A proof-of-concept clinical study examining the NRF2 activator sulforaphane against neutrophilic airway inflammation

Charity G. Duran†, Allison J. Burbank†, Katherine H. Mills1, Heather R. Duckworth1, Maria M. Aleman1, Matthew J. Kesic2, David B. Peden1, Yinghao Pan3, Haibo Zhou3 and Michelle L. Hernandez1,4*

Abstract

Sulforaphane (SFN), a naturally occurring isothiocyanate found in cruciferous vegetables, is implicated as a possible therapy for airway inflammation via induction of the transcription factor NF-E2-related factor 2 (NRF2). In this proof-of-concept clinical study, we show that supplementation of SFN with broccoli sprout homogenate in healthy human subjects did not induce expression of antioxidant genes or protect against neutrophilic airway inflammation in an ozone-exposure model. Therefore, dietary sulforaphane supplementation is not a promising candidate for larger scale clinical trials targeting airway inflammation.

Trial registration: NCT01625130. Registered 19 June, 2012.

Keywords: Sulforaphane, NRF2, Ozone, Airway inflammation, Antioxidants

Introduction

Dear Editor,

Asthma is a heterogeneous chronic disease that can be stratified based on features such as eosinophil or neutrophil predominance, and responsiveness to corticosteroids. Current available therapies including corticosteroids are not as effective for certain forms of the disease, particularly neutrophil-predominant asthma. Furthermore, due to negative perceptions of corticosteroids, the use of complementary and alternative medicine and nutritional interventions for asthma is increasing in the U.S [1]. The naturally occurring isothiocyanate, sulforaphane (SFN), is found in cruciferous vegetables and has been implicated as a possible therapy for airway inflammation via induction of the transcription factor NF-E2-related factor 2 (NRF2), which regulates expression of cytoprotective phase II antioxidant enzymes. The relevance of targeting antioxidant gene expression extends to other airway diseases as well, such as COPD, which is characterized by oxidative stress and dysregulation of antioxidant gene expression [2]. However, there are conflicting reports concerning the ability of SFN to induce antioxidant gene expression, and its effectiveness against airway inflammation [3–5]. In this brief communication, we report our findings from a proof-of-concept study examining if in vivo supplementation with SFN with broccoli sprout homogenate (BSH) is an effective intervention for ozone (O3)-induced airway inflammation, a model of neutrophilic airway inflammation. Oxidative injury is especially relevant for those with asthma, as antioxidant reserve may be impaired in this population. O3 inhalation causes significant airway neutrophilia in healthy non-asthmatic persons [6], making this a useful model for neutrophilic airway disease.

Methods

For this randomized, placebo-controlled, crossover study we recruited 16 non-atopic, non-smoking healthy volunteers between the ages of 18–50 years. The O3 study protocol was approved by the University of North Carolina’s Institutional Review Board, and written informed consent was obtained from all study subjects. All

* Correspondence: michelle_hernandez@med.unc.edu
† Equal contributors
1 UNC Center for Environmental Medicine, Asthma, & Lung Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
2 Division of Allergy, Immunology & Rheumatology, UNC School of Medicine, 104 Mason Farm Road, CB #7310, Chapel Hill, NC 27599-7310, USA
Full list of author information is available at the end of the article

© 2016 The Author(s). Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
subjects underwent a standardized screening protocol including allergy skin testing and methacholine challenge as previously described [7, 8]. Volunteers were randomized in a 1:1 ratio to consume either 200 g of BSH (equivalent to 111 g of commercially available Broccosprouts® (Brassica Protection Products LLC)), or 200 g of alfalfa sprout homogenate (ASH), which lacks SFN. The dose of BSH and ASH were chosen based on results of a prior study that found maximal induction of NRF2-dependent gene expression by BSH with a 3 day 200-g dosing regimen [4]. Subjects received supplements once daily for 3 days during the initial study period, and the alternate treatment during the crossover period. On the third day of supplementation, each subject was exposed to O₃ (0.4 ppm) for 2 h while performing four 15 min sessions of intermittent moderate exercise (defined as minute ventilation or VEₘᵋₜₐₜ = 30–40 L/min) on a treadmill, separated by 15 min of seated rest. Induced sputum was obtained at screening and at 4 h post-O₃ exposure and processed for measurement of cytokines and cell counts as previously described [7, 8]. Blood was collected at screening and post-O₃ for determination of SFN and SFN-conjugate levels by mass spectroscopy. Additionally, blood and nasal epithelial cells (NECs) were collected 4 h post-O₃ to measure NRF2-regulated gene expression (HO-1, NQO-1, GSTM-1). There was a minimum washout period of 14 days between treatment periods.

**Results**

Our primary hypothesis was that NRF2 activation with SFN would decrease %PMNs in induced sputum compared to placebo after O₃ exposure. The primary endpoint for this study was the effect of SFN compared to placebo on the O₃-induced change (post-O₃ minus pre-O₃) in %PMNs in airway sputum. To analyze the treatment effect on sputum cellularity, we compared active (BSH) to placebo (ASH) treatment using a linear mixed model approach [9]. Comparisons between post-O₃ active or placebo treatment to baseline values were carried out using Wilcoxon-Signed rank tests. Criterion for significance was taken to be \( p \leq 0.05 \).

Sixteen subjects were randomized, and fifteen subjects completed all visits. There were no serious adverse events during the course of the study. Three days of supplementation with BSH significantly increased levels of SFN (\( p = 0.001 \)) and its major metabolites, SFN-N-acetyl-L-cysteine (\( p = 0.002 \)) and SFN-glutathione (\( p < 0.001 \)) compared to placebo (Fig. 1a). O₃ exposure significantly increased the quantity of neutrophils in sputum (expressed as neutrophils/mg and %PMN) in both the placebo and BSH treatment groups (Fig. 1b), but the BSH supplementation group showed no significant difference in sputum neutrophilia compared to placebo. Despite significantly increased plasma levels of SFN in the BSH group, post-O₃ gene expression of NRF2 and

![Fig. 1](Plasma levels of SFN and its major metabolites SFN-N-acetyl-L-cysteine and SFN-glutathione following BSH supplementation (a). O₃-induced changes in sputum neutrophil counts with placebo or SFN supplementation (N = 14) (b). Changes are presented as PMNs/mg sputum and %PMNs/mg sputum.)
phase II antioxidant defense genes in NECs and peripheral blood were not significantly different from placebo (Table 1).

**Discussion**

SFN has received significant attention in recent years as a possible intervention for oxidant-induced airway inflammation through induction of NRF2-regulated antioxidant genes, but reports concerning its ability to induce antioxidant gene expression and protect against airway inflammation are conflicting. Possible explanations for these contradictory results include variable dosing, dosage forms, and differential biological responses in diseased versus healthy populations. Our study utilized a similar BSH preparation and dosing schedule as Reidl et al., in which 200 g of BSH was ingested daily for three days by healthy volunteers [4]. This preparation reportedly delivered 102 μmol SFN per dose. In contrast to Reidl et al., we saw no differences in phase II enzyme expression in NECs or peripheral blood. Furthermore, BSH supplementation had no impact on lower airway inflammation, as determined by O3-induced changes in sputum neutrophilia. Our results are in agreement with Sudini et al., in which ingestion of 100 g of whole broccoli sprouts daily by allergic asthmatics for 3 days had no effect on either NRF2-dependent gene expression in NECs and PBMCs, or eosinophilic lower airway inflammation (measured by FENO) [3]. On the other hand, supplementation of SFN using a standardized dose of broccoli sprout extract inhibited nasal inflammatory responses to diesel exhaust in cat-allergic subjects [5]. These contradictory results may be due to differing systemic levels of SFN achieved with dietary supplementation with BSH, which is not standardized. However, because variability exists in the timing and methods of detection for SFN conjugate levels, it is difficult to compare systemic SFN levels across studies.

Similar to our study, other groups have demonstrated marked increases in SFN conjugate levels following in vivo supplementation with BSH with minimal effects on antioxidant gene expression [3, 10]. It is possible that the plasma levels of SFN achieved with our BSH supplementation regimen were not sufficiently high to be biologically active. A dosing study using fresh broccoli sprouts that achieved significantly higher peak plasma levels of SFN metabolites found no significant increases in antioxidant gene expression in whole blood [10]. Although several in vitro studies report induction of NRF2 genes with SFN treatment, it is important to note that many of these studies utilize concentrations of SFN in the micromolar range [11–14]. Plasma levels of SFN achieved in our study are several orders of magnitude lower than those used in vitro; furthermore, levels achieved in target tissues are likely less than those achieved in plasma. Therefore, doses of BSH that can be reasonably consumed by an adult may exhibit little biologic activity.

**Conclusions**

In summary, dietary supplementation of SFN with BSH did not induce expression of NRF2-regulated genes, or have protective effects with O3 exposure, a model of neutrophilic airway inflammation. Collectively, these findings suggest that SFN supplementation with BSH is not a promising candidate for larger scale clinical trials targeting airway inflammation.

**Abbreviations**

ASH, alfalfa sprout homogenate; BSH, broccoli sprout homogenate; NECs, nasal epithelial cells; NRF2, NF-E2-related factor 2; SFN, sulforaphane

**Acknowledgements**

Many thanks to the UNC Biomarker Mass Spectrometry Core Facility (Wanda Bodnar and Leonard Collins) for their assistance [10].

**Funding**

MLH is supported by NIEHS K23-ES021745. AJB is supported by ST32GM086330. This project was supported in part by grants R01ES012706 and P30ES010126 from the National Institute of Environmental Health Sciences. This work was also funded by CR 83578501 from the US Environmental Protection Agency.

**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article.

**Authors’ contributions**

MLH, DBP, and MJK contributed to the conception and design of the study. KHM, MMA, CGD, and HRD were involved in acquisition of data. YP, HZ, MLH, CGD, AJB, and MMA were involved in the analysis and interpretation of the data. All listed authors were involved in drafting and revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

---

**Table 1** % Change antioxidant gene expression in healthy volunteers following O3 exposure

|                     | Placebo | SFN    | *p value |
|---------------------|---------|--------|----------|
| **Nasal Epithelial Cells** |         |        |          |
| GSTM1               | 58.74 ± 57.07 | 49.99 ± 41.76 | 0.9375 |
| HO-1                | 2.041 ± 24.97  | –6.06 ± 16.69 | 0.9097 |
| NQO-1               | 27.65 ± 44.88  | 39.35 ± 41.57 | 0.9097 |
| NRF-2               | 2.521 ± 17.42  | 0.9522 ± 18.52 | 0.9097 |
| ** Peripheral Blood** |         |        |          |
| GSTM1               | 26.29 ± 44.12  | –14.11 ± 20.05 | >0.9999 |
| HO-1                | –5.42 ± 13.93  | –7.61 ± 17.17  | 0.9453 |
| NQO-1               | 13.87 ± 43.43  | –18.18 ± 26.59 | 0.9375 |
| NRF-2               | 22.8 ± 27.20   | –12.59 ± 10.89 | 0.6406 |

Data are shown as mean ± SEM

Changes in GSTM1 expression were performed only on GSTM1 sufficient subjects. For all genes, N = 6–14

*Comparisons between Placebo and SFN groups were carried out using paired Wilcoxon-Signed rank tests.
Consent for publication
Not applicable.

Ethics approval and consent to participate
The O3 study protocol was approved by the University of North Carolina’s Institutional Review Board, and written informed consent was obtained from all study subjects.

Author details
1 UNC Center for Environmental Medicine, Asthma, & Lung Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. 2 Department of Physical Therapy, Methodist University, Fayetteville, NC, USA. 3 Department of Biostatistics, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. 4 Division of Allergy, Immunology & Rheumatology, UNC School of Medicine, 104 Mason Farm Road, CB #7310, Chapel Hill, NC 27599-7310, USA.

Received: 28 May 2016 Accepted: 14 July 2016
Published online: 22 July 2016

References
1. Torres-Llenza V, Bhogal S, Davis M, Ducharme FM. Use of complementary and alternative medicine in children with asthma. Can Respir J. 2010;17:183–7.
2. Kirkham PA, Barnes PJ. Oxidative stress in COPD. Chest. 2013;144:266–73.
3. Sudini K, Diette GB, Breyssse PN, McCormack MC, Bull D, Biswal S, Zhai S, Bereton N, Peng RD, Matsui EC. A Randomized Controlled Trial of the Effect of Broccoli Sprouts on Antioxidant Gene Expression and Airway Inflammation in Asthmatics. J Allergy Clin Immunol Pract. 2016. doi: 10.1016/j.jaip.2016.03.012
4. Redd MA, Saxon A, Diaz-Sanchez D. Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. Clin Immunol. 2009;130:244–51.
5. Heber D, Li Z, Garcia-Lloret M, Wong AM, Lee TY, Thames G, Krak M, Zhang Y, Nel A. Sulforaphane-rich broccoli sprout extract attenuates nasal allergic response to diesel exhaust particles. Food Funct. 2014;5:35–41.
6. Hernandez ML, Harris B, Lay JC, Bromberg PA, Diaz-Sanchez D, Devlin RB, Kleebenger SR, Alexis NE, Peden DB. Comparative airway inflammatory response of normal volunteers to ozone and lipopolysaccharide challenge. Inhal Toxicol. 2010;22:648–56.
7. Hernandez ML, Wagner JG, Kala A, Mills K, Wells HB, Alexis NE, Lay JC, Jiang Q, Zhang H, Zhou H, Peden DB. Vitamin E, gamma-tocopherol, reduces airway neutrophil recruitment after inhaled endotoxin challenge in rats and in healthy volunteers. Free Radic Biol Med. 2013;60:56–62.
8. Hernandez ML, Hestb M, Lay JC, Alexis NE, Brickey WJ, Ting JPY, Zhou HB, Peden DB. Atopic asthmatic patients have reduced airway inflammatory cell recruitment after inhaled endotoxin challenge compared with healthy volunteers. J Allergy Clin Immunol. 2012;130:869.
9. Jones BK, Michael G. Design and Analysis of Crossover Trials. 2nd ed. 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487, USA: Chapman and HallCRC; 2003.
10. Atwell LL, Hsu A, Wong CP, Stevens JF, Bells D, Yu TW, Pereira CB, Lohr CV, Christensen JM, Dashwood RH, Williams DE, Shannon J, Ho E. Absorption and chemopreventive targets of sulforaphane in humans following consumption of broccoli sprouts or a myrosinase-treated broccoli sprout extract. Mol Nutr Food Res. 2015;59:424–33.
11. Bhamre S, Sahoo D, Tibshirani R, Dill DL, Brooks JD. Temporal changes in gene expression induced by sulforaphane in human prostate cancer cells. Prostate. 2009;69:181–90.
12. Kleszczynski K, Ernst IM, Wagner AE, Kruse N, Zillikens D, Rimbach G, Fischer TW. Sulforaphane and phenylethyl isothiocyanate protect human skin against UV-induced oxidative stress and apoptosis: role of Nrf2-dependent gene expression and antioxidant enzymes. Pharmacol Res. 2013;78:28–40.
13. Brooks JD, Patton VG, Videses G. Potent induction of phase 2 enzymes in human prostate cells by sulforaphane. Cancer Epidemiol Biomarkers Prev. 2001;10:949–54.
14. Jiang Q, Chen C, Yang B, Hebbbar V, Kong AN. Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers. Life Sci. 2003;72:2243–53.