Consumption of Broccoli Sprouts Attenuates Intracellular P38 Map Kinase and Reactive Oxygen Species Pro-Inflammatory Activation in Human Leukocytes: A Randomised-Controlled Trial

Abstract

Dietary consumption of green vegetables has a protective association with reduction in cardiovascular diseases. The thiocyanate compound sulforaphane, which is obtained from green vegetables, induces antioxidant genes and can protect against pro-inflammatory cellular activation. Our objective was to investigate the influence of sulforaphane on leukocyte pro-inflammatory activation and assess the impact of a sulforaphane-rich diet on leukocyte activation in vivo. Healthy human volunteers were randomised to consume either broccoli sprout homogenates (BSH) rich in sulforaphane or alfalfa sprout homogenates (ASH) as controls in a double-blind crossover trial. Blood was sampled prior to and then at 1 h, 6 h and 24 h post-homogenate consumption and analysed by flow cytometry for intracellular markers of leukocyte activation, reactive oxygen species (ROS), p38 mitogen activated protein (MAP) kinase phosphorylation and p65 nuclear factor kappa B (NF-κB) phosphorylation. Plasma levels of sulforaphane were determined by LC-MS methods. Six participants were recruited into the study. Plasma concentrations of sulforaphane were 30 ng/ml at one hour following BSH and undetectable following ASH consumption. In the control ASH group, ROS activation and p38 MAP kinase phosphorylation were enhanced one hour following consumption. This increase was attenuated in the BSH group (ROS, p<0.02; p38, p<0.001 comparison between groups). We conclude that ingestion of sulforaphane-rich homogenate can protect against pro-inflammatory activation in circulating leukocytes by attenuation of constitutive ROS and p38 MAP kinase.

Keywords: Sulforaphane consumption; Leukocyte activation; Molecular signalling; Inflammation modulation

Introduction

Epidemiological evidence has revealed that consumption of broccoli and related vegetables is associated with reduced risk of coronary heart disease mortality [1-3]. Sulforaphane is found naturally as the precursor form glucoraphanin in

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cruciferous vegetables belonging to the Brassicaceae family (broccoli, cauliflower, bok choy and cabbage) [4,5]. The enzyme myrosinase, found inherently in plants (particularly in Daikon Japanese white radish) and in the human gut, is responsible for the enzymatic conversion of glucoraphanin into the active form of sulforaphane [6]. In vitro studies have revealed that sulforaphane induces antioxidants via activation of the transcription factor Nrf2 [7-11]. The antioxidant defence genes induced following Nrf2 nuclear translocation, include haem-oxygenase 1 (HO-1), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione peroxidase (GPx) and thyoredoxin (Trx) [12]. These have important inflammatory regulatory effects attenuating activation of p38 mitogen-activated protein kinase (MAPK) [13-17]. In addition, pre-clinical studies from our group and others have demonstrated that sulforaphane suppresses inflammation by inhibiting activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [10,11,18-22]. Despite these insights, the potential effects of sulforaphane on inflammatory signalling pathways have not been fully studied clinically. Given the known role of reactive oxygen species (ROS), p38 MAPK and NF-κB in the transcriptional induction and stability of pro-inflammatory molecules, modifying these with a dietary enriched source of sulforaphane may dampen systemic inflammation. We therefore hypothesised that ingestion of a sulforaphane-rich homogenate would attenuate early activation of pro-inflammatory signalling (e.g. ROS, p38 MAP kinase and NF-κB) in leukocytes. To test this hypothesis we conducted a trial in human volunteers with a sulforaphane-rich meal.

Materials and Methods

Materials

Fluorescent (phycoerythrin (PE)-Cy7-conjugated) antibody that recognises Thr180/Tyr182 phosphorylated p38 and PE-conjugated antibody that recognises Ser529 phosphorylated RelA (NF-κB) were purchased from BD Biosciences (San Jose, CA, USA). Buffers for intracellular staining were also obtained from BD Biosciences. The ROS-sensitive probe 3′-(p-aminophenyl) fluorescein (APF) was obtained from Molecular Probes (Eugene, OR, USA). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Randomised study design

A single-centre longitudinal cross over study was performed (NCT01357070). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the West London Research Ethics Committee (REC reference 11/H0707/10). Informed consent was obtained from all subjects.

Participants

Six healthy individuals were studied on two separate occasions, in random order in a crossover-design trial. Male and female participants were considered eligible for the trial if they were over 18 yrs of age and able to commit for the duration of the trial. Participants were considered ineligible if any of the following circumstances applied: pregnancy; practising vegetarian; history of allergy; current smoker or smoking cessation within the last 3 months; current use of inhaled, topical or systemic corticosteroids or within the last 2 weeks; current use of non-steroidal anti-inflammatory use or within the last 1 week; current use of nutritional or multivitamin supplements or current participation in any other randomised controlled trial. During the study, participants were asked to refrain from eating green vegetables, spices and condiments for 72 h prior to each study meal to minimise the effects of dietary sources of sulforaphane or related compounds.

Study settings

The study took place within the National Heart and Lung Institute, Imperial College London, UK.

Intervention and comparator of the randomised trial: Constitutive leukocyte activity in study participants was measured following the consumption of 200 g of homogenized broccoli sprouts (BSH) or following the consumption of 200 g alfalfa sprouts (ASH, lacking sulforaphane). Additionally, *ex vivo* PMA-enhanced ROS induction and *ex vivo* TNFa-enhanced phosphorylation of p38 MAPK and p65 NF-κB from study participants was measured following homogenate consumption.

Preparation of broccoli or alfalfa sprout homogenates: Homogenates for consumption in the clinical trial were prepared as previously reported [23]. Broccoli sprouts (Discover Fresh, the Netherlands) from a single production lot were used. Sprouts were processed in a designated food product preparation area prior to the expiration date. Sprouts were combined with sterile water in 1:1:2, v:w proportions and then homogenized in a clean blender to eliminate the variable of chewing by subjects. The aliquots of homogenate were then pooled and mixed with daikon sprouts (Green Valley Food Corp, USA). This was accomplished by adding 2% daikon (compared to broccoli sprout mixture based on fresh weight) and homogenizing the preparation in the blender once again. The daikon/broccoli sprout mixture was then incubated at 37°C for 2 h. This process adds excess myrosinase to maximally convert the free glucosinolates of the broccoli sprouts to sulforaphane, the biologically active compound. After incubation, the isothiocyanate and myrosinase mixture was aliquoted into 50 ml sterile Falcon tubes (equivalent to 70 g dry weight per aliquot – single dose) and stored at −20°C using dedicated freezer storage space. The broccoli sprout homogenates were thawed at 4°C, 12-24 h prior to dosing and were not administered if thawed for greater than 24 h. Alfalfa sprout homogenates were prepared using the same procedures; substituting locally purchased organic alfalfa sprouts (Sky Sprouts, Totnes, Devon) for broccoli sprouts, as above.

Participant allocation: Participants were randomised by trials unit personnel not otherwise involved in the study. Randomisation was blocked (six volunteers/block) and, because the preparations were concealed, the study personnel were blind to the allocations until the end of the study.

Intracellular staining for phosphorylated p38 MAPK and p65 NF-κB: Whole blood samples were incubated with Becton Dickinson (BD) Lyse/Fix Buffer (containing phosphatase inhibitors) for 10 min at 37°C. Leukocytes were then isolated by centrifugation (300 g for
5 min) and washed once with PBS. Cells were re-suspended in 0.5 ml pre-chilled BD Perm Buffer III, vortexed, and incubated on ice for 30 min. Following this, cells were washed twice with PBS and re-suspended in 0.5 ml BD Stain Buffer (FBS) prior to incubation for 30 min (at room temperature) with PE-Cy7- or PE-conjugated antibodies that recognised either Ser529 phosphorylated RelA (p65; NF-κB) or Thr180/Tyr182 phosphorylated p38 MAPK or isotype-matched control antibodies, with subsequent washing and analysis by flow cytometry (CyAn ADP, Beckman Coulter, High Wycombe, UK). Fluorescence of PE-conjugated p65 and PE-Cy7-conjugated p38 MAPK antibodies was quantified in granulocytes and mononuclear cells (each identified by forward and side scatter plots) using Summit 4.3 software.

Detection of ROS: Intracellular ROS levels were measured using the redox-sensitive fluorescent dye aminophenylfluorescein (APF). APF resists auto-oxidation with particular sensitivity for neutrophil/granulocyte ROS signalling. To load cells with APF, whole blood samples were combined with the APF dye (10 µM) and incubated for 30 min at 37°C prior to treatment with PMA (200 ng/ml) or TNFa (20 µg/ml) for 30 min. Erythrocytes were then lysed using BD Lyse Buffer for 15 min at 37°C. Leukocytes were then isolated by centrifugation (200 g for 5 min) and washed twice with PBS. ROS induction was quantified in the FITC channel in leukocyte subpopulations (identified by forward and side scatter plots) using Summit 4.3 software.

Plasma sulforaphane assay: Sulforaphane levels were measured in plasma samples by liquid chromatography mass spectrometry (LC-MS). HPLC was performed with a Water 2690 Separation Module system (Milford, MA, USA) equipped with a phenomenex Gemini 3 µ C18 110A column (150 × 2 mm) using an isocratic elution (acetonitrile/0.1% formic acid in water=50:50). The flow rate of the mobile phase and the column oven temperature was set at 0.2 ml/min and 30°C, respectively. The HPLC system was coupled to an API 2000 triple-quadruple mass spectrometer equipped with a turbo ion spray ionization source (AB MDS Sciex, Toronto, Canada). MS-MS detection was achieved using a positive ion multiple reaction monitoring (MRM) mode with an m/z transitions of 177.9 → 114.0 for sulforaphane, and 256.1 → 167.0 for diphenyldimethylamine.

Sample size determination: Based on the following assumptions, a sample size of 6 was required to detect a difference of 1 standard deviation in reactive oxygen species (ROS) between BSH and ASH conditions at a 5% significance level (2-sided) with 90% power. The assumptions were: cross-over trial design, i.e. one sample inference test; 1 baseline measurement of the outcome ROS; 3 post-intervention measurements of the outcome ROS; correlation between baseline and post-intervention ROS measurements=0.50; correlation between post-intervention ROS measurements=0.70.

Statistical methods
Outcome measurements were analysed in a mixed regression model (using STATA v.11) to take into account the repeated measurements over time. The baseline measurements were fitted as a covariate. The sample size calculation was powered to detect a main effect difference between broccoli and alfalfa conditions but the interaction of treatment condition and time of measurement were also estimated and retained in the model if found to be statistically significant at the level of p<0.05. The following predictors were included: main effect of time, fitted categorically (baseline to 24 h after consumption of homogenate) and main effect of the intervention (BSH vs. ASH ingestion). Total number of participants analysed was n=6 in a cross-over fashion.

Results
Reactive oxygen species and p38 MAP kinase were attenuated following ingestion of broccoli sprouts homogenates. Nine participants were recruited into the study. Three participants withdrew from the study and declined to continue after administration of the first homogenate, citing the unpalatable nature of the preparations. Data from the remaining 6 participants are presented; their demographic information is presented in Table 1. They comprised 4 female and 2 male participants with a median age of 26.5 yrs (interquartile range 24.5-29.3 yrs) and a median BMI of 20.2 kg/m² (interquartile range 19.1-21.3 kg/m²). No adverse events were noted from the remaining participants.

Plasma concentrations of sulforaphane were 30.7 ± 2.9 ng/ml (p<0.0001) at 1 h and 10.8 ± 0.9 ng/ml (p<0.0001) at 6 h following ingestion of BSH, becoming undetectable at 24 h when compared to baseline levels (Figure 1). Plasma sulforaphane levels were undetectable following the ingestion of ASH.

Analysis of blood samples from trial participants revealed considerable variation in ROS induction and p38 MAPK and NF-kB phosphorylation in granulocytes and mononuclear cells between individuals that consumed ASH or BSH (Figure 2). Descriptive statistics (means ± SD) are presented in the text between time
Figure 2  Consumption of broccoli sprouts reduced ROS and p38 MAP kinase activation at 1 h. Six healthy volunteers consumed either BSH or ASH homogenates alternatively over a 24 h period. Blood was sampled at 0 (immediately prior to consumption of homogenate), 1, 6 and 24 h afterwards. Leukocytes were loaded with the APF ROS-sensitive probe to determine constitutive levels (A) or stressed with PMA (B) with analysis by flow cytometry. Alternatively blood was immediately fixed and permeabilised prior to staining with antibodies to phospho-p38 MAP kinase (C) or phospho p65 (E) or subject to TNFα 20 ng/ml stress for 30 min and then fixed/permeabilised/stained for phospho-p38 (D) or phospho-p65 (F) with subsequent analysis by flow cytometry. ROS induction was quantified by FITC mean fluorescence intensity (MFI), p38 MAPK phosphorylation was quantified by PE-Cy7 MFI and p65 NF-κB phosphorylation was quantified by PE MFI in arbitrary units (AU). Pooled data (n=6) is shown ± SEM. Statistical results from mixed-regression modelling are shown. *p<0.05; ***p<0.005.
points of interest. For comparative analytical purposes, the data distribution was log-transformed and the log-transformed data was fitted to statistical models, as indicated. Main effects of time; time-plus-homogenate and time plus-homogenate-plus-interactions between-time-and-homogenate consumption were considered in the regression model analysis.

The regression model for the constitutive ROS response included time, intervention and interaction effects (Wald Chi-square=8.59 with 2 df, p=0.014). The results from this model indicated a rise in granulocyte ROS at 1 h that is more pronounced following the consumption of ASH (48.7 ± 43.2 FITC MFI units) compared to the consumption of BSH (30.3 ± 27.3 FITC MFI units); mean difference 0.41 log units, 95% CI 0.06 to 0.75, p=0.02. There were no significant differences between constitutive granulocyte ROS induction at 6 h or 24 h following either ASH or BSH ingestion. Under ex-vivo stress conditions, granulocytes manifest a strong induction of ROS irrespective of the consumption of either ASH or BSH. PMA-enhanced ROS at 1 h following consumption of ASH was 2936 ± 2441 FITC MFI units compared to a mean of 1989 ± 2858 FITC MFI units following BSH consumption. However, the regression models for these PMA-stressed conditions did not reveal any significant differences between consumption of BSH or ASH (p=0.37) (Figure 2B).

The regression model for p38 MAP kinase signalling effects included time, intervention and interaction (Wald Chi-square=8.93 with 2 df, p=0.012). The results from this model indicated that there was a greater rise in p38 MAP kinase phosphorylation at 1 h following ASH ingestion (113.7 ± 94.3 PE-Cy7 MFI units) compared to BSH ingestion (68.0 ± 85.0 PE-Cy7 MFI units); mean difference 1.80 log units, 95% CI (0.52 – 3.09), p=0.006 (Figure 2C). Similar to the ROS findings, differences between groups was not apparent at later time points. Under stress conditions, there was no change between baseline levels of p38 MAP kinase in either BSH and ASH conditions, and the statistical model did not achieve significance (p=0.74) (Figure 2D).

The statistical models for constitutive levels of phosphorylated p65 NF-κB in granulocytes did not reach significance for time or intervention or interaction following either ASH or BSH consumption (p=0.26) (Figure 2E). However, the models for levels of stress-induced p65 NF-κB showed a strong time effect (Wald chi-square p=0.0001), but no effect from interaction. There was a decline in levels of p65 NF-κB over time with a strong effect seen at 24 h compared to baseline for both groups but no statistical interaction, thus implying no differences between homogenates being consumed; but an effect of consumption itself. Effects in monocytes, lymphocytes and mononuclear cells did not reach significance in statistical modelling of the parameters of interest.

Discussion

In this study we evaluated for the first time the effect of ingestion of broccoli sprouts, which are rich in glucosinolates, the precursors of sulforaphane, on leukocyte activation in healthy volunteers. We have shown that in the early phase following ingestion of BSH (1 h-6 h), biologically significant levels of sulforaphane are detectable in the plasma. This is associated with attenuation of ROS and p38 MAPK in leukocytes from volunteers at the earliest time-point following ingestion (1 h).

Sulforaphane was selected for study because it is a potent indirect antioxidant that induces numerous endogenous anti-oxidant enzymes (e.g. HO-1, ferritin) via the transcription factor Nrf2 [24,25]. Sulforaphane suppresses arterial inflammation in rodents and inhibits MAP kinases and NF-κB in cultured vascular cells and leukocytes [6-10,16-18]. We have shown previously that sulforaphane pre-treatment protects against renal damage and systemic inflammation in response to cardiopulmonary bypass in a porcine model [26].

Consumption of broccoli sprouts has been shown to generate sulforaphane plasma concentrations that reached peak values 1-1.5 h after feeding and then dropped with first-order kinetics [4,5,21]. This is consistent with our observed plasma concentration data. We have shown that the influence of BSH was most prominent at 1 h following ingestion. Early pre- and co-treatment with sulforaphane appears to have relatively fast biological effects within 1-2 h. For example, following iv administration of 25 mg/kg sulforaphane into wild-type male Sprague-Dawley rats, maximal induction of NQO1 mRNA expression in lymphocytes was observed at 1.6 h; GPx at 0.72 h; and HO-1 at 0.56 h. One-hour pretreatment of aortic endothelial cells with sulforaphane (1-4 μM) suppressed TNFα-induced MCP-1 and VCAM-1 pro-inflammatory gene transcription as well as protein levels with suppression of p38 MAP kinase phosphorylation but no effect on NF-κB. Co-treatment of raw macrophages with Sulforaphane (5 μM) and LPS (500 ng/ml) revealed down-regulation of inducible iNOS and Cox-2 within 2 h. Similarly, Sulforaphane (10-20 μM) pre-treatment for 45 min in macrophages exhibited reduced NF-κB activation following LPS stress. Together, these observations suggest that Nrf2 is a fast-acting transcription factor (not requiring synthesis but moreover, stabilisation and nuclear translocation) or that the anti-inflammatory effects observed with short/early treatments with sulforaphane occur independently of genes activated via the Nrf2/ARE pathway. Further studies are required to distinguish between these possibilities.

The enzyme myrosinase (thioglucoside glucohydrolase) is responsible for the conversion of the precursor glucoraphanin into the active form of sulforaphane and is inherently present within plants and also present in the bacterial flora of the human intestine [24]. In our study, we utilised daikon sprouts to enhance the levels of myrosinase in BSH preparations in addition to homogenising the plant material to eliminate the variable of chewing. The dose of 200 g broccoli sprouts was chosen because in previous studies it was tolerated well in humans and led to maximal induction of antioxidant enzymes [23].

Although sulforaphane is rapidly cleared from plasma, antioxidant enzymes can be induced for at least 24 h in leukocytes of healthy

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volunteers [21]. We therefore designed our trial to measure the effects on leukocytes up to 24 h following consumption of homogenate and included a generous wash-out period of two weeks between each arm of the study. However, at later timepoints (6 h and 24 h), there was no effect in leukocytes despite the induction and persistence of anti-inflammatory gene expression reported in the literature [21]. It is difficult to interpret data at these late timepoints because participants were exposed to varied diets and other environmental factors at these intervals. The constitutive levels measured can be influenced throughout the day by multiple events. There were no restrictions on what participants did throughout the duration of the study after the homogenates were consumed. For example, ingestion of curcumin from curries or consumption of red wine containing resveratrol [27-29] could potentially lead to variable responses between individuals. There are many nutriceutical factors that can influence the results and with further research taking place to characterise their importance. Ex vivo stress was applied to leukocytes from homogenate trial participants to test their reactivity to a known inflammatory stimulus. Patterns of activation appear to be different but because of the variability of individual responses it was difficult to draw sound and meaningful conclusions on the basis of the data.

It is also plausible that other additional compounds in BSH (and ASH) are responsible for some part of these observations. Individuals may exhibit differing pharmacokinetic and pharmacodynamic profiles to the ingestion of these preparations with influences from genetic polymorphisms of metabolism pathways.

The primary site of absorption of sulforaphane occurs within the jejenum. Due to sulforaphane’s lipophilic profile and small molecular size, it is passively absorbed via enterocytes in the liver from the entero-portal system [30,31]. Sulforaphane is conjugated in the liver with glutathione by the action of glutathione-s-transferase and excreted in the urine [32]. Investigators have reported that high cellular accumulation of sulforaphane occurs in mammalian cells noting cellular concentrations of 4.4-13.3 mM following 30 min incubation with 0.028-0.28 mM sulforaphane, suggesting a plasma-tissue difference of 47-145 fold accumulation [33]. Genetic polymorphisms in Nrf2 have been reported to be associated with increased risk of developing acute lung injury following trauma, in patients [34]. Additionally, it was also possible that study participants were able to discriminate between homogenates, and in doing so modify subsequent behaviour. Despite this potential for bias, allocation concealment was found to be adequate, judging from a post-study electronic questionnaire.

An important aspect of the human study was the palatability of the homogenates. The appearance, smell and taste of the preparations of the homogenate aliquots were unappealing to some trial participants. This needs to be improved in future studies using homogenate preparations (e.g. with other flavourings), or abrogated using purified forms of this agent. Reassuringly, there were no adverse effects reported, but clearly the study was not powered for untoward events.

Conclusion

To conclude, our data suggest that consumption of sulforaphane-containing vegetables may reduce inflammatory activation of circulating leukocytes. Thus dietary modifications may have clinical utility for the modulation of leukocyte activity in patients and protect against development of cardiovascular disease. More extensive studies are now required to examine whether BSH or related preparations can have beneficial effects in patients with established heart disease.

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References


