Sulforaphane Augments Glutathione and Influences Brain Metabolites in Human Subjects: A Clinical Pilot Study

Thomas W. Sedlak^a Leslie G. Nucifora^a Minori Koga^a Lindsay S. Shaffer^a
Cecilia Higgs^a Teppei Tanaka^a Anna M. Wang^b Jennifer M. Coughlin^a
Peter B. Barker^b Jed W. Fahey^c Akira Sawa^a

^aDepartment of Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, USA; ^bDepartment of Radiology and Radiological Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, USA; ^cDepartment of Clinical Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

Keywords

Sulforaphane · Glutathione · Oxidative stress

Abstract

Schizophrenia and other neuropsychiatric disorders await mechanism-associated interventions. Excess oxidative stress is increasingly appreciated to participate in the pathophysiology of brain disorders, and decreases in the major antioxidant, glutathione (GSH), have been reported in multiple studies. Technical cautions regarding the estimation of oxidative stress-related changes in the brain via imaging techniques have led investigators to explore peripheral GSH as a possible pathological signature of oxidative stress-associated brain changes. In a preclinical model of GSH deficiency, we found a correlation between whole brain and peripheral GSH levels. We found that the naturally occurring isothiocyanate sulforaphane increased blood GSH levels in healthy human subjects following 7 days of daily oral administration. In parallel, we explored the potential influence of sulforaphane on brain GSH levels in the anterior cingulate cortex, hippocampus, and thalamus via 7-T magnetic resonance

spectroscopy. A significant positive correlation between blood and thalamic GSH post- and pre-sulforaphane treatment ratios was observed, in addition to a consistent increase in brain GSH levels in response to treatment. This clinical pilot study suggests the value of exploring relationships between peripheral GSH and clinical/neuropsychological measures, as well as the influences sulforaphane has on functional measures that are altered in neuropsychiatric disorders.

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Introduction

Oxidative stress underlies the pathophysiology of multiple human disorders, ranging from cardiovascular syndromes and diabetes to neurodegenerative conditions such as Alzheimer and Parkinson disease [1–5]. Furthermore, recent studies have suggested that oxidative stress also plays a role in neuropsychiatric conditions such as schizophrenia (SZ) [6–9].

Glutathione (GSH) is one of the most important regulators of oxidative stress and redox balance. This antioxidant tripeptide exists at low millimolar intracellular levels and is directly utilized by over 20 enzymes to exert its antioxidant and cytoprotective effects. Involvement of GSH in pathological conditions has been reported for many diseases [10–13].

An obstacle to the study and treatment of brain disorders is access to biological specimens, which are more readily available for diseases of the periphery. Thus, many studies rely upon more accessible blood and cerebral spinal fluid samples to characterize the molecular underpinnings of neuropsychiatric conditions [14–23]. Although there is variability between subjects, multiple studies have reported a significant reduction of GSH in SZ in peripherally accessible tissues and fluids, such as blood [6, 18, 19, 24–29].

However, the molecular disposition of GSH in the brain associated with SZ is unclear. In vivo magnetic resonance spectroscopy (MRS) is an analytical technique used to measure levels of brain metabolites [30]. Successful application of MRS has shown alterations in brain glutamate (Glu) and γ -aminobutyric acid (GABA) levels in SZ patients [31–34]. In contrast, the data for GSH have been less consistent [19, 35, 36]. The limited sensitivity of 3-T MRS in detecting GSH levels may be the underlying source of this inconsistency, and studies with 7-T MRS should improve detection and yield more consistent results. Therefore, at present we should be cautious about interpreting MRS-based measures of GSH. The current MRS methodology may not be sensitive enough to detect subtle but critical changes in association with pathological alterations. As a result, many investigators are exploring the potential utility of peripheral GSH as a possible signature of oxidative stress-associated brain changes.

Sulforaphane is a natural phytochemical compound abundant in the seeds and sprouts of cruciferous plants such as broccoli. It has shown promise in preclinical models of traumatic brain and spinal cord injury [37, 38]. Sulforaphane activates the transcription factor NF-E2-related factor 2 (Nrf2) via its ability to bind to and disassociate its cytosolic inhibitor kelch-like ECH-associated protein 1 (KEAP1) [39, 40]. Thus, sulforaphane is expected to facilitate proper redox balance and protection against oxidative stress [41]. Because of its presence in consumable food, sulforaphane is an attractive candidate for further human study. Indeed, its potential utility has been demonstrated in the treatment of asthma, air pollution injury, benign prostate hyperplasia, UV-induced erythema, diabetes, the control of *Helicobacter pylori* (a

stomach carcinogen), and potentially in the prevention of a number of different cancers [42–44]. Furthermore, recent reports have suggested that administration of sulforaphane leads to beneficial outcomes of human brain disorders such as autism and SZ [45–47]. Nevertheless, it remains elusive how sulforaphane may mechanistically interface with the pathogenesis of neuropsychiatric conditions.

We hypothesized that the administration of sulforaphane to human subjects could augment blood and brain GSH levels, and that peripheral blood GSH levels may reflect the oxidative stress- and redox-associated physiology and pathophysiology in the brain. Given that sulforaphane may be a candidate treatment, studying the influence of sulforaphane on GSH may be the first step in defining markers for predicting beneficial effects.

Methods

Mouse Model

Excitatory amino acid carrier-1 knockout (*Eaac1* KO) [48] and wild-type (WT) mice, both having a CD1 background, were used in this study and housed in a controlled facility ($23 \pm 1^\circ\text{C}$; $50 \pm 5\%$ humidity; light and dark cycles started at 7 a.m. and 7 p.m., respectively) with free access to food and water. GSH levels were quantified in whole brain from 3-month-old male mice (*Eaac1* KO mice, $n = 4$; WT mice, $n = 5$). We utilized male animals as rodent GSH enzyme levels fluctuate up to 50% during estrus [49]. Three-month-old animals are free from the age-dependent decline in GSH levels observed in rodents over 1 year of age [50].

Broccoli Sprout Extract (Sulforaphane)

The broccoli sprout extract used in this study is a highly standardized formulation of sulforaphane [51, 52]. The extract was standardized to 100 μmol sulforaphane in the form of 2 gel capsules per day over 7 consecutive days, as described previously [45, 53–58]. The dose of 100 μmol sulforaphane and the 7-day treatment duration were determined empirically from previous studies, including a trial demonstrating induction of antioxidant enzymes in nasal lavage cells of human subjects [59] and improved excretion of air pollutants [56]. Our formulation of sulforaphane is 80% bioavailable [58], reaches peak plasma levels 1 h after oral ingestion, declines with first-order kinetics, and is actively excreted in urine (60% at 8 h) [58, 60].

Human Study Participants

Nine healthy volunteers (5 male and 4 female) were recruited at the Johns Hopkins Schizophrenia Center, Baltimore, MD, USA. The demographic information is presented in Table 1. GSH levels are similar in men and women, though they start to decline over 60 years of age [61]. The present study included younger participants (aged 21–26 years, with 1 participant aged 56 years). Subjects were excluded if they had a personal or immediate family history of severe psychiatric illness, a history of a recent nosocomial infection, chronic neurological disorders, a traumatic head injury re-

Table 1. Clinical and demographic characteristics

Sex, <i>n</i> (%)	
Male	5 (56)
Female	4 (44)
Mean age $\pm$ SD, years	27.8 $\pm$ 10.67
Race/ethnicity, <i>n</i> (%)	
Caucasian	2 (22)
African American	3 (34)
Asian	2 (22)
Other	2 (22)
Education, <i>n</i> (%)	
Advanced degree	5 (56)
Undergraduate	4 (44)
Smoking, <i>n</i> (%)	
Smoker	0 (0)
Nonsmoker	9 (100)

sulting in loss of consciousness, or active substance abuse. We excluded tobacco smokers from this study, as smoking is a known inducer of oxidative stress [62, 63]. Patients with contraindications to MRI scanning, such as dependence on benzodiazepine medication, prohibitive claustrophobia, metallic implants or prosthetics, pacemakers, or any medical conditions that would constitute a safety risk to the participant (e.g., middle ear disorder), were also excluded.

Clinical Study Design

The participants completed two visits, scheduled 7 days (1 week) apart. The participants were given 100 μ mol sulforaphane as standardized broccoli sprout extract in the form of 2 gel capsules, and instructed to ingest the extract each morning for 1 week. The research coordinators contacted the participants by telephone to provide daily reminders on taking the extract. Urine and blood specimens were collected prior to the first dose of broccoli sprout extract and within 4 h of the final dose. MRS scans were performed prior to the first dose and within 4 h of ingesting the final dose. The participants completed a log of the dates and times of taking the extract and documented any potential discomforts or ailments they experienced during the study. Three participants indicated mild-to-moderate nausea or abdominal pain when the extract was taken on an empty stomach. The study design is summarized in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000487639).

Measurement of GSH Levels in Blood and Brain Tissue (Murine and Human)

Total GSH (the sum of GSH and glutathione disulfide [GSSG]) was measured in human blood cells using modifications of Tietze's method [64]. We refer to "total GSH" simply as "GSH." We have quantified GSH and oxidative stress in multiple studies using these methodologies [18, 65, 66]. Nonmonocytic blood cells were resuspended in a buffer containing 200 mM 2-(N-morpholino)ethanesulfonic acid and 1 mM EDTA (ethylenediaminetetraacetic acid) and sonicated. The lysed cell suspension was then spun at 10,000 *g* for 15 min at 4°C. The resultant supernatant was deproteinized by

adding 50% v/v of freshly prepared 10% metaphosphoric acid followed by a 5-min incubation at room temperature. The deproteinized samples were then spun at 2,000 *g* for 2 min, and the supernatant was stored at –20°C until assaying. The kinetic assay was performed by adding 5 μ L of freshly prepared 4 M triethanolamine to 100 μ L of metaphosphoric acid-treated cell lysates. GSH was then measured using a kit (Cayman Chemical Company; 703002) in strict accordance with the manufacturer's instructions. The rate of increase in absorbance at 415 nm, which measures the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) by GSH, reflects the total GSH content. The kinetic assay was allowed to run for 20 min, with data collected at every 1-min interval. The concentration of total GSH in plasma was reported as nmol/mL. A standard curve from 0 to 16 nmol/mL was used to calibrate the assay. Each reported GSH value was determined using the average of 2 independent measurements.

7-T Magnetic Resonance Spectroscopy

All participants were scanned using a 7-T scanner (Philips Achieva; Philips, Best, The Netherlands) equipped with a 32-channel head coil (Nova Medical, Wilmington, MA, USA). 3D T1-weighted images were acquired using a magnetization-prepared rapid acquisition with gradient echo sequence (FOV = 220 $\times$ 220 $\times$ 180 mm). Spectra were recorded from the anterior cingulate cortex (ACC; 30 $\times$ 30 $\times$ 30 mm), left hippocampus (HP; 35 $\times$ 15 $\times$ 15 mm), and bilateral thalamus (THAL; 20 $\times$ 30 $\times$ 15 mm) using a stimulated echo acquisition mode sequence (TE/TM/TR = 14/33/3,000 ms, NEX = 128), except for 2 participants where spectra were only recorded from the ACC with a voxel size of 38 $\times$ 30 $\times$ 30 mm (online suppl. Fig. 2).

Not all the remaining participants had MRS data acquired for all three brain regions. VAPOR (variable power and optimized relaxation delays) water suppression was used to minimize the dominant water signal [67]. In addition, a reference scan without water suppression was also acquired from each voxel (NEX = 16). Spectra were analyzed with the LCModel software package [68] and a basis set was simulated from the VeSPA project [69]. Metabolite concentrations were normalized using the unsuppressed water signal as the reference. Fitting errors were assessed by CRLBs (Cramér-Rao lower bounds). Metabolite concentrations were only included in further statistical analyses when the corresponding CRLB values were below 20%.

The goal of our study was to determine changes in brain GSH levels upon sulforaphane treatment, possibly in correlation with levels of blood GSH; therefore GSH was our primary outcome measure. GABA, glutamine (Gln), Glu, and N-acetylaspartate (NAA) served as secondary outcome measures. Differences between pre- and posttreatment measurements in concentrations of GABA, Gln, Glu, GSH, and NAA were compared using a paired *t* test.

Analysis and Statistics

Statistical analyses were performed using R version 3.3.0 for Windows. The correlative relationship between blood GSH levels and brain metabolite levels measured by MRS was calculated using the pre- and post-sulforaphane blood GSH ratios and metabolite ratios. The blood GSH ratios were nonparametric as determined by the Shapiro-Wilk normality test; therefore, Spearman's correlation coefficient was used to determine the relationship between blood GSH and brain MRS data. For nonparametric data, the

Fig. 1. Measurement of glutathione (GSH) levels in blood cells and brain tissue in wild-type (WT) and *Eaac1* knockout (+/–) mice. **a** The levels of total GSH in lymphocytes were significantly reduced in the 3-month-old male *Eaac1*^{+/-} mice ($n = 4$) compared with the WT littermates ($n = 5$). **b** The levels of total GSH in whole brain were significantly reduced in the *Eaac1*^{+/-} mice compared

with the WT littermates. **c** The levels of total GSH in the lymphocytes and those in the brain were different between individual animals (white circles, WT mice; black circles, *Eaac1*^{+/-} mice). Nevertheless, the ratio of the level of total GSH in the lymphocytes to that in the brain was constant.

Mann-Whitney test was used to perform two group comparisons between total GSH levels in WT and *Eaac1* KO mice in both lymphocytes and brain. Spearman's correlation coefficient was used to determine the relationship between total GSH levels in the brain and those in lymphocytes of mice.

Spearman's correlation analysis and a pair-wise, two-tailed t test were used to determine the influence of sex on the sulforaphane response in blood GSH levels. Wilcoxon rank sum testing was used to determine whether racial categories were covariates of GSH levels in blood. Pearson's correlation analysis was used to determine the influence of age on the sulforaphane response in blood GSH levels. In order to perform a thorough analysis, a univariate and a multivariate linear regression model were used to determine the influence of race and age and of sex and race, respectively, on the sulforaphane response in THAL GSH levels. The Shapiro-Wilk test was applied to confirm normality (parametric or nonparametric). p values were adjusted using the method of Benjamini and Hochberg. All data are expressed as means $\pm$ standard deviation (SD) and an alpha of $p < 0.05$.

Results

Peripheral and Brain GSH Levels Observed in the Mouse Models

We examined whether peripheral GSH levels may reflect pathophysiological changes in brain GSH levels in animal models. We initially utilized a mouse model system to study the effects of GSH deficiency in blood and brain within the controlled genetic and environmental setting afforded by these models. Since studying the influence of sulforaphane administration on GSH levels was our main interest and human studies typically have some

level of genetic and environmental variability, we hoped that the mouse models could guide our human study in examining the relationship of peripheral to brain GSH levels within a controlled genetic and environmental system. We used *Eaac1* KO mice, which have reduced GSH levels in the brain owing to deficient synthesis [48], although alterations in GSH in the periphery remain elusive. First, we replicated the previous finding [48] that levels of brain GSH were decreased in *Eaac1* KO mice compared with WT mice (Fig. 1a). We now report that peripheral circulating lymphocytes of *Eaac1* KO mice had greatly reduced GSH levels compared to WT mice ($p = 0.008$) (Fig. 1b). Importantly, brain and peripheral GSH levels were strongly correlated in both WT and KO mice ($\rho = 0.967$; $p < 0.001$) (Fig. 1c). Thus, although we need to be cautious about generalizing these observations, the measurement of peripheral GSH levels might be a potentially useful index of brain GSH levels.

Increased Levels of Blood GSH in Human Subjects following Daily Administration of Sulforaphane for 7 Consecutive Days

Pre- and post-dose urine analysis confirmed the presence of urinary sulforaphane metabolites, affirming the participants' adherence to the oral sulforaphane protocol (data not shown). Following 1-week administration of sulforaphane, the study participants demonstrated a significant augmentation of GSH in non-monocytes that include a mixture of T cells, B cells, and NK cells (Fig. 2). The GSH level was 9.22 nmol/mL before sulforaphane

Fig. 2. Measurement of glutathione (GSH) levels in human blood cells. Cellular GSH was significantly increased following a 1-week administration of sulforaphane (SFN) (two-tailed, paired *t* test; $p = 0.02$).

administration and 12.2 nmol/mL following sulforaphane administration, a 32% increase. Neither the participants' age ($r = -0.1936$; $p = 0.62$) nor their sex ($p = 0.087$; $p = 0.825$) was found to be an explanatory covariate for the effect of sulforaphane on GSH. Race was not found to be an explanatory variable either, as demonstrated by Wilcoxon rank sum testing of the four groups ($p = 0.318$).

Measuring Brain Metabolites by MRS

In this study we obtained measurements of five metabolites (GSH, GABA, Gln, Glu, and NAA) in three different brain regions (THAL, HP, and ACC). We specifically chose these three brain regions as they underlie the key structural and functional connectivity affected in SZ [70–77]. Our primary hypothesis in the present study is that sulforaphane may affect levels of GSH in these brain regions, and may possibly be correlated with GSH levels in peripheral blood.

We identified an increase in HP GSH from 1.11 ± 0.34 to 1.38 ± 0.28 mM ($p = 0.041$; paired, two-tailed *t* test; online supplementary Figure 2). The change in levels of GSH in the THAL and ACC did not reach significance. A representative pair-wise analysis of THAL GSH levels before and after sulforaphane administration is shown in online supplementary Figure 3. Regarding the other brain metabolites quantified for the non-primary hypothesis of the present study, the level of Gln in the THAL increased from 1.21 ± 0.22 to 1.49 ± 0.26 mM ($p = 0.017$; paired, two-tailed *t* test). Due to the limited sample size, the increase did not remain significant following post hoc correction

for multiple comparisons, but the result remains suggestive when considering the small number of participants.

The sulforaphane response in brain GSH levels is not influenced by age, sex, or race, as determined by linear regression analysis. We performed a representative analysis of the GSH levels measured in the THAL because this region yielded results most significantly correlated with the biochemical measurement of GSH levels in the blood before and after sulforaphane ingestion. The respective *p* values, for example, for the GSH levels in the THAL before and after sulforaphane administration for age, sex, and race were 0.582, 0.900, and 0.567, respectively, in the multivariate linear regression analysis, and $p = 0.406$ in the univariate analysis for race. Taken together, age, sex, and race are not covariates associated with sulforaphane response in brain GSH levels.

We observed a significantly positive correlation between GSH levels in blood and those in the THAL brain region ($\rho = 0.943$; $p = 0.017$; $q = 0.051$). For transparency we also report the correlative results obtained for all five metabolites measured within the three different brain regions (online suppl. Table 1). Following sulforaphane administration, the increase in blood GSH was positively correlated with GABA, Gln, Glu, and GSH in the THAL. Although these correlations were not significant following multiple comparison, they remain suggestive. Power analysis calculations suggest that a sample size of $n = 50$ would yield a significant result, and this will be the focus of a future study.

Discussion

We report that a short-term administration of sulforaphane was sufficient to significantly increase peripheral GSH levels in human subjects. We found an increase in GSH in the HP, but not elsewhere in the brain regions assessed. The peripheral GSH ratio had a strong and significantly positive correlation with brain GSH levels in the THAL upon sulforaphane treatment, consistent with the results obtained from our genetically and environmentally homogeneous preclinical GSH deficiency mouse model study.

Within this study, we performed statistical analyses to verify that age, sex, and race do not influence the sulforaphane response in GSH levels in our participant population. Although *GCLC* GAG TNR polymorphism reportedly influences GSH levels in the European Caucasian population [78], we reported that specific genotypes may not have affected the levels in our study population

(the group of North Americans that include African Americans) [79]. It remains elusive which genetic and/or environmental factors potentially influence the sulforaphane response in GSH levels.

As has been reported for cardiovascular and cerebrovascular diseases, longer treatment duration and/or higher dosages may be warranted. For example, in a clinical trial involving human subjects with type 2 diabetes, improvements in oxidative stress were reported with a dose of sulforaphane of 10 g/day (~225 μ mol) and a duration of 4 weeks, each substantially more than in the present study [80]. This may account for the fact that we only observed suggestive changes in brain metabolites following sulforaphane treatment in the present study. Nevertheless, our short-term dosing strategy was well tolerated, which will further facilitate subsequent sulforaphane protocols in an attempt to link clinical biomarkers with patient-oriented outcomes.

Peripheral and brain GSH levels were correlated in mice when both were quantified by a standard biochemical assay. Nonetheless, it is less clear what the sulforaphane-elicited augmentation of GSH in the blood implies for the brain, at least for the present dosage and duration of sulforaphane use. Estimating GSH concentrations in the brain via MRS in human subjects has limitations or requires caution at least at present [81]. Sulforaphane likely crosses the blood-brain barrier, although its potency and necessary dosing for doing so is not yet established in humans. In rats, peripheral administration of sulforaphane can induce the anti-inflammatory *HO-1* gene in the brain, and beneficial effects have been noted on stroke, traumatic brain injury, and phencyclidine-induced hyperlocomotion [37, 82, 83].

Outcome studies for sulforaphane interventions will benefit from connecting clinical phenotypes to objectively measured biomarkers that may reflect disease patho-

physiology [47]. For example, in a submitted study, we will report that peripheral GSH levels may be correlated with cognitive functions. We thus posit the significance of exploring the possible correlations between peripheral GSH and clinical/neuropsychological measures and the influence of sulforaphane on such functional measures that are altered in neuropsychiatric disorders. The present study is a key first step toward such future studies.

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Statement of Ethics

All animal protocols and experiments were approved by the Johns Hopkins Animal Care and Use Program and in accordance with the guidelines for the care and use of laboratory animals issued by the National Institutes of Health.

All human participants provided written informed consent. All methods and experimental protocols used in this study were approved by the Johns Hopkins University Institutional Review Board (IRB# NA_00073385; principal investigator: T.W.S.). All experiments were performed in accordance with the guidelines and regulations established by the abovementioned IRB.

Disclosure Statement

We have no conflict of interest to declare.

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