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Off-target effects of sulforaphane include the derepression of long terminal repeats through histone acetylation events☆

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Abstract

Sulforaphane is a naturally occurring isothiocyanate in cruciferous vegetables. Sulforaphane inhibits histone deacetylases, leading to the transcriptional activation of genes including tumor suppressor genes. The compound has attracted considerable attention in the chemoprevention of prostate cancer. Here we tested the hypothesis that sulforaphane is not specific for tumor suppressor genes but also activates loci such as long terminal repeats (LTRs), which might impair genome stability. Studies were conducted using chemically pure sulforaphane in primary human IMR-90 fibroblasts and in broccoli sprout feeding studies in healthy adults. Sulforaphane (2.0 μM) caused an increase in LTR transcriptional activity in cultured cells. Consumption of broccoli sprouts (34, 68 or 102 g) by human volunteers caused a dose dependent elevation in LTR mRNA in circulating leukocytes, peaking at more than a 10-fold increase. This increase in transcript levels was associated with an increase in histone H3 K9 acetylation marks in LTR 15 in peripheral blood mononuclear cells from subjects consuming sprouts. Collectively, this study suggests that sulforaphane has off-target effects that warrant further investigation when recommending high levels of sulforaphane intake, despite its promising activities in chemoprevention.

Keywords: Histone deacetylases; Long terminal repeats; Off target effects; Sulforaphane

1. Introduction

Cruciferous vegetables such as broccoli and cauliflower contain a large number of glucosinolates including glucoerucin, glucoiberin and glucoraphanin [1]. When unheated vegetables are processed by chopping or chewing, the enzyme myrosinase is released from myrosin grains in myrosin cells, and glucosinolates are released from adjacent S cells [2–4]. Myrosinase catalyzes the hydrolytic removal of the glucose moiety in glucoraphanin, followed by nonenzymatic release of a hydrogen sulfate moiety and spontaneous rearrangement of the unstable intermediate to form the aliphatic isothiocyanate sulforaphane (SFN) [1]. SFN has attracted considerable attention due to its putative role in cancer prevention [5].

Various, not mutually exclusive, mechanisms have been proposed to explain the chemopreventive activities of SFN. One theory is that SFN enhances drug-mediated cytotoxicity against cancer cells including cancer stem cells [6–8]. The significance of these observations is not limited to the chemotherapy of cancer but extends to the prevention of cancer through enhancing cellular sensitivity to cell death signals in tumor initiation. SFN-dependent inhibition of antiapoptotic NF-κB signaling pathways appears to play a major role in the elimination of abnormal cells [9,10]. A second theory is that SFN-dependent inhibition of histone deacetylases (HDACs) causes an increase in the expression of tumor suppressor genes such as p21 and Bax, leading to cell cycle arrest and apoptosis [11,12]. Evidence suggests that SFN inhibits Class I and Class II HDACs [13]. The locus (gene) specificity of SFN is uncertain, despite a general consensus that chemoprevention needs to pursue gene-specific gene expression through the modulation of epigenetic marks in distinct genomic loci [14]. Gene-specific epigenetic editing can, theoretically, be achieved by fusing enzymes or inhibitors to gene-specific DNA binding domains.

In this paper we tested the hypothesis that the inhibition of HDACs by SFN does not only de-repress tumor suppressor genes but also has undesirable off-target effects, meditated by derepression of genes other than tumor suppressor genes. Studies were conducted in both cell cultures and healthy adults to take advantage of the small interindividual variation in cell cultures and to capture effects of biotransformation in human studies. As model loci for detecting off-target effects, we used long terminal repeats (LTRs), based on the following rationale.

LTRs make up about 8% of the human genome, and at least 51 LTRs are transcriptionally competent [15]. Repetitive elements such as

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LTRs pose a burden to genome stability, as their mobilization facilitates recombination between nonhomologous loci, leading to chromosomal deletions and translocations [16,17]. Mobilization of LTR transposons is associated with 10% of all spontaneous mutations in mice [18]. The transcriptional activity of LTRs is controlled by histone acetylation and other epigenetic marks; inhibition of HDACs leads to an increase in LTR transcription [19]. Derepression of LTRs may impair genome stability through insertional mutagenesis, recombination events that cause translocations and other rearrangements, de-regulation of genes in the host genome mediated by LTR promoter activity and antisense effects if transcription extends into exon sequence downstream of the transposon [20].

2. Methods and materials

2.1. Cell cultures

Primary human IMR-90 lung fibroblasts from a female Caucasian were obtained from American Type Culture Collection (ATCC CCL-186; Manassas, VA, USA). IMR-90 fibroblasts are primary human cells that have not been transformed or immortalized in any way. Therefore, the genetic make-up of IMR-90 fibroblasts is identical to that of human tissues, leaving no room for uncertainties regarding possible impacts of altered genetics as encountered in immortalized cell lines. Fibroblasts (from passages 32–36) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 0.1% sodium pyruvate, 100,000 U/L penicillin,100 mg/L streptomycin and 0.1% nonessential amino acids (final concentrations). R,S-SFN was obtained from LKT Laboratories (St. Paul, MN, USA) and was dissolved in dimethyl sulfoxide to prepare a stock solution containing 40 mmol/l SFN. Aliquots were frozen at −20°C until use. SFN concentrations in cell culture media were adjusted to a final concentration of 2.0 μmol/l; controls were treated with solvent. Samples were collected at timed intervals.

2.2. Human feeding study

Eight apparently healthy adults (four male and four female) participated in a broccoli sprout feeding study approved by the Institutional Review Board at the University of Nebraska-Lincoln. Exclusion criteria included pregnancy, smoking, self-reported health problems and use of SFN supplements. Participants in this study included six Caucasians (two Hispanics) and two Asians ages 19–30 years. Subjects were instructed not to consume cruciferous vegetables in the 48 h leading up to the study and during the 8-h period in which blood samples were collected. Each subject consumed three doses (34 g, 68 g and 102 g) of BroccoSprouts® broccoli sprouts from a local supermarket (HyVee, Lincoln, NE, USA). Sprouts were consumed with a bagel and cream cheese [11]. Each treatment was separated by a 2-week washout period, and the order of doses was randomized. Thirty milliliters of blood were collected before sprout consumption (baseline, time 0 h) and at timed intervals (2, 4 and 8 h) after consumption. Peripheral blood mononuclear cells (PBMCs) and plasma were purified using Histopaque and gradient centrifugation as described previously [21]; aliquots were frozen at −80°C until analysis.

2.3. Quantitation of SFN

SFN was extracted and quantitated by high performance liquid chromatography (HPLC) as previously described [22]. Briefly, 1 g of BroccoSprouts® was combined with 20 ml of water acidified with 0.1-M hydrochloric acid and homogenized for 10–15 s with a Tissue-Tearor model 985370 hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK, USA). Extracts were transferred to a 45°C water bath for 2 h and then cooled to room temperature. The solution was extracted twice with 20-ml dichloromethane, and the solvent phases were collected and combined. The solvent was dried through sodium sulfate and then loaded onto a Supelclean™ LC-Florisil® SPE column (Supelco). The column was washed with 3-ml ethyl acetate, and SFN was collected with 3-ml methanol. Samples were passed through a 0.2-μm filter and stored at −20°C until analysis. Determination of SFN concentration was performed by injection on a Waters (Milford, MA, USA) 600S HPLC system equipped with a VyDac C18 (Grace, Deerfield, IL, USA) column and a Waters 2996 Photodiode Array detector. Separation was achieved with an isocratic flow rate of 1.0 ml/min using water and acetonitrile (30:70 v/v). A sample volume of 20 μl was injected.

2.4. Quantitative real-time polymerase chain reaction (PCR) (qRT-PCR)

The abundance of p21 mRNA and LTR mRNA (transcript R/U5) was quantified by qRT-PCR as previously described using the cycle threshold method; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize for PCR efficiency [23,24]. PCR primer sequences for p21 were 5'-AGGCGGTTATGAAATTCACC-3' (forward) and 5'-CCCTTCAAAGTGCCATCTG-3' (reverse). LTR primer sequences were the same as previously reported [25]. Note that the values for LTR mRNA represent the grand total of all transcriptionally active LTRs due to near-identical sequences in these repeats [25]. Areas under the curves (AUCs) for LTR mRNA were calculated using the linear trapezoidal rule and were corrected for baseline levels [26].

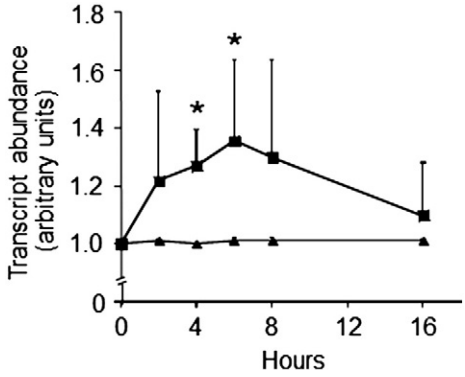

Fig. 1. SFN increases LTR transcription in IMR-90 fibroblast cultures. Fibroblasts were treated with 2.0-μmol/l SFN (squares) or solvent (triangles). *Significantly different compared with vehicle control at the same collection time ($p<0.05$ by Wilcoxon Signed-Rank test, $N=4–7$).

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described [23]. Antibody against K9-acetylated histone H3 (H3K9ac; ab10812) was purchased from Abcam (Cambridge, MA, USA). Data were normalized for nucleosomal occupancy using an antibody to the C-terminus in histone H3. The enrichment of H3K9ac marks in LTR15 (nomenclature as per [15]) were quantified by qRT-PCR, using GAPDH as a control. PCR primer sequences were the same as in our previous studies [25]. In case of ChIP assays, as opposed to mRNA quantification, individual LTRs can be distinguished by having one PCR primer anneal with sequences in the host genome adjacent to the LTR of interest.

2.6. Statistics

Homogeneity of variances was tested using Bartlett's Test. If variances were heterogeneous, data were log transformed before analysis. Data from IMR90 cell cultures were analyzed by using the Wilcoxon Signed-Rank Test. Data from PBMC experiments were analyzed using one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) for posthoc comparisons [27] for gene expression data and Wilcoxon Signed-Rank Test for ChIP data. Differences were considered statistically significant if $p<0.05$. StatView 5.0.1 was used for conducting statistical analyses (SAS Institute; Cary, NC, USA).

3. Results

3.1. LTR transcript levels increase after SFN treatment in IMR-90 fibroblast cultures

SFN derepressed LTRs in IMR-90 fibroblasts (Fig. 1). A significant increase in LTR mRNA was detectable at t = 4 h, and peak values were achieved at t = 6 h compared with vehicle controls. LTR expression

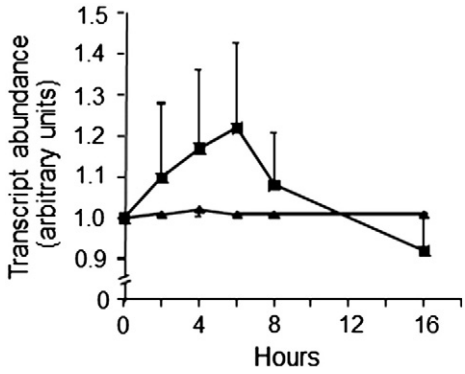

Fig. 2. SFN had no significant effect on p21 transcription in IMR-90 fibroblast cultures. Fibroblasts were treated with 2.0-μmol/l SFN (squares) or solvent (triangles). *Significantly different compared with vehicle control at the same collection time ($p<0.05$ by Wilcoxon Signed-Rank test, $N=4–7$).