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

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ABSTRACT

Increased neuroinflammation and oxidative stress resulting from heightened microglial activation are associated with age-related cognitive impairment. The objectives of this study were to examine the effects of the bioactive sulforaphane (SFN) on the nuclear factor E2-related factor 2 (Nrf2) pathway in BV2 microglia and primary microglia, and to evaluate proinflammatory cytokine expression in lipopolysaccharide (LPS)-stimulated primary microglia from adult and aged mice. BV2 microglia and primary microglia isolated from young adult and aged mice were treated with SFN and LPS. Changes in Nrf2 activity, expression of Nrf2 target genes, and levels of proinflammatory markers were assessed by quantitative PCR and immunoassay. SFN increased Nrf2 DNA-binding activity and upregulated Nrf2 target 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 Nrf2 target genes and attenuated IL-1β, IL-6, and iNOS induced by LPS. These data indicate that SFN is a potential beneficial supplement that may be useful for reducing microglial mediated neuroinflammation and oxidative stress associated with aging.

1. 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 (Eggen et al., 2013; Hickman et al., 2013). 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 (Crain et al., 2013). This shift in microglial phenotype results in chronic, low grade neuroinflammation which is considered a contributing factor to some aspects of cognitive aging and other aging-related diseases. 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 (Godbout et al., 2005; Henry et al., 2009). 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 (Itoh et al., 1997). Involvement of Nrf2 in regulation of inflammation and microglial activation has also been reported (Innamorato et al., 2008; Rojo et al., 2010). Because the Nrf2 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 (Barger et al., 2007; de Vries et al., 2008; Innamorato et al., 2008).

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 (Fahey et al., 1997; Talalay et al., 1995). Clinical and pre-clinical studies have established that SFN from broccoli is readily absorbed (Vermeulen et al., 2008) and has a low toxicity profile, making it suitable to be provided in supplement form (Shapiro et al., 2006; Ye et al., 2002). 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 (Townsend et al., 2014). Other studies suggest that Nrf2 signaling might be disrupted during aging, leading to decreased endogenous antioxidant response (Duan

Abbreviations: ARE, antioxidant response element; GCLM, glutamate–cysteine ligand modifier subunit; HMOX1, heme oxygenase I; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NQO1, NAD(P)H quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor; qPCR, real-time quantitative RT-PCR; SFN, sulforaphane.

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et al., 2009; Suh et al., 2004), 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.

2. Materials and methods

2.1. BV2 microglia culture and treatment

The immortalized murine microglia cell line, BV2, has been used as a model to investigate the neuroimmune system (Bocchini et al., 1992; Jang et al., 2008). 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 plated in 6-well culture dishes, then treated with vehicle (complete medium) or 2.5 μM SFN (LKT Laboratories, St. Paul, MN). Cells were harvested after 3, 6, 9, and 24 h. In subsequent experiments, cells were treated for 1 h with SFN or vehicle, then with vehicle ± lipopolysaccharide (LPS, 100 ng/mL) (Sigma, St. Louis, MO; 0127:B8) for 8 h. Each treatment was replicated 3 times in separate but identical trials.

2.2. CD11b$^+$ primary microglia cell isolation and treatment

Adult (4–5 month-old) and aged (18–20 month-old) male BALB/c mice from our in-house colony were individually housed in polypropylene cages in a temperature controlled environment (21 °C) with a reversed phase 12 h light:dark cycle (lights out at 09:00 h). Mice were given ad libitum access to rodent chow and water. Mice were euthanized using CO_2 asphyxiation and brains rapidly removed for microglia isolation. All studies were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Illinois Institutional Animal Care and Use Committee.

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 (Nikodemova and Watters, 2012). Cells that were positive for CD11b were isolated from brains of young adult (n = 16) and aged (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 ×*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 ×*g*. After centrifugation, myelin and percoll were aspirated and the remaining cells were washed with PEB solution consisting of sterile phosphate-buffered saline (PBS), 0.2 mM EDTA, and 0.5% BSA. 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 a medium (DMEM, 10% FBS) containing 10 ng/mL granulocyte–macrophage colony stimulated factor 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 vehicle ± LPS (10 ng/mL) for 8 h.

2.3. 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 ×*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.

2.4. Markers of inflammation and oxidative stress

Total RNA was isolated from BV2 cells using E.Z.N.A. total RNA kits (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 NAD(P)H quinone oxidoreductase 1 (NQO1, Mm.PT.56a.9609207), heme oxygenase 1 (HMOX1, Mm.PT.56a.9675808), and glutamate–cysteine ligase, modifier subunit (GCLM, Mm.PT.56a.11654780), and proinflammatory markers interleukin (IL)-1β (Mm.PT.56a.41616450), IL-6 (Mm.PT.56a.13354106), and inducible nitric oxide synthase (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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm.PT.39.a.1) and the $2^{-\Delta\Delta Ct}$ calculation method as previously described (Jurgens et al., 2012). 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.

2.5. Statistical analysis

All data were analyzed using Statistical Analysis System (Cary, NC). Data from BV2 cells were subjected to analysis of variance (ANOVA) to assess main effects of SFN at each time point, or effects of SFN, LPS and the SFN × LPS interaction. Similarly, data from primary microglia were subjected to ANOVA to assess main effects of Age, SFN, LPS, and all interactions. 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. Results

3.1. 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 NQO1, HMOX1, and GCLM (Thimmulappa et al., 2002). We assessed NQO1, HMOX1, and GCLM mRNA at four time points to determine the optimal time of gene induction. As shown in Fig. 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 ($P < 0.0001$,