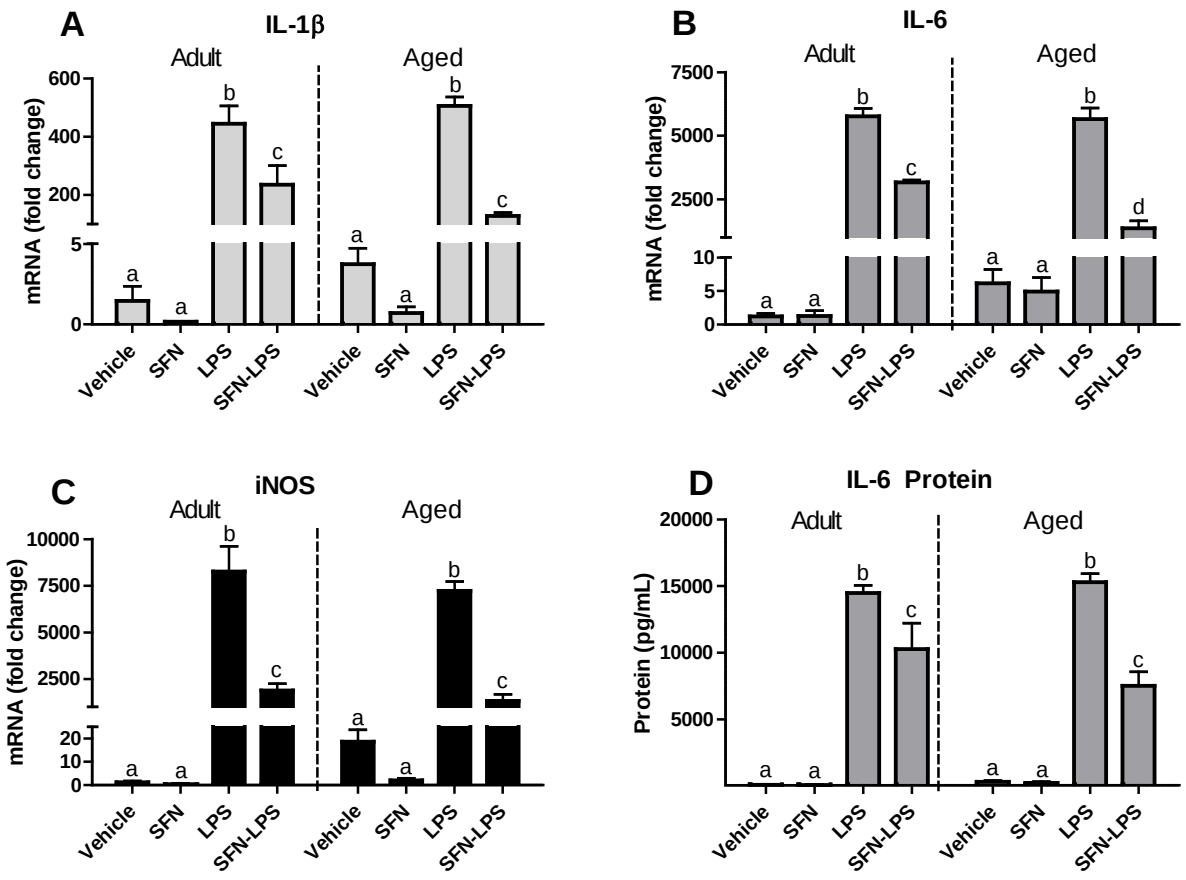

Figure 3.5. *SFN reduced proinflammatory markers in primary microglia from adult and aged mice.* SFN decreased LPS-induced IL-1β, IL-6, and iNOS mRNA. Bars represent means ± SEM (n = 4). Means with different letters are significantly different from each other (SFN x LPS, $p < 0.0001$).

3.7 References

[1] Eggen BJ, Raj D, Hanisch UK, Boddeke HW. Microglial phenotype and adaptation. J Neuroimmune Pharmacol. 2013;8:807-23.

[2] Hickman SE, Kingery ND, Ohsumi TK, Borowsky ML, Wang L-c, Means TK, et al. The microglial sensome revealed by direct RNA sequencing. Nat Neurosci. 2013;16:1896-905.

[3] Crain JM, Nikodemova M, Watters JJ. Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult central nervous system in male and female mice. J Neurosci Res. 2013;91:1143-51.

[4] Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J. 2005;19:1329-31.

[5] Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain Behav Immun. 2009;23:309-17.

[6] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997;236:313-22.

[7] Innamorato N, Rojo, AI, Garcia-Yague, AJ, Yamamoto, M, de Ceballos, ML, Cuadrado, A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol. 2008;181:680-9.

[8] Rojo AI, Innamorato NG, Martín-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. Glia. 2010;58:588-98.

[9] Barger SW, Goodwin ME, Porter MM, Beggs ML. Glutamate release from activated microglia requires the oxidative burst and lipid peroxidation. J Neurochem. 2007;101:1205-13.

[10] de Vries HE, Witte M, Hondius D, Rozemuller AJM, Drukarch B, Hoozemans J, et al. Nrf2-induced antioxidant protection: A promising target to counteract ROS-mediated damage in neurodegenerative disease? Free Radic Biol Med. 2008;45:1375-83.

[11] Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci. 1997;94:10367-72.

[12] Talalay P, Fahey JW, Holtzclaw WD, Prestera T, Zhang Y. Chemoprotection against cancer by Phase 2 enzyme induction. Toxicol Lett. 1995;82–83:173-9.

[13] Vermeulen M, Klöpping-Ketelaars IWAA, van den Berg R, Vaes WHJ. Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. J Agr Food Chem. 2008;56:10505-9.

[14] Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta. 2002;316:43-53.

[15] Shapiro TA, Fahey JW, Dinkova-Kostova AT, Holtzclaw WD, Stephenson KK, Wade KL, et al. Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study. Nutr Cancer. 2006;55:53-62.

[16] Townsend BE, Chen Y-J, Jeffery EH, Johnson RW. Dietary broccoli mildly improves neuroinflammation in aged mice but does not reduce lipopolysaccharide-induced sickness behavior. Nutr Res. 2014;34:990-9.

[17] Duan W, Zhang R, Guo Y, Jiang Y, Huang Y, Jiang H, et al. Nrf2 activity is lost in the spinal cord and its astrocytes of aged mice. In Vitro Cellular & Developmental Biology - Animal. 2009;45:388-97.

[18] Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu R-M, et al. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci. 2004;101:3381-6.

[19] Bocchini V, Mazzolla R, Barluzzi R, Blasi E, Sick P, Kettenmann H. An immortalized cell line expresses properties of activated microglial cells. J Neurosci Res. 1992;31:616-21.

[20] Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proc Natl Acad Sci. 2008;105:7534-9.

[21] Nikodemova M, Watters J. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. J Neuroinflam. 2012;9:147.

[22] Jurgens HA, Amancherla K, Johnson RW. Influenza infection induces neuroinflammation, alters hippocampal neuron morphology, and impairs cognition in adult mice. J Neurosci. 2012;32:3958-68.

[23] Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. 2002;62:5196-203.

[24] Konwinski RR, Haddad R, Chun JA, Klenow S, Larson SC, Haab BB, et al. Oltipraz, 3H-1,2-dithiole-3-thione, and sulforaphane induce overlapping and protective antioxidant responses in murine microglial cells. Toxicol Lett. 2004;153:343-55.

[25] Brandenburg L-O, Kipp M, Lucius R, Pufe T, Wruck C. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. Inflamm Res. 2010;59:443-50.

[26] Godbout JP, Johnson RW. Age and neuroinflammation: a lifetime of psychoneuroimmune consequences. Immunol Allergy Clin. 2009;29:321-37.

[27] Liu J, Zhao ML, Brosnan CF, Lee SC. Expression of type II nitric oxide synthase in primary human astrocytes and microglia: role of IL-1beta and IL-1 receptor antagonist. J Immunol. 1996;157:3569-76.

[28] Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, et al. Increased cytokine production in mononuclear cells of healthy elderly people. Eur J Immunol. 1993;23:2375-8.

[29] Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. J Neuroinflamm. 2004;1:14.

[30] Lucin KM, Wyss-Coray T. Immune activation in brain aging and neurodegeneration: too much or too little? Neuron. 2009;64:110-22.

[31] Dröge W, Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. Aging Cell. 2007;6:361-70.

[32] Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004;3:205-14.

[33] Liu B, Hong J-S. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther. 2003;304:1-7.

[34] Shih P-H, Yen G-C. Differential expressions of antioxidant status in aging rats: the role of transcriptional factor Nrf2 and MAPK signaling pathway. Biogerontology. 2007;8:71-80.

[35] L'Episcopo F, Tirolo C, Testa N, Caniglia S, Morale MC, Impagnatiello F, et al. Aging-Induced Nrf2-ARE Pathway Disruption in the Subventricular Zone Drives Neurogenic Impairment in Parkinsonian Mice via PI3K-Wnt/β-Catenin Dysregulation. J Neurosci. 2013;33:1462-85.

[36] Lin W, Wu RT, Wu T, Khor T-O, Wang H, Kong A-N. Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol. 2008;76:967-73.

[37] Chen X-L, Dodd G, Thomas S, Zhang X, Wasserman MA, Rovin BH, et al. Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. Am J Physiol Heart Circ Physiol. 2006;290:H1862-H70.

[38] Koh K, Cha Y, Kim S, Kim J. tBHQ inhibits LPS-induced microglial activation via Nrf2-mediated suppression of p38 phosphorylation. Biochem Bioph Res Co. 2009;380:449-53.

[39] Floyd RA, Hensley K. Oxidative stress in brain aging: Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging. 2002;23:795-807.

[40] Dawson VL, Brahmbhatt HP, Mong JA, Dawson TM. Expression of inducible nitric oxide synthase causes delayed neurotoxicity in primary mixed neuronal-glial cortical cultures. Neuropharmacology. 1994;33:1425-30.

[41] Wong ML, Rettori V, Al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, et al. Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nat Med. 1996;2:581-4.

[42] Fenn AM, Henry CJ, Huang Y, Dugan A, Godbout JP. Lipopolysaccharide-induced interleukin (IL)-4 receptor-alpha expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice. Brain Behav Immun. 2012;26:766-77.

[43] Frank MG, Barrientos RM, Watkins LR, Maier SF. Aging sensitizes rapidly isolated hippocampal microglia to LPS ex vivo. J Neuroimmunol. 2010;226:181-4.

[44] Abraham J, Jang S, Godbout JP, Chen J, Kelley KW, Dantzer R, et al. Aging sensitizes mice to behavioral deficits induced by central HIV-1 gp120. Neurobiol Aging. 2008;29:614-21.

[45] Abraham J, Johnson RW. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuv Res. 2009;12:445-53.

[46] Berg BM, Godbout JP, Chen J, Kelley KW, Johnson RW. α-Tocopherol and selenium facilitate recovery from lipopolysaccharide-induced sickness in aged mice. J Nutr. 2005;135:1157-63.

Chapter 4- Sulforaphane reduces lipopolysaccharide-induced proinflammatory markers in hippocampus and liver but does not improve sickness behavior

4.1 Abstract

Acute peripheral infection is associated with central and peripheral inflammation, increased oxidative stress, and behavioral components of sickness that include reduction in body weight and food intake, and decreased activity. Prolonged inflammation can lead to maladaptive behavioral symptoms including depressive-disorders and anxiety. The nutritional bioactive sulforaphane (SFN) displays potent anti-inflammatory properties against lipopolysaccharide (LPS)-induced inflammation *in vitro*, but has not been previously assessed *in vivo* during peripheral infection as a potential treatment for sickness behavior. Sulforaphane activates the nuclear factor E2-related factor 2 (Nrf2) pathway, which upregulates antioxidant response element genes (ARE) and lowers inflammation. In this study, LPS was used to mimic a peripheral infection. SFN was administered prior to LPS injection to examine whether SFN inhibited proinflammatory cytokines and reduced sickness behavior. We hypothesized that SFN would increase antioxidant response, reduce expression of proinflammatory cytokines in hippocampus and liver, and inhibit sickness behavior in mice challenged with LPS. In support of our hypothesis, SFN elevated ARE genes and reduced expression of proinflammatory mediators in hippocampus and liver, but surprisingly did not improve LPS-induced sickness response. These data indicate that SFN has an anti-inflammatory effect in both brain and periphery, but that longer exposure to SFN may be necessary to reduce sickness behavior.

4.2 Introduction

Peripheral inflammation has a well-established impact on the brain [1]. During acute infection, circulating cytokines trigger neuroinflammation through neural and humoral signaling pathways, resulting in increased cytokine expression in the brain that leads to behavioral changes indicative of a suppressed motivational state. These behavioral and physiological changes include decreased activity, lowered interest in socialization and pleasurable activities, and reduced food intake and body weight [2,3]. Although these adaptive sickness behaviors are a normal component of the innate immune response, amplified or prolonged neuroinflammation can lead to maladaptive sickness responses, increased absenteeism, and general feeling of discomfort [4]. Notably, previous studies have demonstrated that dietary interventions such as resveratrol and α-tocopherol reduced central inflammation and alleviated sickness behavior in mice treated with LPS [5,6]. These studies and others suggest that nutritional supplements are useful to mitigate the molecular and behavioral changes that occur during acute infection [5,7].

Oxidative stress results from reactive oxygen and nitrogen species that are generated during peripheral infection by complexes such as inducible nitric oxide synthase (iNOS) and NADPH oxidase 2 (NOX2), thus triggering the activation of redox-responsive inflammatory pathways. The brain is highly sensitive to oxidative damage, and minimizing oxidative stress through nutritional or pharmacological means has been proposed as a method of promoting long-term brain health [8]. Sulforaphane (SFN), a bioactive derived from broccoli, increases endogenous antioxidant response by upregulating genes containing the antioxidant response element (ARE) through activation of the transcription factor nuclear factor E2-related factor 2

(Nrf2). Elevated ARE gene expression in the periphery and brain is protective against cellular and tissue damage associated with and environmental toxins and other highly oxidative conditions [9,10]. In addition to its well-described antioxidant properties, the Nrf2/ARE pathway has also been reported to have anti-inflammatory effects in cell culture and animal models [11]. SFN is readily absorbed and can be administered in dietary or supplemental form, making it a potential therapeutic candidate for mitigating inflammation and oxidative stress associated with peripheral infection. However, the effects of SFN on acute sickness response have not been reported. The objective of this study was to determine whether SFN treatment reduces acute peripheral and central inflammation and inhibits sickness behavior following a LPS challenge. We hypothesized that SFN would upregulate ARE genes, and improve LPS-induced sickness behavior by reducing proinflammatory cytokines in periphery and brain.

4.3 Materials and Methods

Animals and experimentation

Adult (4-6 month-old) male BALB/c mice reared in-house were individually housed in a temperature controlled environment with a reversed phase 12 h light:dark cycle (lights out at 09:00 h). Mice were handled 1-2 min per day for one week prior to behavior testing. All studies were carried out in accordance with United States National Institutes of Health guidelines and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Sulforaphane (LKT Laboratories, St. Paul, MN) was dissolved in sterile saline immediately prior to experimentation. To assess if SFN upregulated ARE genes in liver and hippocampus in a time-dependent manner, a single dose of SFN (50 mg/kg) was administered i.p. and mice were

euthanized 2, 4, 6, or 8 h after injection. In subsequent studies, SFN (50 mg/kg) or saline was administered i.p. daily for 3 days with injections 24 h apart. On day 3, SFN and LPS (1 μg, i.p.) were co-administered. *Escherichia coli* LPS (serotype 0127:B8, Sigma, St. Louis, MO) was dissolved in sterile saline prior to injection. Treatments were administered during the first hour after onset of the dark phase of the light:dark cycle.

Sickness response

Lipopolysaccharide injection mimics peripheral infection, resulting in adaptive sickness response [6]. To determine whether SFN inhibited LPS-induced sickness response, we assessed food intake, body weight, and locomotor activity. Home cage locomotor activity was assessed 6 h after LPS. Mice were maintained in their home cage and locomotor activity was video-recorded for 5 min. The cage was divided into four equal quadrants on the video records for scoring, and the number of line crossings (all 4 paws crossing into a new quadrant) and rearings (2 paws off the ground) were counted by an investigator blinded to the treatments.

Tissue collection and analysis

Animals were euthanized via CO_2 asphyxiation 6 h after LPS and perfused with sterile ice-cold saline. Brain and liver were rapidly removed and dissected sections were flash frozen. All tissues were stored at -80°C until further processing for analysis.

To assess changes in gene expression, RNA was isolated from tissues using E.Z.N.A Total RNA kits according to manufacturer's instructions (Omega Biotek, Norcross, GA). Synthesis of cDNA was carried out using a high capacity RT kit (Applied Biosystems, Grand Island, NY). Real-

time quantitative RT-PCR (qPCR) was performed to detect changes in mRNA expression of ARE genes NQO1 (Mm.PT.58.9609207) and HMOX1 (Mm.PT.58.9675808). IL-1β (Mm.PT.58.41616450), IL-6 (Mm.PT.58.13354106), iNOS (Mm.PT.58.5680554), and CYBB (Mm.PT.58.11318181) were used to detect if proinflammatory mediators were reduced by SFN. All genes were analyzed using PrimeTime qPCR Assays (Integrated DNA Technologies, Coralville, IA) and were compared to the housekeeping control gene GAPDH (Mm.PT.39.a.1) using the $2^{-\Delta\Delta Ct}$ calculation method as previously described [12]. Data are expressed as fold change relative to controls.

Protein was extracted by homogenizing tissue in lysis buffer containing 20 mM Tris-Cl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA). IL-1β protein was quantified using a commercially available ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analyses

All data were analyzed using Statistical Analysis System (SAS, Cary, NC). Data were subjected to two-way analysis of variance (ANOVA) for main effects of SFN, LPS, and 2-way interactions. When ANOVA revealed a significant interaction, *post hoc* Student's t test using Fisher's least significant differences was used to determine mean separation. All data are expressed as means ± SEM.

4.4 Results

SFN increased ARE genes in liver and hippocampus

Increased endogenous antioxidant response following SFN exposure is thought to provide cellular protection [13]. Previous studies have indicated that SFN upregulates ARE genes in liver and brain through Nrf2 activation [14-16]. Consistent with these reports, the current study revealed that a single injection of SFN increased transcription of the NQO1 in the liver at 2 h ($p < 0.05$) and 4 h ($p < 0.01$) and increased HMOX1 at 2 h ($p < 0.0001$), 4 h ($p < 0.001$), and 8 h ($p < 0.001$) (Figure 4.1). In hippocampus, SFN increased NQO1 and HMOX1 at 8 h ($p < 0.05$ for each).

SFN reduced LPS-induced proinflammatory mediators in liver

The liver is the primary detoxification organ for LPS, where hepatic macrophages scavenge LPS [17]. LPS evoked a robust increase in proinflammatory cytokines IL-1β ($p < 0.0001$) and IL-6 ($p < 0.001$) in the liver (Figure 4.2). SFN reduced LPS induced IL-1β (SFN x LPS, $p < 0.05$) and IL-6 mRNA (SFN x LPS, $p < 0.01$). In order to assess if the changes in mRNA were reflected by changes in protein, IL-1β protein was measured. SFN reduced LPS-induced IL-1β protein in liver (SFN, $p < 0.05$; LPS $p < 0.01$).

LPS also upregulated cytochrome b-245 β (CYBB; $p < 0.05$), a functional subunit of the superoxide-producing NOX2 complex that is involved in the phagocytic capacity of macrophages. While CYBB mRNA appeared to be reduced in SFN-treated mice, the effect was not significant ($p = 0.17$).

SFN reduced proinflammatory mediators in hippocampus

Intraperitoneal injection of LPS is associated with elevated proinflammatory cytokine expression in the hippocampus which can cause cognitive and behavioral impairment [18]. To determine whether peripherally administered SFN could attenuate inflammation in the hippocampus, IL-1β and IL-6 were measured 6 h after a peripheral LPS injection. LPS increased IL-1β ($p < 0.0001$) and IL-6 ($p < 0.05$) mRNA in hippocampus (Figure 4.3). SFN reduced LPS-induced IL-1β (SFN x LPS interaction, $p < 0.05$) but did not affect IL-6. SFN also lowered basal and LPS-induced iNOS in the hippocampus ($p < 0.05$). We were unable to detect Il-1β and IL-6 protein in the brain due to concentrations lower than the assay detection limit.

SFN did not improve sickness response in LPS-treated mice

Because inflammatory cytokine signaling in the brain causes adaptive sickness behaviors, we assessed food intake, body weight, and locomotor activity 6 h after LPS (Figure 4.4). Food intake decreased in all mice treated with LPS ($p < 0.0001$). The combination of SFN and LPS also resulted in lower food intake (SFN x LPS $p < 0.05$). Body weight was decreased 6 h after LPS ($p < 0.05$). Locomotor activity was decreased in mice in mice treated with LPS ($p < 0.0001$). SFN did not improve locomotor activity in LPS-treated mice.

4.5 Discussion

The immunological changes that occur during acute peripheral infection cause neuroinflammation and sickness behaviors that negatively impact productivity and general physical and psychological well-being [19,20]. The dietary bioactive SFN is a potent inducer of

endogenous antioxidant response through the Nrf2 pathway, and is known to have anti-inflammatory effects *in vitro* [21,22]. The present study provides additional evidence that SFN upregulates antioxidant Nrf2 target genes and reduces central and peripheral inflammation. However, despite the lowered inflammatory mediators, SFN did not mitigate acute LPS-induced sickness behavior.

Liver macrophages are critical immune cells for detoxification and production of proinflammatory signaling mediators. In the present study, a robust inflammatory response to LPS was evident in the liver, resulting in increased IL-1β, IL-6, and cytochrome-b 245 β. Importantly, SFN reduced hepatic IL-1β and IL-6, demonstrating its ability to decrease peripheral inflammatory mediators. Previous studies have reported that SFN activates the Nrf2 pathway in liver, resulting in cellular defense against oxidative damage, environmental toxins, and carcinogens [16,23,24]. The present data provide additional evidence that SFN has an anti-inflammatory effect on the liver during acute LPS exposure.

Because neuroinflammation during peripheral infection has a pronounced effect on sickness behavior, we were particularly interested to see whether SFN lowered neuroinflammation. In this study, SFN reduced LPS-increased hippocampal IL-1β mRNA, supporting our hypothesis that SFN has anti-inflammatory effects on the brain. Because central IL-1β also indirectly increases oxidative stress by upregulating iNOS activity, we measured iNOS mRNA and found that SFN lowered both basal and LPS-induced expression of iNOS. Surprisingly, this reduction in neuroinflammation did not improve sickness behavior. This is likely attributed to the fact that although a significant reduction in neuroinflammatory markers was apparent, SFN did not completely inhibit neuroinflammation (i.e. IL-6 was still elevated). Additionally, while it has

been reported that peripherally administered SFN crosses the blood brain barrier and can be detected in hippocampus within minutes of injection [25], it is possible that the concentrations that the brain is exposed to are too low or transient to influence the neuroinflammatory pathways that mediate sickness behavior. Another possible reason for the lack of improvement in sickness behavior is that while SFN reduced IL-1β in hippocampus, this reduction may not have occurred in other regions of the brain. In this study, we chose to focus on the hippocampus, as the hippocampus is densely populated with microglia, a cell type that is known to be involved in the proinflammatory cytokine production that facilitates sickness behavior [26]. Furthermore, microglia are highly sensitive to the anti-inflammatory effects of SFN *in vitro* [27]. Distinct regional differences in ARE gene expression and neuroinflammation following SFN treatment have not been reported and may merit further study.

The bidirectional signaling aspect of the immune and nervous systems implies that reduction of peripheral inflammation can reduce neuroinflammation [19,28]. Several studies have reported that the dietary antioxidants α-tocopherol, resveratrol, or luteolin administered to mice prior to a peripheral immune stimulus mitigated neuroinflammation and inhibited sickness behavior [5,6,29,30], In contrast, while SFN also reduced neuroinflammatory markers, it did not improve locomotor activity or other measures of sickness response. While it is tempting to speculate that longer exposure to SFN may be able to overcome the challenges of acute inflammatory response, previously we demonstrated that a 4 week exposure to dietary broccoli (a known source of SFN) did not improve sickness behavior in mice 24 h after LPS [31]. However, dietary broccoli did not reduce elevated brain IL-1β, and it is possible that

supplemental SFN, such as administered in the present studies, would have a greater effect on sickness behavior during the 24 h recovery period following LPS.

In summary, the data presented here indicate that the anti-inflammatory peripheral effects of SFN may be more impactful than the effects in the brain. SFN's peripheral effects may be especially relevant to the chronic inflammatory conditions encountered during stress and normal aging.

4.6 Figures

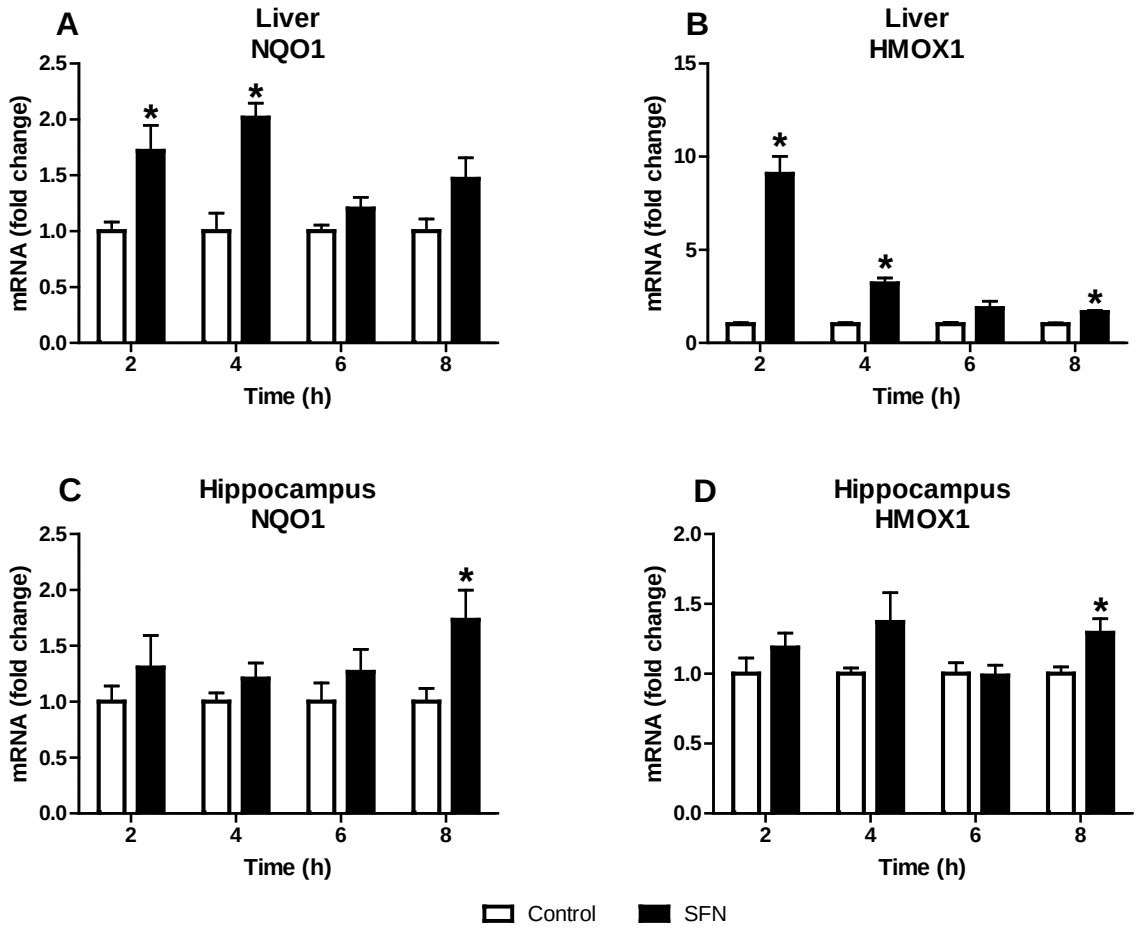

Figure 4.1. *SFN increased ARE genes in liver and hippocampus in a time-dependent manner.* (A-B) SFN increased NQO1 and HMOX1 in liver. (C-D) SFN increased NQO1 and HMOX1 in hippocampus. Bars represent means ± SEM (n = 6-7). * indicates significant main effect of LPS ($p < 0.05$).

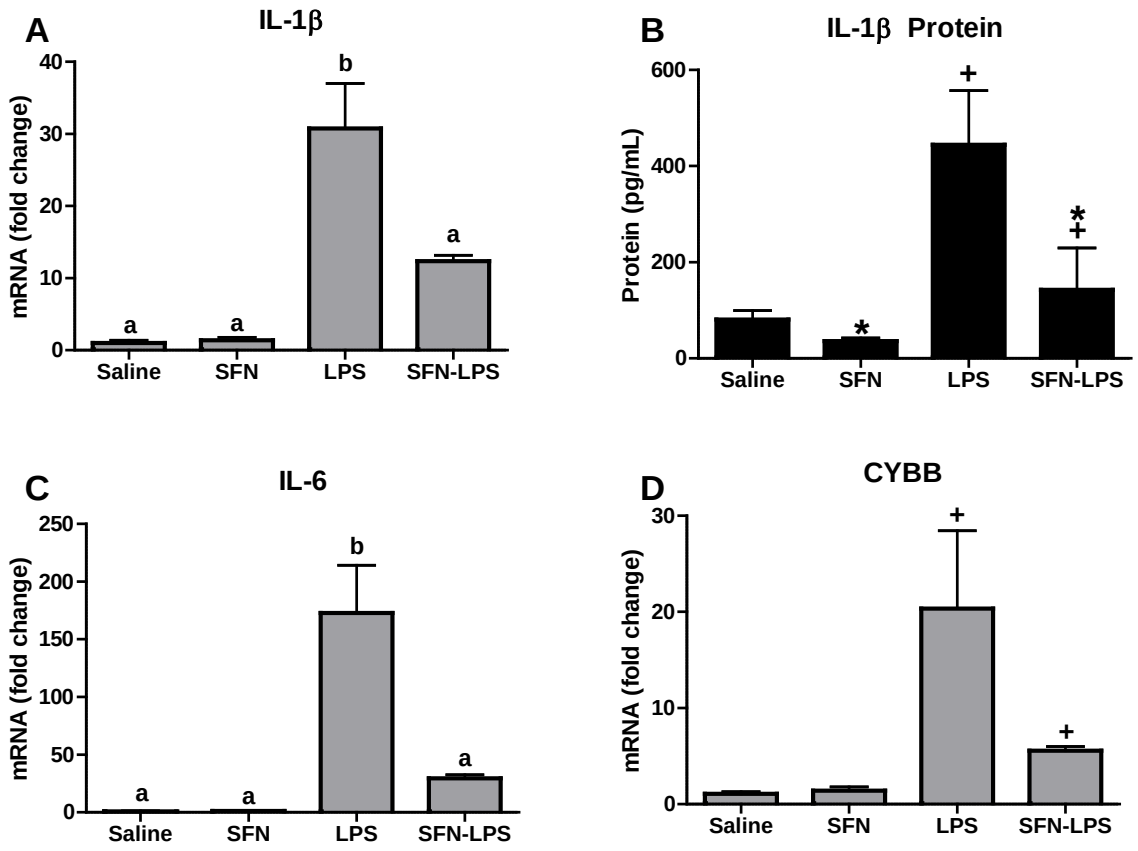

Figure 4.2. *SFN reduced LPS-induced IL-1β and IL-6 in liver*. (A-B) SFN reduced LPS-increased IL-1β mRNA and protein in liver. (C) SFN reduced LPS-induced IL-6. (D) LPS increased CYBB. Bars represent means ± SEM (n = 6-8). * indicates significant main effect of SFN and + signifies main effect of LPS ($p < 0.05$). Means with different letters are significantly different from each other ($p < 0.05$).

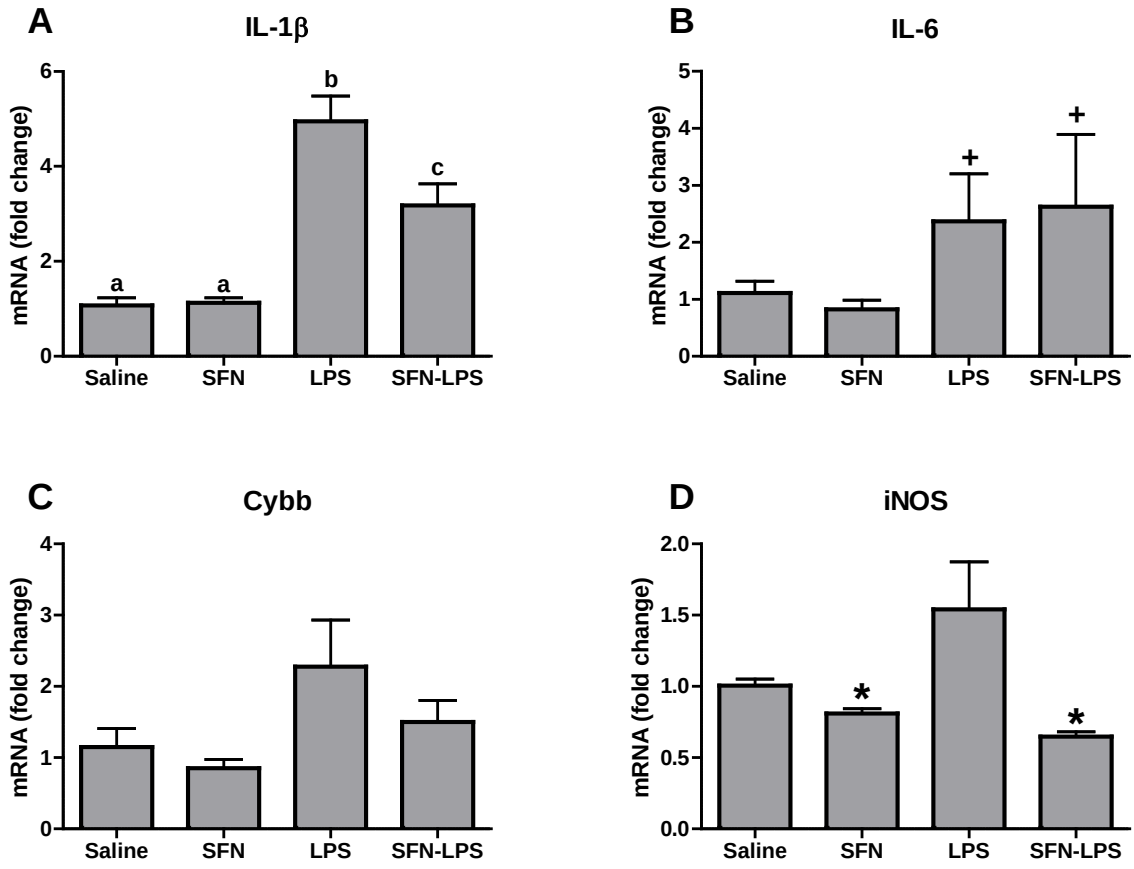

Figure 4.3. *SFN reduced IL-1β and iNOS mRNA in hippocampus*. (A) SFN reduced LPS-induced IL-1β mRNA hippocampus. (B) LPS increased IL-6. (C) CYBB was not affected by SFN or LPS. (D) SFN reduced iNOS in hippocampus. Bars represent means ± SEM (n = 6-8). * indicates significant main effect of SFN and + signifies main effect of LPS ($p < 0.05$). Means with different letters are significantly different from each other ($p < 0.05$).

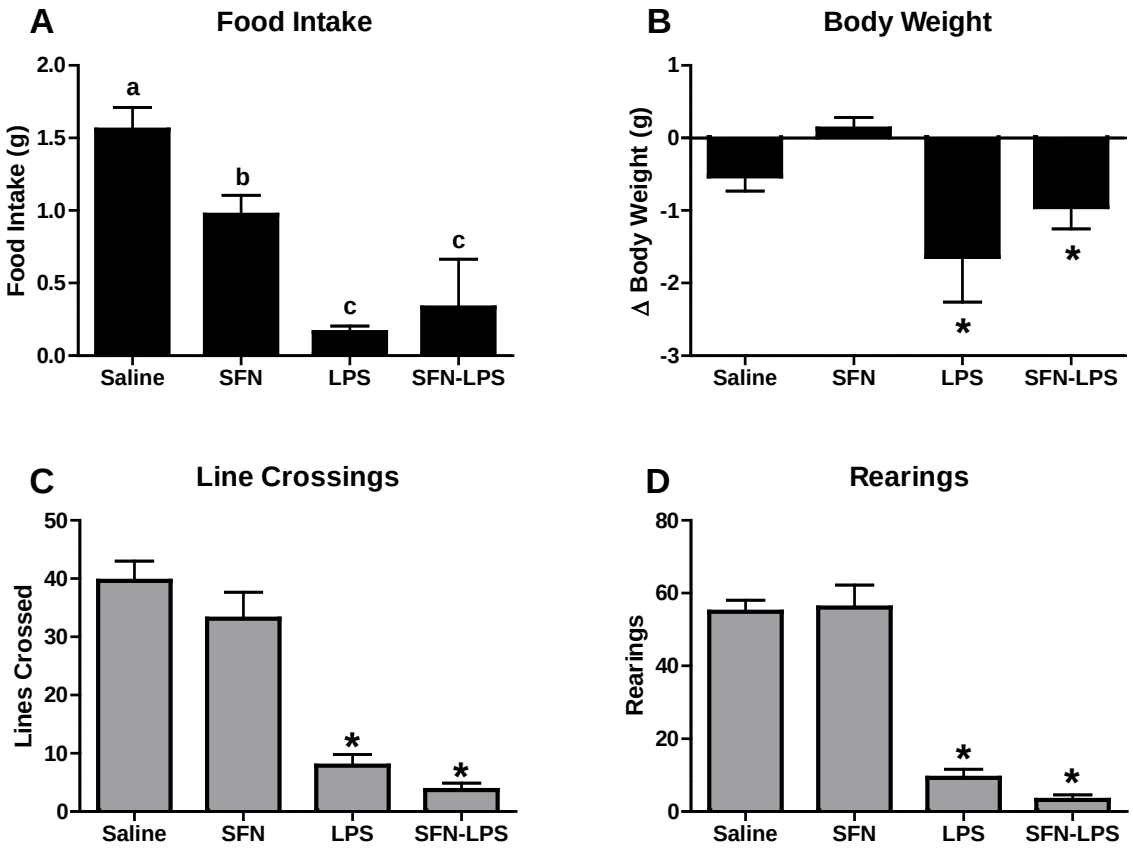

Figure 4. 4. *SFN did not improve sickness response after LPS.* (A) Food intake was reduced by LPS. (B) Body weight was decreased in LPS-treated mice. (C-D) Locomotor activity was reduced in LPS-treated mice. Bars represent means ± SEM (n = 6-8). * indicates main effect of LPS ($p < 0.05$). Means with different letters are significantly different from each other ($p < 0.05$).

4.7 References

[1] Dantzer R, Kelley K. Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun. 2007;21:153 - 60.

[2] Dantzer R, BluthÉ R-M, LayÉ S, Bret-Dibat J-L, Parnet P, Kelley KW. Cytokines and Sickness Behavior. Ann N Y Acad Sci. 1998;840:586-90.

[3] Dantzer R, Konsman J-P, Bluthé R-M, Kelley KW. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton Neurosci. 2000;85:60-5.

[4] Smith AP. Twenty-five years of research on the behavioural malaise associated with influenza and the common cold. Psychoneuroendocrino. 2013;38:744-51.

[5] Abraham J, Johnson RW. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuv Res. 2009;12:445-53.

[6] Berg BM, Godbout JP, Kelley KW, Johnson RW. Alpha-tocopherol attenuates lipopolysaccharide-induced sickness behavior in mice. Brain Behav Immun. 2004;18:149-57.

[7] Godbout JP, Berg BM, Krzyszton C, Johnson RW. α-Tocopherol attenuates NFκB activation and pro-inflammatory cytokine production in brain and improves recovery from lipopolysaccharide-induced sickness behavior. J Neuroimmunol. 2005;169:97-105.

[8] Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004;3:205-14.

[9] Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke–induced emphysema in mice. J Clin Invest. 2004;114:1248-59.

[10] Ping Z, Liu W, Kang Z, Cai J, Wang Q, Cheng N, et al. Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme. Brain Res. 2010;1343:178-85.

[11] Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. The Journal of Clinical Investigation. 2006;116:984-95.

[12] Jurgens HA, Amancherla K, Johnson RW. Influenza infection induces neuroinflammation, alters hippocampal neuron morphology, and impairs cognition in adult mice. J Neurosci. 2012;32:3958-68.

[13] Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. 2002;62:5196-203.

[14] Hu R, Hebbar V, Kim B-R, Chen C, Winnik B, Buckley B, et al. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther. 2004;310:263-71.

[15] Keck A-S, Qiao Q, Jeffery EH. Food matrix effects on bioactivity of broccoli-derived sulforaphane in liver and colon of F344 rats. J Agr Food Chem. 2003;51:3320-7.

[16] Zhao H-D, Zhang F, Shen G, Li Y-B, Li Y-H, Jing H-R, et al. Sulforaphane protects liver injury induced by intestinal ischemia reperfusion through Nrf2-ARE pathway. World J Gastroenterol. 2010;16:3002-10.

[17] Luster MI, Germolec DR, Yoshida T, Kayama F, Thompson M. Endotoxin-induced cytokine gene expression and excretion in the liver. Hepatology. 1994;19:480-8.

[18] Richwine AF, Parkin AO, Buchanan JB, Chen J, Markham JA, Juraska JM, et al. Architectural changes to CA1 pyramidal neurons in adult and aged mice after peripheral immune stimulation. Psychoneuroendocrinology. 2008;33:1369-77.

[19] Dantzer R, O'Connor J, Freund G, Johnson R, Kelley K. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci. 2008;9:46 - 56.

[20] Franche R-L, Krause N. Readiness for Return to Work Following Injury or Illness: Conceptualizing the Interpersonal Impact of Health Care, Workplace, and Insurance Factors. J Occup Rehabil. 2002;12:233-56.

[21] Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem. 2000;275:16023-9.

[22] Lin W, Wu RT, Wu T, Khor T-O, Wang H, Kong A-N. Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol. 2008;76:967-73.

[23] Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, et al. Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (−/−) mice. Cancer Lett. 2006;243:170-92.

[24] Sun X, Mi L, Liu J, Song L, Chung F-L, Gan N. Sulforaphane prevents microcystin-LR-induced oxidative damage and apoptosis in BALB/c mice. Toxicol Appl Pharmacol. 2011;255:9-17.

[25] Innamorato N, Rojo, AI, Garcia-Yague, AJ, Yamamoto, M, de Ceballos, ML, Cuadrado, A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol. 2008;181:680-9.

[26] Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT, et al. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. J Neuroinflamm. 2008;5:1-14.

[27] Brandenburg L-O, Kipp M, Lucius R, Pufe T, Wruck C. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. Inflamm Res. 2010;59:443-50.

[28] Kent S, Bluthé R-M, Kelley KW, Dantzer R. Sickness behavior as a new target for drug development. Trends Pharmacol Sci. 1992;13:24-8.

[29] Berg BM, Godbout JP, Chen J, Kelley KW, Johnson RW. α-Tocopherol and selenium facilitate recovery from lipopolysaccharide-induced sickness in aged mice. J Nutr. 2005;135:1157-63.

[30] Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. J Nutr. 2010;140:1892-8.

[31] Townsend BE, Chen Y-J, Jeffery EH, Johnson RW. Dietary broccoli mildly improves neuroinflammation in aged mice but does not reduce lipopolysaccharide-induced sickness behavior. Nutr Res. 2014;34:990-9.

Chapter 5- Dietary broccoli mildly improves neuroinflammation in aged mice but does not reduce lipopolysaccharide-induced sickness behavior[1]

5.1 Abstract

Aging is associated with oxidative stress and heightened inflammatory response to infection. Dietary interventions to reduce these changes are therefore desirable. Broccoli contains glucoraphanin, which is converted to sulforaphane (SFN) by plant myrosinase during cooking preparation or digestion. SFN increases antioxidant enzymes including NAD(P)H quinone oxidoreductase (NQO1) and heme oxygenase I (HMOX1) and inhibits inflammatory cytokines. We hypothesized that dietary broccoli would support an antioxidant response in brain and periphery of aged mice and inhibit lipopolysaccharide-induced inflammation and sickness. Young adult and aged mice were fed control or 10% broccoli diet for 28 days prior to an intraperitoneal LPS injection. Social interactions were assessed 2, 4, 8, and 24 h following LPS, and mRNA quantified in liver and brain at 24 h. Dietary broccoli did not ameliorate LPS-induced decrease in social interactions in young or aged mice. Interleukin (IL)-1β expression was unaffected by broccoli consumption but was induced by LPS in brain and liver of adult and aged mice. Additionally, IL-1β was elevated in brain of aged mice without LPS. Broccoli consumption decreased age-elevated cytochrome b-245 β, an oxidative stress marker, and reduced glial activation markers in aged mice. Collectively, these data suggest that 10% broccoli diet provides a modest reduction in age-related oxidative stress and glial reactivity, but is

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insufficient to inhibit LPS-induced inflammation. Thus, it is likely that SFN would need to be provided in supplement form to control the inflammatory response to LPS.

5.2 Introduction

Aging is accompanied by chronic low-grade inflammation and increased oxidative stress, both of which are common factors in the pathology of chronic diseases [1,2]. Chronic inflammation leads to cognitive deficits and increases likelihood of developing neurodegenerative disease [3]. The aging brain is highly sensitive to inflammatory mediators generated in the periphery, evidenced by the molecular and behavioral changes that follow a peripheral immune stimulus such as infection, lipopolysaccharide (LPS) endotoxin, or stress [4-6]. In fact, LPS-challenged aged mice exhibit exacerbated inflammation in the brain compared to adult mice [6,7]. Exaggerated expression of inflammatory mediators associated with immune activation in the aged signifies a need to identify interventions that attenuate age-related inflammation and oxidative stress both centrally and peripherally.

The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is the primary transcriptional regulator of the cellular antioxidant response and is increasingly implicated in longevity and protection from inflammation. Declining Nrf2 activity may also be involved in the deleterious neurocognitive decline associated with aging [8-10]. The broccoli-derived bioactive sulforaphane (SFN) elicits activation of the Nrf2 antioxidant pathway, which protects tissues from toxic and carcinogenic insult by promoting transcription of genes containing the antioxidant response element (ARE) [11-13]. Due to the cytoprotective nature of Nrf2, activation of the Nrf2 pathway may be a good therapeutic target for reducing oxidative and

immune stress associated with chronic low-grade inflammation. In addition to evoking a Nrf2-dependent antioxidant response, SFN also displays anti-inflammatory effects *in vitro*, which generates further interest in SFN and foods rich in SFN as potential therapeutic candidates for chronic inflammatory diseases [14,15]. As highlighted in a recent review article, the beneficial effects of sulforaphane have also been demonstrated in a number of experimental animal models, with evidence strongly suggesting that sulforaphane is a versatile treatment for inflammation and oxidative stress [16].

Significant advances have been made in understanding the biochemical mechanisms underlying SFN-mediated activation of Nrf2 and its physiological effects, but minimal research has examined whether whole broccoli consumption influences age-associated inflammation. Broccoli provides a rich dietary source of vitamins, minerals, and flavonoids, but the unique nature of its health-promoting benefits, including cancer prevention and increased endogenous antioxidant production, have been associated with its naturally high levels of glucoraphanin [17-19]. Glucoraphanin is enzymatically hydrolyzed to the bioactive isothiocyanate SFN during crushing, chewing, or digestion of broccoli. Frequent intake of broccoli is associated with lowered risk of cancer and elevation of antioxidant enzymes [20,21]. Therefore, clinical research involving dietary supplementation with broccoli has focused primarily on chemoprevention and detoxification through activation of Phase II enzymes. Despite the accumulating evidence that SFN reduces inflammatory markers in cell culture and protects against oxidative stress during brain injury *in vivo*, the effects of dietary broccoli on peripheral and central inflammation in adult and aged animals have not been thoroughly investigated. Our objective was to examine whether dietary broccoli reduces LPS-induced inflammatory

markers in brain or liver of aged mice, and whether dietary broccoli could alter the sickness behavior response to LPS. We hypothesized that dietary broccoli would support an antioxidant response in brain and periphery of aged mice and inhibit LPS-induced inflammation and sickness behavior. In order to test this hypothesis, we used a pre-clinical murine model to investigate whether four weeks of dietary supplementation was sufficient to decrease markers of inflammation and reduce sickness behavior in adult and aged mice challenged with LPS. Sickness behavior and molecular inflammatory response has been well-characterized in our model of LPS-challenged aged mice, and these measurements will provide useful information for determining if broccoli supplementation attenuates behavioral complications of inflammation. A reduction in LPS-induced proinflammatory markers in the broccoli-supplemented mice would indicate that broccoli is a suitable dietary addition to temper inflammation.

5.3 Methods and Materials

Animals and experimental diets

Adult (4-month-old) and aged (18-month-old) BALB/c mice reared in-house were individually housed in a temperature controlled environment with a reversed phase light:dark cycle (lights on 8:00 pm). During the 28-day experimental period, mice were given ad libitum access to water and diet consisting of AIN-93M or AIN-93M + 10% freeze-dried broccoli (Table 1). Soy oil was replaced with corn oil to mitigate any potential anti-inflammatory effects derived from increased omega-3 fatty acid content of soy oil. The broccoli used in the diet provided 5.22 µmol SFN/g as determined by laboratory hydrolysis using the methods described by Dosz

and Jeffery [22]. Therefore, it is estimated that mice fed the 10% broccoli diet were exposed to 0.5 µmol glucoraphanin per g of diet consumed, providing up to 0.5 µmol SFN/g, depending upon the extent of glucoraphanin hydrolysis. To diminish the potential for degradation of glucosinolates from the broccoli-containing diet, both diets were replaced every other day. Body weight (BW) was recorded weekly. Mice were handled 1-2 min per day for one week prior to behavior testing. All studies were carried out in accordance with United States National Institutes of Health guidelines and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Immune challenge

Escherichia coli LPS (serotype 0127:B8, Sigma, St. Louis, MO) was dissolved in sterile saline prior to experimentation. On day 29 of dietary intervention, mice from each diet group (n = 7) were given LPS (0.33 mg/kg BW) or saline intraperitoneally (i.p.). Treatments were administered during the first hour after onset of the dark phase of the light:dark cycle.

Behavioral testing

To determine whether broccoli diet reduced sickness behavior, social exploratory behavior was assessed in all mice 2, 4, 8, and 24 h after treatment, as previously described in detail [23]. Base-line social exploratory behavior was determined 24 h prior to treatment and was used as a basis of comparison for calculating percent baseline time spent investigating a novel juvenile. A novel juvenile conspecific mouse was placed inside a protective cage before being placed in the home cage of the experimental mouse. Social interactions were video-recorded for 5 min and

scored by an experimenter blinded to the treatments. Social exploration is determined as the amount of time spent investigating the juvenile (sniffing, in close proximity to the juvenile) and is reported as percent of baseline.

Tissue collection and analysis

Animals were euthanized via CO_2 asphyxiation 24 h after treatment, perfused with sterile ice-cold saline, then brain and liver tissues were dissected and flash frozen. All tissue samples were stored at -80°C until further processing for analysis.

RNA was isolated using E.Z.N.A. Total RNA kits according to the manufacturer's instructions (Omega Biotek, Norcross, GA). Synthesis of cDNA was carried out using a high capacity RT kit (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions. Real-time quantitative RT-PCR (qPCR) was performed to detect changes in mRNA expression of ARE genes NQO1 (Mm.PT.56a.9609207) and HMOX1 (Mm.PT.56a.9675808) and the transcription factor Nrf2 (Mm.PT.56a.29108649M). The inflammatory cytokine IL-1β (Mm.PT.56a.41616450) was used as a marker to detect if inflammatory cytokine production was reduced in animals fed the broccoli diet. The glial activation markers GFAP (Mm.PT.56a.6609337.q), CD11b (Mm.PT.56a.9189361), MHC-II (Mm.PT.56a.43429730), and CX3CR1 (Mm.PT.56a.17555544) were used to determine whether astrocyte and microglial activation was affected by dietary intervention. All genes were analyzed using PrimeTime qPCR Assays (Integrated DNA Technologies, Coralville, IA) and were compared to the housekeeping control gene GAPDH (Mm.PT.39.a.1) using the $2^{-\Delta\Delta Ct}$ calculation method as previously described [24]. Data are expressed as fold change versus control diet mice treated with saline.

Statistical analyses

All data were analyzed using Statistical Analysis System (SAS, Cary, NC). Data were subjected to three-way analysis of variance (ANOVA) for main effects of age, diet, and LPS, and all 2- and 3-way interactions. Where ANOVA revealed a significant interaction, *post hoc* Student's t test using Fisher's least significant differences was used to determine mean separation. All data are expressed as means ± standard error of mean (SEM).

5.4 Results

LPS increased glial markers while broccoli diet reduced GFAP and increased CX3CR1 mRNA in aged mice

ARE gene expression is elevated in glial cells treated with SFN, indicating that glia may be sensitive to the protective benefits of SFN [25-27]. Because glial cells are also the predominant producers of proinflammatory mediators in brain, we measured expression of several markers of glial reactivity. Glial fibrillary acidic protein (GFAP) was elevated in brain of aged mice ($p < 0.001$). Interestingly, broccoli diet lowered expression of GFAP in aged mice (Age x Diet interaction, $p < 0.05$) (Figure 5.1). To determine whether dietary broccoli could decrease neuroinflammation in response to systemic LPS, we measured markers of glia reactivity in LPS or saline treated mice. GFAP was increased in mice given LPS ($p < 0.05$). The microglial activation markers MHC-II and CD11b were quantified to examine reactivity in microglia. MHC-II expression was not changed 24 h following LPS, but LPS did induce higher CD11b expression ($p < 0.0001$). Diet had no effect on the expression of these microglial activation markers at 24 h after LPS treatment.

Fractalkine receptors (CX3CR1) expressed on microglia provide a regulatory mechanism by which microglia activity is regulated in brain. Aged mice reportedly have decreased CX3CR1 resulting in decreased immunoregulatory signals to microglia leading to prolonged activation state following LPS [28]. CX3CR1 was induced by LPS ($p < 0.05$). Broccoli diet increased CX3CR1 mRNA in aged mice (Age x Diet interaction; $p < 0.05$), suggesting another potential role of dietary broccoli in reducing glial reactivity.

Broccoli diet did not attenuate LPS-induced reduction in social interactions

To evaluate whether dietary broccoli reduced sickness after an acute peripheral immune challenge, we used the social exploratory test. LPS decreased social investigation 2, 4, 8, and 24 h after LPS (LPS x Time interaction, $p < 0.05$) (Figure 5.2). Broccoli diet did not significantly influence social behavior.

Age and LPS increased proinflammatory IL-1β mRNA in liver and brain

Because IL-1β is known to play a significant role in sickness behavior, IL-1β expression was quantified in both central and peripheral tissues [29,30]. Aged mice had elevated basal IL-1β in brain (Figure 5.3). These results are consistent with previous studies that demonstrated increased IL-1β in aged mice and exaggerated expression to LPS [3,6,31]. LPS significantly increased IL-1β mRNA in liver and brain of both adult and aged mice ($p < 0.001$). The broccoli diet did not affect brain IL-1β levels in control or LPS-treated mice. Although broccoli diet appeared to decrease IL-1β in control and LPS-treated aged mice, there was no main effect of diet and the Age x Diet interaction was not significant ($p = 0.12$).

CYBB was elevated in liver of aged mice

NADPH oxidase generates neurotoxic and hepatotoxic reactive oxygen species that have been implicated in development of chronic disease [32,33]. Cytochrome B-245 β (CYBB) is a functional component of the NADPH oxidase system that contributes to release of free radicals from phagocytic cells. We examined whether broccoli attenuated CYBB expression. An Age x Diet interaction revealed that CYBB expression was increased in the liver of aged control animals compared to adult control animals ($p < 0.05$), and broccoli diet tended to prevent the elevation in CYBB in aged mice ($p < 0.10$) (Figure 5. 4).

Broccoli did not significantly influence the Nrf2 pathway

Several studies demonstrate that broccoli consumption increases Nrf2-inducible antioxidant enzyme activity in colon and liver tissue, presumably through SFN absorbed from dietary broccoli [34]. Importantly, SFN also induces Nrf2 antioxidant pathway in brain leading to increased ARE gene expression that provides neuroprotective benefits [35,36]. To determine whether this anti-inflammatory effect is exhibited in brain or periphery of aged mice following dietary broccoli consumption, we measured Nrf2 gene expression after four weeks of control or broccoli diet in mice given LPS or saline. Broccoli diet marginally increased Nrf2 expression in brain of LPS-treated mice, although this increase did not reach significance ($p < 0.10$). LPS did not induce Nrf2 expression at 24 h post-treatment (Fig. 5). Neither diet, treatment, nor age effected Nrf2 expression in liver.

NQO1 increased in liver of aged mice ($p = 0.05$). Analysis of brain tissue revealed an Age x Diet x Treatment interaction ($p < 0.05$), where increased NQO1 expression was most evident in

mice fed broccoli diet and given LPS. LPS increased HMOX1 expression in brain and liver ($p < 0.01$), but dietary broccoli had no affect (Figure 5.6).

5.5 Discussion

Dietary interventions that reduce aging-related inflammation garner significant research interest. While broccoli and broccoli sprouts are drawing increased interest from medical and nutritional scientists, much of the research focus has been centered on the benefits of dietary broccoli for cancer treatment and prevention. In the present studies, we focused on the anti-inflammatory properties of compounds found in whole broccoli and sought to determine whether a broccoli-supplemented diet was beneficial for attenuating systemic and central inflammation in aged mice. In these studies, four weeks of feeding a 10% freeze-dried broccoli diet mildly improved markers of glial reactivity in aged mice and tended to prevent age-induced increase in hepatic CYBB. In contrast to *in vitro* studies in which supra-physiological concentrations of SFN reduced LPS-induced proinflammatory cytokines, dietary broccoli did not reduce proinflammatory cytokines in mice that were challenged with LPS.

CYBB expression is regulated by a number of transcription factors, including the redox sensitive nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Our data and others suggest that CYBB expression increases with age, which may contribute to increased oxidative stress that occurs with age [33,37]. While CYBB expression levels are not a direct indication of ROS, transcriptional regulation of CYBB has a marked impact on ROS production [38,39]. We demonstrate that dietary broccoli may prevent the age-induced elevation in CYBB, which may hold significance for reducing increased oxidative stress associated with aging.

Using both *in vitro* and *in vivo* models, SFN conveys Nrf2-dependent neuroprotective effects to cultured astrocytes and microglia and to brain regions including hippocampus, striatum, and cortex [36,40,41]. Consistent with previously published data, we saw transcriptional increases in GFAP in aged mice, suggesting increased astrocyte reactivity [42]. Interestingly, broccoli diet down-regulated LPS-induced GFAP expression in aged mice suggesting that astrocytes may be sensitive to low circulating levels of SFN achieved from consuming dietary broccoli. This finding may demonstrate that based on the juxtaposition of astrocytes with brain blood vessels, astrocytes may be better positioned to respond to the anti-inflammatory effects of SFN. To our knowledge, this is the first evidence to suggest that dietary broccoli influences GFAP. In light of this, it would be interesting to further examine the effects of feeding a broccoli-supplemented diet to mice on changes in surface expression of glial reactivity markers in primary culture. This has been tested to some extent with SFN, but to our knowledge, not with dietary broccoli. We also observed evidence of microglia or perivascular macrophage reactivity. Increased expression of the genetic marker for microglia/macrophage activation, CD11b, was expectedly increased in animals treated with LPS. Expression of CD11b was unaffected by diet suggesting that neither microglia nor brain resident macrophages were responsive to the beneficial effects of a broccoli diet in our model. This was surprising, given that microglia and macrophages are robust producers of reactive oxygen and nitrogen species during inflammatory stimulation. However, these cells are also quite sensitive to LPS induced inflammation and the dose of LPS used may have overwhelmed the beneficial effects of dietary broccoli. These data indicate that gliosis induced by a peripheral stimulus is aggravated by age, and that dietary broccoli may reduce aging-associated glial reactivity.

The fractalkine ligand (CX3CL1) and fractalkine receptor (CX3CR1) is an important regulatory system for tempering the microglial response following activation from endogenous and exogenous immune stimuli. Indeed, mice with a genetic deletion of CX3CR1 have an exaggerated microglial inflammatory response and increased duration of sickness behavior compared to wild-type mice. CX3CR1 knockout mice have a similar response to LPS treatment as to that observed in aged animals [28,43,44]. Additionally, it has been demonstrated that LPS decreases CX3CR1 at both the mRNA and protein level in microglia [28]. We observed an LPS-induced decrease in CX3CR1 expression in our model that was prevented in aged animals given LPS and fed broccoli diet. These data suggest that aged animals that consume dietary broccoli may have suppressed microglial activation compared to animals that do not consume broccoli in the diet and therefore may have improved long term brain health, e.g. improved neuron survival and increase in neurogenesis, when confronted with infectious disease due to potential suppression of microglial hyperactivity that has been described in aged mice [28,45]. Consumption of broccoli has not been previously reported to upregulate CX3CR1 and it is important to note that additional research is required to determine if the increase in mRNA translates to cell surface expression on primary microglia and whether this change promotes long term brain health due to attenuated microglia activation.

Increased glia reactivity is accompanied by elevated IL-1β [44,46]. Since broccoli diet decreased markers of glial reactivity in aged mice, we examined IL-1β expression to determine if broccoli diet attenuated additional inflammatory mediators. IL-1β is a key inflammatory cytokine in both the peripheral and central immune response [47]. IL-1β induces sickness behaviors such as anorexia, decreased locomotion, and social activity when exogenously

administered while inhibition of IL-1β signaling attenuates sickness behaviors in response to LPS treatment [30,48,49]. For these reasons, we hypothesized that broccoli diet would exert an anti-inflammatory benefit by inhibiting IL-1β expression and this would attenuate LPS-induced sickness behaviors. In the present study, decreased social behavior was paralleled by increased IL-1β in brain, but there was no evidence that broccoli diet moderated LPS-induced sickness behavior. It is possible that the dose of LPS used (0.33 mg/kg) overwhelmed the anti-inflammatory dietary effects of consuming broccoli. It is also likely that the anorexic effect of LPS-induced sickness limited broccoli intake, resulting in lowered SFN exposure. Indeed, we observed that 24 h food consumption was decreased in LPS-treated mice compared to saline controls (data not shown). SFN is metabolized and excreted rapidly following broccoli consumption, and metabolites are not retained in tissue past 24 h [50-52]. It seems plausible that diminished intake of broccoli during the 24 h sickness period could account for the lack of effectiveness against acute peripheral inflammation. Our findings are in contrast to other studies where dietary luteolin, resveratrol, or α-tocopherol and selenium improved LPS-induced sickness behavior in aged mice. Collectively these nutritional interventions suggest that dietary supplements are a viable therapeutic vehicle to ameliorate prolonged sickness in aged models [31,53,54]. A more successful approach may be to incorporate SFN in supplement form into the diet. In agreement with this approach, some studies have demonstrated reduced neuroinflammation using purified SFN given intraperitoneally at doses of 50 mg/kg, which is several-fold higher than could be reasonably obtained through the 10% broccoli diet [36,41]. It remains to be determined whether the concentrations of SFN that were necessary to achieve

reductions in inflammatory markers in these studies can be obtained through voluntary dietary consumption.

Broccoli was selected for this study because it is a frequently consumed glucoraphanin-containing vegetable [55,56]. Although the SFN-precursor glucoraphanin is more concentrated in broccoli sprouts compared to broccoli, high glucosinolate content adds additional bitterness to the taste of the sprout, making it less palatable than broccoli [12,57]. Preparation of freeze-dried broccoli has been optimized to preserve glucosinolates and prevent inactivation of myrosinase. This is particularly important because SFN is not stable and is more bioactive when fed to rats in its glucosinolate precursor form than when hydrolyzed prior to being fed to rodents [34]. Addition of 10-20% freeze-dried broccoli to rodent diet has been reported to increase activity of hepatic and colonic ARE enzymes [58-60]. In contrast to these reports, 10% broccoli diet utilized in our studies did not increase ARE genes in brain or liver tissue of aged mice. However, in this study, HMOX1 was induced by LPS, suggesting that this gene is activated in response to increased oxidative stress generated by LPS-induced inflammation [61]. HMOX1 is an endogenous antioxidant that inhibits inducible nitric oxide synthase in LPS-stimulated macrophages, and higher HMOX1 mRNA and protein is associated with an anti-inflammatory macrophage phenotype [62-64]. Although HMOX1 is notable as part of the antioxidant cascade activated by Nrf2, HMOX1 mRNA expression was also responsive to inflammation induced by LPS. Induction of HMOX1 by LPS in our model was an expected component in agreement with findings indicating that, in addition to containing a Nrf2-inducible ARE promoter region, HMOX1 is upregulated by the proinflammatory NFκB transcriptional pathway that is strongly activated by LPS [65]. Based on our findings, HMOX1 appears to be more transcriptionally responsive to

activation of NFκB during inflammation than to 10% broccoli diet. A 10% broccoli diet may be insufficient to elevate SFN levels in circulation to temper acute inflammation in mice. In agreement with this suggestion, Innamorato et al. reported that HMOX1 protein is induced in the brain by a high dose of SFN injected i.p. [36], but there are no published data reporting *in vivo* induction of HMOX1 transcription and translation following low doses of SFN such as that obtained when consuming broccoli supplemented diet. A clinical study that examined gene expression in gastric mucosa after consumption of broccoli soup reported that while several antioxidant genes were elevated in gastric mucosa, only a fraction of genes previously induced by SFN *in vitro* were altered by the broccoli soup [66]. It is evident that additional pre-clinical and clinical studies are needed to determine effective timing and dosage of broccoli inclusion in the diet.

Another explanation for the lack of ARE gene expression induced by broccoli diet is that other peripheral tissues, such as intestine or resident macrophages of the peritoneum, may be more sensitive to broccoli supplemented diet. Several phamacokinetic studies report that SFN metabolites are widely distributed in tissues following orally administered SFN, but bioactivity in these tissues is not well-established and distribution of SFN does not appear to correlate with tissue-specific bioactivity [52,67]. Based on our findings, dietary broccoli is insufficient to upregulate HMOX1 and NQO1 in liver and brain. Future studies will help to determine if broccoli supplemented diets are more beneficial in low-grade peripheral inflammatory conditions than acute conditions such as LPS.

An apparent limitation to this study is that reduced food intake is part of the natural sickness response to LPS. Decreased intake of dietary broccoli in LPS-injected mice on the final

day of the study may have interfered with acute effects that would have been apparent if the mice ate as usual. The overall lack of effects due to dietary broccoli may have been due to reduced food intake.

In summary, we have demonstrated that consumption of a 10% broccoli diet mildly reduced neuroinflammation in aged mice by preventing upregulation reactive glia markers. However, we did not find evidence to support our hypothesis that LPS-induced inflammatory markers and sickness behavior could be attenuated by dietary broccoli. While these data do not support a role for broccoli consumption in suppressing sickness behaviors associated with an LPS-induced acute inflammatory response, they do not rule out that components found in broccoli, such as SFN, may be beneficial when consumed in pharmacological doses via supplementation. Taken together, our data suggest potential health benefits for the aged human population using dietary broccoli to improve the low-grade neuroinflammation that is associated with aging.

5.6 Figures and Tables

	AIN-93M	AIN-93M + 10% Broccoli
Protein	14.7%	14.7%
Carbohydrate	75.9%	75.9%
Fat	9.4%	9.4%
Freeze-dried broccoli (*Brassica oleracea L. cv "*Green Magic")	--	100 g
Casein	140 g	113.6 g
Corn Starch	495.7 g	473.8 g
Maltodextrin 10	125 g	110.1 g
Sucrose	100 g	99.1 g
Cellulose	50 g	25.7 g
L-Cystine	1.8 g	1.8 g
Mineral Mix	35 g	35 g
Vitamin Mix	10 g	10 g
Choline Bitartrate	2.5 g	2.5 g
Corn oil	40 g	36.5 g

Table 5.1. Ingredient composition of the diets fed to mice[a,b]

[a] Broccoli was grown at the University of Illinois, Urbana. All other diet ingredients were purchased from Harlan Laboratories (Indianapolis, IN).

[b] Nutrient content of broccoli was obtained from the USDA Nutrient Database for Standard Reference

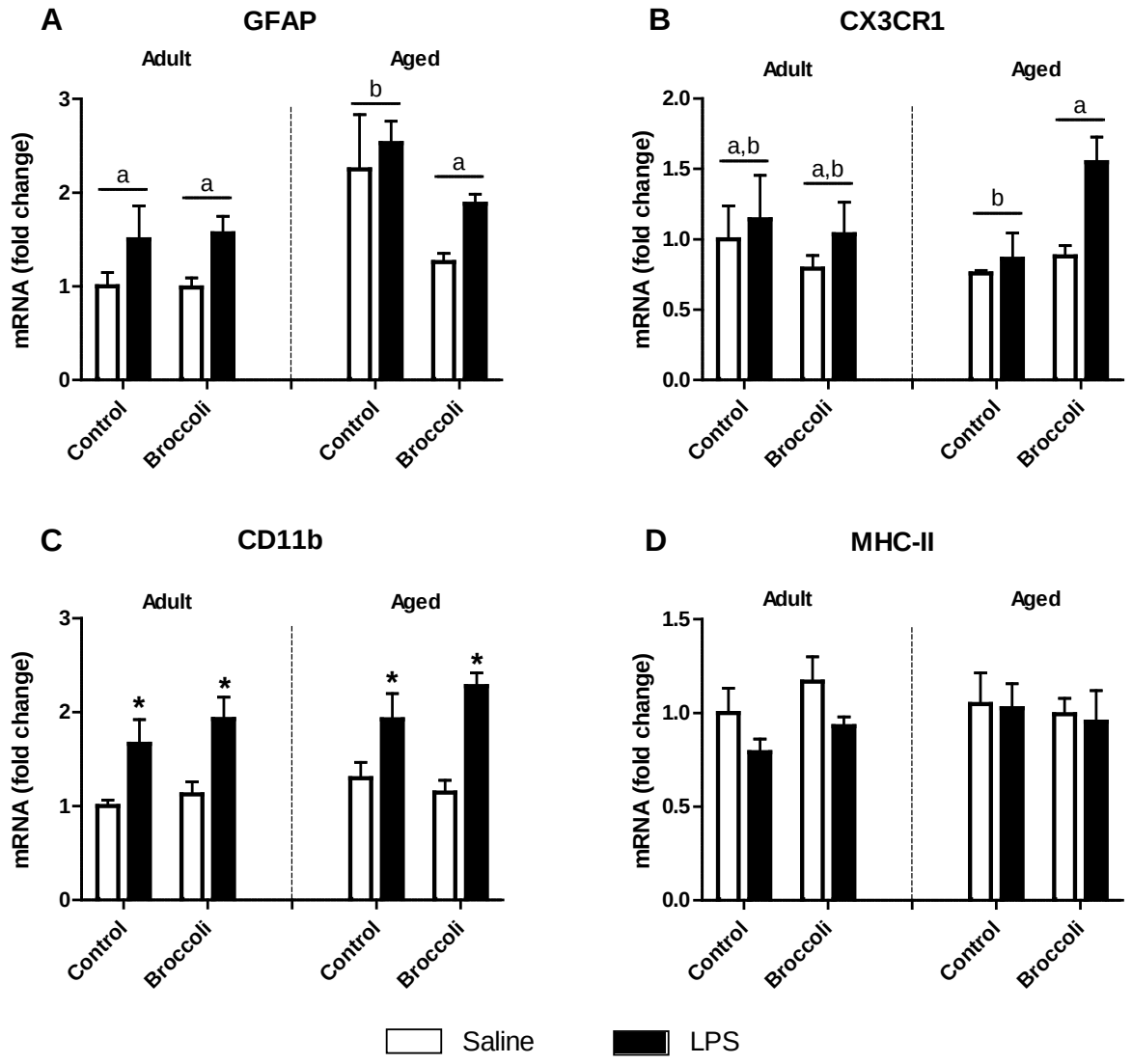

Figure 5.1. *LPS increased glial markers while* b*roccoli diet reduced GFAP and increased CX3CR1 mRNA in aged mice. (*A) GFAP was elevated by age ($p < 0.001$) and by LPS ($p < 0.05$). Broccoli diet decreased GFAP in aged mice. (B) CX3CR1 increased with LPS ($p < 0.05$). Broccoli diet increased CX3CR1 in aged mice compared to adult controls. (C) CD11b was elevated by LPS. (D) No change was evident in MHC-II after LPS. Data are presented as means ± SEM (n = 5-7). Means with different letters are statistically significant. * $p < 0.05$ compared to adult saline controls.

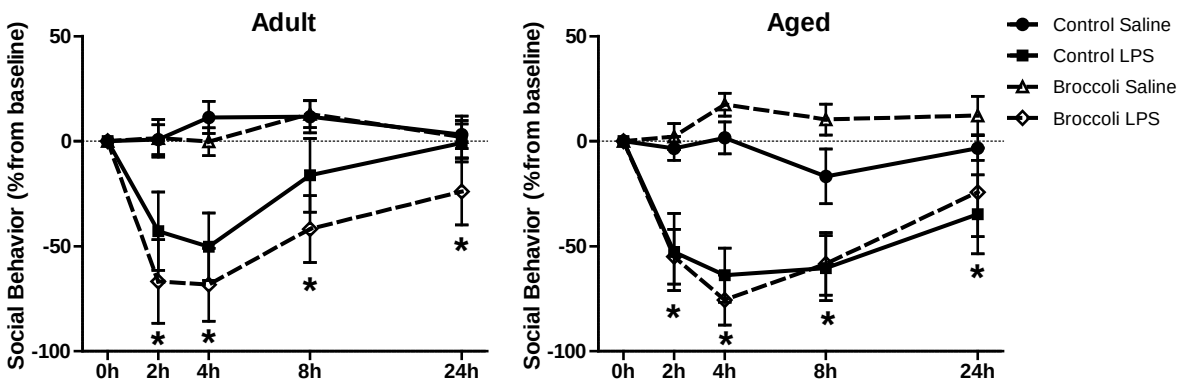

Figure 5.2. *LPS decreased social interactions similarly in mice fed control or broccoli supplemented diet.* Data are means ± SEM (n = 5-7). * indicates LPS x time effect $p < 0.05$ compared to saline controls.

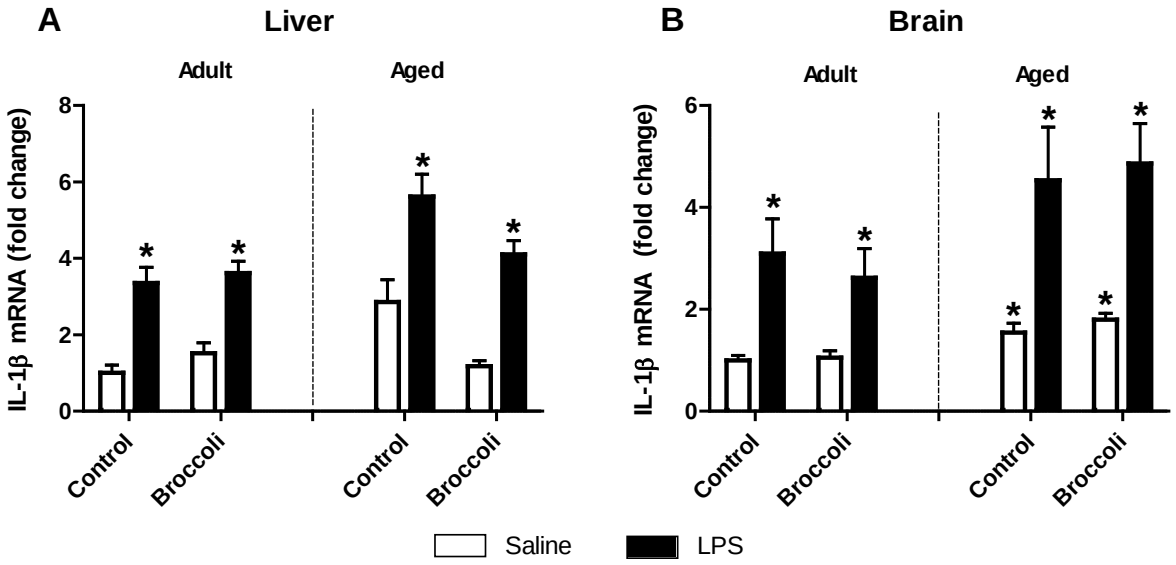

Figure 5.3. *IL-1β mRNA was increased in aged mice and all mice given LPS in liver and brain*. (A) IL-1β was increased 24 h after LPS in liver ($p < 0.001$) (B) IL-1β was elevated in brain of aged mice ($p < 0.01$) and increased 24 h after LPS ($p < 0.0001$). Bars represent means ± SEM (n = 5-7). * $p < 0.05$ compared to adult saline controls.

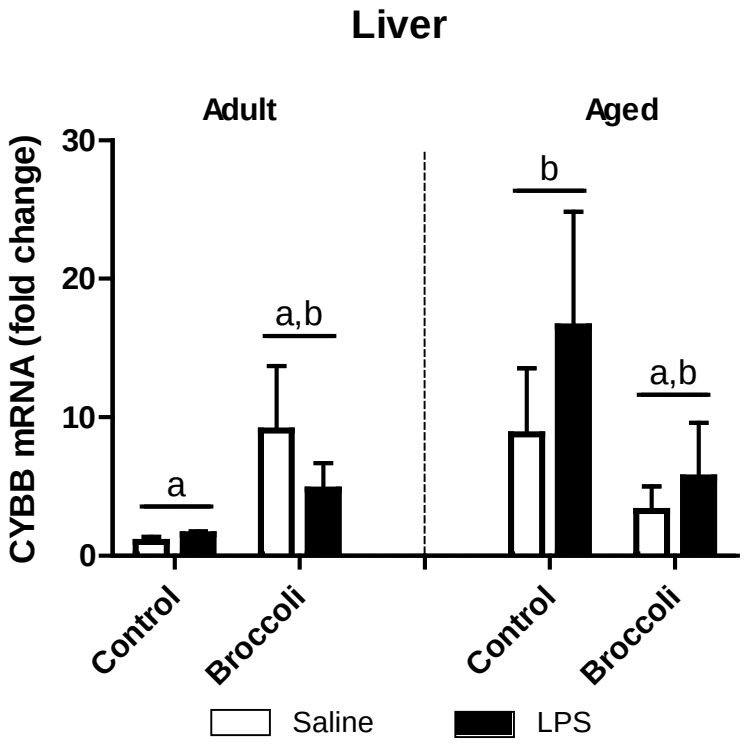

Figure 5.4. *CYBB was elevated in liver of aged mice.* CYBB was elevated in the aged control diet group compared to adult controls (Age x Diet interaction, $p < 0.05$). In aged mice, broccoli diet tended to decrease CYBB compared to control diet ($p < 0.10$). Values are means ± SEM (n = 5-7).

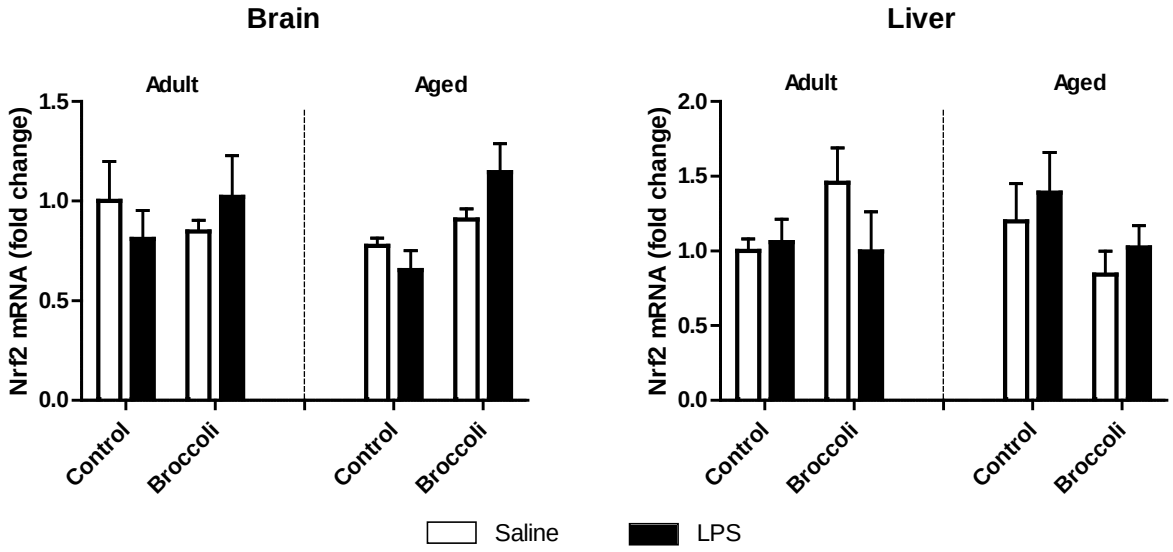

Figure 5.5. *Broccoli diet did not increase Nrf2 mRNA*. Broccoli and LPS tended to increase Nrf2 in brain 24 h after LPS (Age x Diet interaction $p < 0.10$). Data are represented as means ± SEM (n = 5-7).

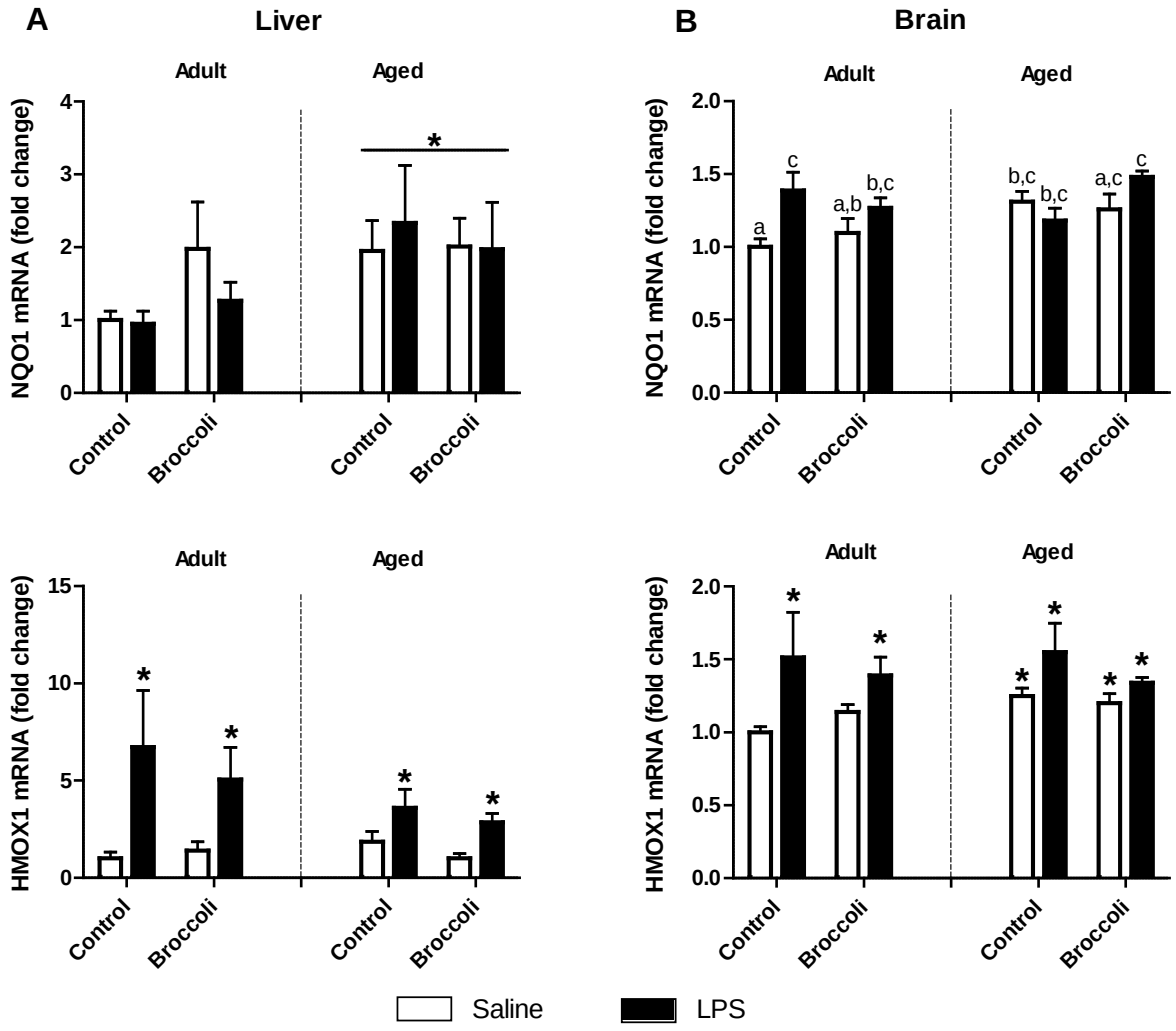

Figure 5.6. *NQO1and HMOX1 in brain tissue were increased in aged mice and all LPS-treated mice.* (A) NQO1 was increased in liver of aged mice ($p = 0.05$). HMOX1 was increased in liver 24 h after LPS ($p < 0.01$). (B) NQO1 was increased in brain of aged mice ($p < 0.05$) and was elevated 24 h after LPS ($p < 0.01$). Means with different letters are statistically significant (Age x Diet x LPS interaction $p < 0.05$). Bars represent means ± SEM ($n = 5$-7). * $p < 0.05$ compared to adult saline controls.

5.7 References

[1] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci. 1993;90:7915-22.

[2] Harman D. Free radicals in aging. Mol Cell Biochem. 1988;84:155-61.

[3] Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behav Immun. 2008;22:301-11.

[4] Buchanan JB, Sparkman NL, Chen J, Johnson RW. Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice. Psychoneuroendocrino. 2008;33:755-65.

[5] Effros RB, Walford RL. Diminished T-cell response to influenza virus in aged mice. Immunology. 1983;49:387-92.

[6] Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J. 2005;19:1329-31.

[7] Dilger RN, Johnson RW. Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. J Leukocyte Biol. 2008;84:932-9.

[8] Calkins MJ, Johnson DA, Townsend JA, Vargas MR, Dowell JA, Williamson TP, et al. The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. Antioxid Redox Sign. 2009;11:497-508.

[9] Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu R-M, et al. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci. 2004;101:3381-6.

[10] Ungvari Z, Bailey-Downs L, Gautam T, Sosnowska D, Wang M, Monticone RE, et al. Age-associated vascular oxidative stress, Nrf2 dysfunction, and NF-κB activation in the nonhuman primate Macaca mulatta. J Gerontol A-Biol. 2011;66A:866-75.

[11] Riedl MA, Saxon A, Diaz-Sanchez D. Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. Clin Immunol. 2009;130:244-51.

[12] Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci. 1997;94:10367-72.

[13] Talalay P, Fahey JW, Healy ZR, Wehage SL, Benedict AL, Min C, et al. Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation. Proc Natl Acad Sci. 2007;104:17500-5.

[14] Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhäuser C. Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem. 2001;276:32008-15.

[15] Lin W, Wu RT, Wu T, Khor T-O, Wang H, Kong A-N. Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol. 2008;76:967-73.

[16] Guerrero-Beltran CE, Calderon-Oliver M, Pedraza-Chaverri J, Chirino YI. Protective effect of sulforaphane against oxidative stress: recent advances. Exp Toxicol Pathol. 2012;64:503-8.

[17] Jeffery E, Araya M. Physiological effects of broccoli consumption. Phytochem Rev. 2009;8:283-98.

[18] London SJ, Yuan J-M, Chung F-L, Gao Y-T, Coetzee GA, Ross RK, et al. Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. Lancet. 2000;356:724-9.

[19] Koh E, Wimalasiri KMS, Chassy AW, Mitchell AE. Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli. Journal of Food Composition and Analysis. 2009;22:637-43.

[20] Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J Nutr. 2004;134:1134-8.

[21] Tse G, Eslick GD. Cruciferous vegetables and risk of colorectal neoplasms: a systematic review and meta-analysis. Nutr Cancer. 2014;66:128-39.

[22] Dosz EB, Jeffery EH. Modifying the processing and handling of frozen broccoli for increased sulforaphane formation. J Food Sci. 2013;78:H1459-63.

[23] Park S-E, Dantzer R, Kelley K, McCusker R. Central administration of insulin-like growth factor-I decreases depressive-like behavior and brain cytokine expression in mice. J Neuroinflammation. 2011;8:12.

[24] Jurgens HA, Amancherla K, Johnson RW. Influenza infection induces neuroinflammation, alters hippocampal neuron morphology, and impairs cognition in adult mice. J Neurosci. 2012;32:3958-68.

[25] Bergström P, Andersson HC, Gao Y, Karlsson J-O, Nodin C, Anderson MF, et al. Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage. Neuropharmacology. 2011;60:343-53.

[26] Kraft AD, Johnson DA, Johnson JA. Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. J Neurosci. 2004;24:1101-12.

[27] Konwinski RR, Haddad R, Chun JA, Klenow S, Larson SC, Haab BB, et al. Oltipraz, 3H-1,2-dithiole-3-thione, and sulforaphane induce overlapping and protective antioxidant responses in murine microglial cells. Toxicol Lett. 2004;153:343-55.

[28] Wynne AM, Henry CJ, Huang Y, Cleland A, Godbout JP. Protracted downregulation of CX3CR1 on microglia of aged mice after lipopolysaccharide challenge. Brain Behav Immun. 2010;24:1190-201.

[29] Kelley KW, Hutchison K, French R, Bluthe RM, Parnet P, Johnson RW, et al. Central interleukin-1 receptors as mediators of sickness. Ann NY Acad Sci. 1997;823:234-46.

[30] Abraham J, Johnson RW. Central inhibition of interleukin-1beta ameliorates sickness behavior in aged mice. Brain Behav Immun. 2009;23:396-401.

[31] Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. J Nutr. 2010;140:1892-8.

[32] Kono H, Rusyn I, Yin M, xE, bele E, Yamashina S, et al. NADPH oxidase–derived free radicals are key oxidants in alcohol-induced liver disease. J Clin Invest. 2000;106:867-72.

[33] Qin L, Liu Y, Hong J-S, Crews FT. NADPH oxidase and aging drive microglial activation, oxidative stress, and dopaminergic neurodegeneration following systemic LPS administration. Glia. 2013;61:855-68.

[34] Keck A-S, Qiao Q, Jeffery EH. Food matrix effects on bioactivity of broccoli-derived sulforaphane in liver and colon of F344 rats. J Agr Food Chem. 2003;51:3320-7.

[35] Dash PK, Zhao J, Orsi SA, Zhang M, Moore AN. Sulforaphane improves cognitive function administered following traumatic brain injury. Neurosci Lett. 2009;460:103-7.

[36] Innamorato N, Rojo, AI, Garcia-Yague, AJ, Yamamoto, M, de Ceballos, ML, Cuadrado, A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol. 2008;181:680-9.

[37] Kim D, You B, Jo E-K, Han S-K, Simon MI, Lee SJ. NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. Proceedings of the National Academy of Sciences. 2010;107:14851-6.

[38] Guichard C, Moreau R, Pessayre D, Epperson TK, Krause KH. NOX family NADPH oxidases in liver and in pancreatic islets: a role in the metabolic syndrome and diabetes? Biochem Soc Trans. 2008;36:920-9.

[39] Anrather J, Racchumi G, Iadecola C. NF-κB Regulates Phagocytic NADPH Oxidase by Inducing the Expression of gp91phox. J Biol Chem. 2006;281:5657-67.

[40] Danilov CA, Chandrasekaran K, Racz J, Soane L, Zielke C, Fiskum G. Sulforaphane protects astrocytes against oxidative stress and delayed death caused by oxygen and glucose deprivation. Glia. 2009;57:645-56.

[41] Jazwa A, Rojo AI, Innamorato NG, Hesse M, Fernandez-Ruiz J, Cuadrado A. Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism. Antioxid Redox Sign. 2011;14:2347-60.

[42] O'Callaghan JP, Miller DB. The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. Neurobiol Aging. 1991;12:171-4.

[43] Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, et al. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci. 2006;9:917-24.

[44] Corona AW, Huang Y, O'Connor JC, Dantzer R, Kelley KW, Popovich PG, et al. Fractalkine receptor (CX3CR1) deficiency sensitizes mice to the behavioral changes induced by lipopolysaccharide. J Neuroinflamm. 2010;7:93.

[45] Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain Behav Immun. 2009;23:309-17.

[46] Lee SC, Dickson DW, Brosnan CF. Interleukin-1, nitric oxide and reactive astrocytes. Brain Behav Immun. 1995;9:345-54.

[47] Goshen I, Yirmiya R. Interleukin-1 (IL-1): a central regulator of stress responses. Front Neuroendocrin. 2009;30:30-45.

[48] Segreti J, Gheusi G, Dantzer R, Kelley KW, Johnson RW. Defect in interleukin-1β secretion prevents sickness behavior in C3H/HeJ mice. Physiol Behav. 1997;61:873-8.

[49] Burgess W, Gheusi G, Yao J, Johnson RW, Dantzer R, Kelley KW. Interleukin-1beta-converting enzyme-deficient mice resist central but not systemic endotoxin-induced anorexia. Am J Physiol. 1998;274:R1829-33.

[50] Veeranki OL, Bhattacharya A, Marshall JR, Zhang Y. Organ-specific exposure and response to sulforaphane, a key chemopreventive ingredient in broccoli: implications for cancer prevention. Br J Nutr. 2013;109:25-32.

[51] Li Y, Zhang T, Li X, Zou P, Schwartz SJ, Sun D. Kinetics of sulforaphane in mice after consumption of sulforaphane-enriched broccoli sprout preparation. Mol Nutr Food Res. 2013;57:2128-36.

[52] Clarke J, Hsu A, Williams D, Dashwood R, Stevens J, Yamamoto M, et al. Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice. Pharm Res. 2011;28:3171-9.

[53] Abraham J, Johnson RW. Consuming a diet supplemented with resveratrol reduced infection-related neuroinflammation and deficits in working memory in aged mice. Rejuv Res. 2009;12:445-53.

[54] Berg BM, Godbout JP, Chen J, Kelley KW, Johnson RW. α-Tocopherol and selenium facilitate recovery from lipopolysaccharide-induced sickness in aged mice. J Nutr. 2005;135:1157-63.

[55] Latté KP, Appel K-E, Lampen A. Health benefits and possible risks of broccoli – An overview. Food Chem Toxicol. 2011;49:3287-309.

[56] McNaughton SA, Marks GC. Development of a food composition database for the estimation of dietary intakes of glucosinolates, the biologically active constituents of cruciferous vegetables. Br J Nutr. 2003;90:687-97.

[57] Drewnowski A, Gomez-Carneros C. Bitter taste, phytonutrients, and the consumer: a review. Am J Clin Nutr. 2000;72:1424-35.

[58] Matusheski NV, Jeffery EH. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. J Agr Food Chem. 2001;49:5743.

[59] Ramsdell HS, Eaton DL. Modification of aflatoxin B1 biotransformation in vitro and DNA binding in vivo by dietary broccoli in rats. J Toxicol Env Health. 1988;25:269-84.

[60] Vang O, Mehrota K, Georgellis A, Andersen O. Effects of dietary broccoli on rat testicular xenobiotic metabolizing enzymes. Eur J Drug Metab Ph. 1999;24:353.

[61] Rushworth SA, MacEwan DJ, O'Connell MA. Lipopolysaccharide-induced expression of NAD(P)H:quinone oxidoreductase 1 and heme oxygenase-1 protects against excessive inflammatory responses in human monocytes. J Immunol. 2008;181:6730-7.

[62] Sierra-Filardi E, Vega MA, Sánchez-Mateos P, Corbí AL, Puig-Kröger A. Heme Oxygenase-1 expression in M-CSF-polarized M2 macrophages contributes to LPS-induced IL-10 release. Immunobiology. 2010;215:788-95.

[63] Soares MP, Marguti I, Cunha A, Larsen R. Immunoregulatory effects of HO-1: how does it work? Curr Opin Pharmacol. 2009;9:482-9.

[64] Turcanu V, Dhouib M, Poindron P. Nitric oxide synthase inhibition by haem oxygenase decreases macrophage nitric-oxide-dependent cytotoxicity: a negative feedback mechanism for the regulation of nitric oxide production. Res Immunol. 1998;149:741-4.

[65] Lavrovsky Y, Song CS, Chatterjee B, Roy AK. Age-dependent increase of heme oxygenase-1 gene expression in the liver mediated by NFkappaB. Mech Ageing Dev. 2000;114:49-60.

[66] Gasper AV, Traka M, Bacon JR, Smith JA, Taylor MA, Hawkey CJ, et al. Consuming broccoli does not induce genes associated with xenobiotic metabolism and cell cycle control in human gastric mucosa. J Nutr. 2007;137:1718-24.

[67] Hu R, Hebbar V, Kim B-R, Chen C, Winnik B, Buckley B, et al. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther. 2004;310:263-71.

Chapter 6 – Summary and Significance

Inflammation and oxidative stress are viewed by many to be the two most important factors that determine whether a person will experience healthy aging or pathological aging [1]. Inflammation and oxidative stress contribute to the pathogenesis of many chronic diseases of aging, including cardiovascular disease, diabetes, atherosclerosis, neurodegenerative disease, and others [2,3]. The striking prevalence of inflammation and altered redox balance in aging-related disease pathology suggests that these factors may underlie molecular changes that occur during the normal aging process. In support of this concept, research has demonstrated that low-grade inflammation is also apparent in otherwise healthy aged individuals, dramatically increasing their risk of cognitive impairment, frailty, and chronic disease [1,4,5].

The rapidly aging global population and parallel increase in aging-related disease has stimulated scientists to elucidate the molecular mechanisms that define aging [6,7]. Consequently, the volume of publications related to aging and inflammation has greatly expanded over the past two decades, advancing our understanding of the aging process and leading to more targeted approaches to promote healthy aging. The molecular changes that are evident during both pathological and non-pathological aging suggest that increasing age leads to excess proinflammatory signaling that is not countered by a sufficient anti-inflammatory response [8,9]. Furthermore, increased oxidative damage during aging indicates that endogenous antioxidant mechanisms do not provide adequate antioxidant activity to protect tissues from prolonged oxidative stress [10,11]. While the underlying causes of immune dysregulation in the elderly are not fully understood, it seems that this imbalance contributes

to a variety of physiological and psychological effects that lower the quality of life and increase the prevalence of chronic disease.

Increased inflammation and oxidative stress are apparent in the aging brain as well as the peripheral immune system, leading to cognitive and behavioral impairment, delayed recovery time following an illness, and limited ability for self-care in the elderly population, Collectively, these factors increase hospitalizations and place a greater burden on family members and healthcare providers [12-14]. Clarifying the mechanisms that result in age-related immune imbalance and identifying preventative interventions are therefore critical aspects of aging research. This dissertation describes antioxidant and anti-inflammatory effects of the broccoli-derived bioactive SFN on brain and behavior and implications for future aging research.

The first aim of this study examined the effects of SFN on adult and aged primary microglia that were stimulated *ex vivo* with LPS. The results demonstrated that SFN increased expression of Nrf2 target genes and decreased LPS-induced proinflammatory mediators in adult and aged microglia. This is a significant finding, because microglial involvement is increasingly implicated in neuroinflammatory disorders related to aging, cognitive decline, and neurodegeneration [15-17]. These results open the door to the possibility that SFN exposure may be able to alter the aged microglial phenotype *in vivo*. While our data indicated that the Nrf2/ARE pathway in aged microglia is sensitive to the antioxidant-enhancing properties of SFN, additional *in vivo* studies are needed to determine if stimulation of the Nrf2 pathway in the brain of aging mice supports an anti-inflammatory microglial phenotype and promotes long-term brain health.

Based on our data demonstrating that SFN decreased proinflammatory mediators in microglia, the second aim of this study was to determine if SFN reduced neuroinflammation in

mice that had been injected with LPS. Sulforaphane influences the central and peripheral immune response, as indicated by reduced expression of the proinflammatory cytokine IL-1β in the hippocampus and liver of LPS-treated mice. While our data demonstrated that SFN reduced proinflammatory markers, it did not completely inhibit them, which likely explains why we did not see a change in sickness behavior. However, this study focused only on the acute molecular and behavioral changes in response to LPS, and the effects of supplemental SFN on chronic low-grade inflammation that is associated with aging and cognitive impairment may be an interesting area for further investigation.

The third aim of the study focused on the effects of broccoli-supplemented diet on peripheral and neuroinflammation in LPS-stimulated adult and aged mice. This study indicated that dietary broccoli had no overall effect on inflammatory markers in adult mice, but suggested that broccoli diet may lower markers of glial reactivity in aged mice. In the previous study, we demonstrated that multiple doses of peripherally administered SFN lowered LPS-induced IL-1β expression in hippocampus of adult mice. Here, it is important to note that SFN obtained through dietary means has not been measured in the brain, and it is possible that SFN directly reaches the brain only when administered in high concentrations. However, bi-directional communication between the immune system and the central nervous system is an important determinant of neuroinflammatory status during peripheral infection. Proinflammatory mediators that are generated in the periphery reach the brain through the neural and humoral signaling pathways, evoking a neuroinflammatory response [18,19]. This well-established communication network implies that inhibition of proinflammatory peripheral mediators also reduces the inflammatory signals that are sent to the brain. It is possible that

longer exposure to supplemental SFN could inhibit peripheral inflammatory mediators, thereby reducing neuroinflammation. This is of potential interest in aging studies, where chronic dietary exposure to a bioactive that reduces peripheral and central inflammation could preserve cognitive function.

Anti-inflammatory dietary bioactives such as SFN can be valuable interventions to promote healthier aging. More research is needed in this area to identify additional molecular targets of SFN that may be beneficial in reducing inflammation, and to investigate anti-inflammatory effects using SFN combined with other Nrf2-activiating bioactives.

6.1 References

[1] Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, et al. Molecular inflammation: Underpinnings of aging and age-related diseases. Ageing Res Rev. 2009;8:18-30.

[2] Khansari N, Shakiba Y, Mahmoudi M. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. Recent Pat Inflamm Allergy Drug Discov. 2009;3:73-80.

[3] McGeer EG, Klegeris A, McGeer PL. Inflammation, the complement system and the diseases of aging. Neurobiol Aging. 2005;26:94-7.

[4] Krabbe KS, Pedersen M, Bruunsgaard H. Inflammatory mediators in the elderly. Exp Gerontol. 2004;39:687-99.

[5] Singh T, Newman AB. Inflammatory markers in population studies of aging. Ageing Res Rev. 2011;10:319-29.

[6] World Population Ageing 2013. Department of Economic and Social Affairs, Population Division, United Nations. 2013.

[7] Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: the challenges ahead. The Lancet.374:1196-208.

[8] Franceschi C, BonafÈ M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging: an evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000;908:244-54.

[9] Sparkman NL, Johnson RW. Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. Neuroimmunomodulation. 2008;15:323-30.

[10] Dröge W, Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. Aging Cell. 2007;6:361-70.

[11] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408:239-47.

[12] Ginaldi L, Loreto MF, Corsi MP, Modesti M, De Martinis M. Immunosenescence and infectious diseases. Microbes Infect. 2001;3:851-7.

[13] Johnston M, Wakeling A, Graham N, Stokes F. Cognitive impairment, emotional disorder and length of stay of elderly patients in a district general hospital. Brit J Med Psychol. 1987;60:133-9.

[14] Wofford JL, Loehr LR, Schwartz E. Acute cognitive impairment in elderly ED patients: Etiologies and outcomes. Am J Emerg Med. 1996;14:649-53.

[15] Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. J Neuroinflamm. 2004;1:14.

[16] Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behav Immun. 2008;22:301-11.

[17] Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J. 2005;19:1329-31.

[18] Dantzer R, Konsman J-P, Bluthé R-M, Kelley KW. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton Neurosci. 2000;85:60-5.

[19] Dantzer R, BluthÉ R-M, LayÉ S, Bret-Dibat J-L, Parnet P, Kelley KW. Cytokines and Sickness Behavior. Ann N Y Acad Sci. 1998;840:586-90.