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

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Abstract

Objectives—Acute peripheral infection is associated with central and peripheral inflammation, increased oxidative stress, and adaptive sickness behaviors. Sulforaphane (SFN) activates the transcription factor nuclear factor E2-related factor 2 (Nrf2), which upregulates antioxidant genes and lowers inflammation. The objectives of this study were to examine the effects of SFN on proinflammatory markers and Nrf2 target genes in hippocampus and liver of mice challenged with lipopolysaccharide (LPS), and to evaluate sickness response following the LPS immune challenge.

Methods—Adult Balb/c mice received SFN (50 mg/kg, i.p.) for 3 d before being injected i.p. with LPS (1 µg) to mimic an acute peripheral infection. Sickness behaviors were measured at baseline and 6 h after LPS. Expression of proinflammatory mediators and antioxidant genes were analyzed in hippocampus and liver 6 h after LPS.

Results—SFN elevated Nrf2 target genes and reduced expression of proinflammatory mediators in hippocampus and liver, but did not improve LPS-induced sickness response.

Discussion—The nutritional bioactive SFN displays potent anti-inflammatory properties against LPS-induced inflammation *in vitro*, but has not been previously assessed *in vivo* during peripheral infection as a potential treatment for sickness behavior. These data indicate that SFN has anti-inflammatory effects in both brain and periphery, but that longer exposure to SFN may be necessary to reduce sickness behavior.

Keywords

Lipopolysaccharide; Neuroinflammation; Peripheral infection; Sickness behavior; Sulforaphane

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 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 The bioactive 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.

Methods

Animals and experimentation

Adult (4–6 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 had *ad libitum* access to rodent chow and water. 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 Guide for the Care and Use of Laboratory Animals, and were approved by the University of Illinois Institutional Animal Care and Use Committee.

Immediately prior to experimentation, SFN (LKT Laboratories, St. Paul, MN) was dissolved in sterile saline. 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 or saline was administered 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 responses.⁶ To determine whether SFN inhibited the LPS-induced sickness response, food intake, body weight, and locomotor activity were assessed. Spontaneous locomotor activity was assessed 6 h after LPS as previously described.¹² 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 CO₂ asphyxiation 6 h after LPS and transcardially perfused with sterile ice-cold saline. Hippocampus, hypothalamus, and liver were rapidly dissected and immediately frozen in liquid nitrogen. All tissues were stored at -80°C until further processing for analysis.

To assess changes in gene expression, RNA was isolated from hippocampus, hypothalamus, and liver 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 NAD(P)H quinone oxidoreductase 1 (NQO1, Mm.PT.58.9609207) and heme oxygenase-1 (HMOX1, Mm.PT.58.9675808). Expression of interleukin (IL)-1β (Mm.PT.58.41616450), IL-6 (Mm.PT.58.13354106), iNOS (Mm.PT.58.5680554), and cytochrome b-245 β (CYBB, Mm.PT.58.11318181) was 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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm.PT.39.a.1) using the 2^{-Ct} calculation method as previously described.¹³ 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 CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and protease inhibitor cocktail. All chemical reagents were purchased from Sigma (St. Louis, MO).

Protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA). IL-1 β protein was quantified using a commercially available ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

All data were analyzed using Statistical Analysis System (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 $\pm$ SEM.

Results

SFN increased ARE genes in liver and hippocampus

Increased endogenous antioxidant response following SFN exposure is thought to provide cellular protection.¹⁴ Previous studies have indicated that SFN upregulates ARE genes in liver and brain through Nrf2 activation.^{15–17} Consistent with these reports, the current study revealed that a single injection of SFN increased transcription of 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 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.¹⁸ LPS evoked a robust increase in proinflammatory cytokines IL-1 β ($P<0.0001$) and IL-6 ($P<0.001$) in the liver (Figure 2). SFN reduced LPS induced IL-1 β (SFN $\times$ LPS, $P<0.05$) and IL-6 mRNA (SFN $\times$ LPS, $P<0.01$). In order to assess if the changes in mRNA were reflected by changes in protein, IL-1 β protein was measured. SFN reduced LPS-induced IL-1 β 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 but not the hypothalamus

Intraperitoneal injection of LPS is associated with elevated proinflammatory cytokine expression in the hippocampus which can cause cognitive and behavioral impairment.¹⁹ To determine whether peripherally administered SFN could attenuate inflammation in the hippocampus, IL-1 β and IL-6 were measured 6 h after a peripheral LPS injection. LPS increased IL-1 β ($P<0.0001$) and IL-6 ($P<0.05$) mRNA in hippocampus (Figure 3A–D). SFN reduced LPS-induced IL-1 β (SFN $\times$ 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-1 β and IL-6 protein in the brain due to levels that were lower than the assay detection limit. Surprisingly, analysis of the hypothalamus revealed that IL-1 β mRNA was

increased by LPS ($p < 0.01$), but there was no main effect of SFN or interaction between SFN and LPS (Figure 3E).

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). 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 $\times$ LPS, $P < 0.05$). Body weight was decreased 6 h after LPS ($P < 0.05$). Interestingly, our data indicates a negative effect of SFN on food intake. However, previous unpublished data suggests that the significant negative impact of SFN on food intake at 6 h is completely resolved by 24 h after injection, suggesting that this is a not a long-term effect of SFN treatment. Locomotor activity was decreased in mice treated with LPS ($P < 0.0001$). SFN did not improve locomotor activity in LPS-treated mice.

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.^{20, 21} 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*.^{22, 23} 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-1 β , IL-6, and cytochrome-b 245 β . Importantly, SFN reduced hepatic IL-1 β 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.^{17, 24, 25} The present data provide supporting evidence that SFN has an anti-inflammatory effect on the liver during acute LPS exposure.

Because neuroinflammation during peripheral infection is paralleled by sickness behavior, we were particularly interested to see whether SFN lowered neuroinflammation. In this study, SFN reduced LPS-increased hippocampal IL-1 β mRNA, supporting our hypothesis that SFN has anti-inflammatory effects on the brain. Because central IL-1 β 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 in the hippocampus, SFN did not completely inhibit neuroinflammation (i.e. IL-6 was still elevated). Additionally, while it has been previously reported that peripherally administered SFN crosses the blood brain barrier and can be detected in hippocampus within minutes of injection,²⁶ 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-1 β in hippocampus, this reduction was not evident in the hypothalamus and may not have occurred in other regions of the brain. In this study, we chose to focus primarily 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.²⁷ Furthermore, microglia are highly sensitive to the anti-inflammatory effects of SFN *in vitro*.²⁸ 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.^{20, 29} 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, 30, 31} 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.³² The dietary broccoli in our previously published study³² provided up to 0.52 μ mol of SFN/g diet consumed. In contrast, the mice in the present study were given 50 mg SFN per kg body weight, which is equivalent to 282 μ mol/kg. Although this dose may seem high, it should be noted that it is not uncommon for clinical studies to provide human subjects with broccoli or broccoli sprout preparations containing 200 μ mol of isothiocyanates (i.e. SFN) with no reports of adverse effects.^{33, 34, 35} However, dietary broccoli did not reduce elevated brain IL-1 β , and it is possible that supplemental SFN, such as administered in the present study, would have a greater effect on sickness behavior during the recovery period following LPS. In support of this, there is some evidence that during acute peripheral inflammation, the Nrf2 pathway is involved in attenuating the duration of the proinflammatory immune response without affecting peak inflammatory levels.³⁶

The current data indicate that SFN reduced IL-1 β and other proinflammatory markers in LPS-treated mice, consistent with *in vitro* evidence that SFN lowers LPS-induced inflammation in peripheral and brain-derived cells.^{28, 37, 38} Interestingly, the anti-inflammatory effects of SFN are lost in Nrf2 knockout models, suggesting that a functional Nrf2 pathway is an important component in regulating inflammation.^{23, 26, 39} Furthermore, in comparison to wild-type controls, Nrf2 knockout mice are more sensitive to the inflammatory effects of toxins, bacterial LPS, and carcinogens.^{11, 40, 41} Additional studies are needed to further characterize the role of Nrf2 in response to acute and chronic immune stimuli.

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

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Figure 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 $\pm$ SEM (n=6–7). * indicates main effect of LPS (P<0.05).

Figure 2. SFN reduced LPS-induced IL-1 β and IL-6 in liver
 (A–B) SFN reduced LPS-increased IL-1 β mRNA and protein in liver. (C) SFN reduced LPS-induced IL-6. (D) LPS increased CYBB. Bars represent means $\pm$ SEM (n=6–8). * indicates main effect of SFN and + signifies main effect of LPS (P<0.05). Means with different letters are statistically different from each other (P<0.05).

Figure 3. SFN reduced IL-1 β and iNOS mRNA in hippocampus but not in hypothalamus

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

Figure 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 $\pm$ SEM (n=6–8). * indicates main effect of LPS ($P < 0.05$). Means with different letters are statistically different from each other ($P < 0.05$).