Transcriptome Analysis of Human Colon Caco-2 Cells Exposed to Sulforaphane\textsuperscript{1–3}

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ABSTRACT Sulforaphane (SF), a dietary phytochemical obtained from broccoli, has been implicated in several physiological processes consistent with anticarcinogenic activity, including enhanced xenobiotic metabolism, cell cycle arrest, and apoptosis. In this study, we report changes in global gene expression in Caco-2 cells exposed to physiologically appropriate concentrations of SF, through the use of replicated Affymetrix array and RT-PCR experiments. After exposure to 50 \( \mu \text{mol/L} \) SF, 106 genes exhibited a \( >2 \)-fold increase in expression and 63 genes exhibited a \( >2 \)-fold decrease in expression. There were fewer changes in gene expression at lower SF concentrations. The majority of these genes had not previously been shown to be modulated by SF, suggesting novel mechanisms of possible anticarcinogenic activity, including induction of differentiation and modulation of fatty acid metabolism. The changes in the expression of 10 of these genes, together with 4 additional genes of biological interest, were further quantified in independent studies with RT-PCR. These genes include several that have recently become associated with carcinogenesis, such as Krüppel-like factor (KLF)\textsubscript{4}, a gut-enhanced transcription factor associated with induction of differentiation and reduction in cellular proliferation; DNA (cytosine-5-) -methyltransferase 1, associated with methylation; and \( \alpha \)-methylene-CoA racemase (AMACR), a marker associated with the development of colon and prostate cancer. The expression of 5 of these genes [caudal type homeobox transcription factor 2 (CDX-2), KLF\textsubscript{4}, KLF\textsubscript{5}, cyclin-dependent kinase inhibitor 1A (p21), and AMACR] was additionally studied after in vitro exposure to SF of surgically resected healthy and cancerous colon tissue from each of 3 patients. The study suggests the complex effects that SF has on gene expression and highlights several potential mechanisms by which the consumption of broccoli may reduce the risk of carcinogenesis. J. Nutr. 135: 1865–1872, 2005.

KEY WORDS: • colon cancer • chemoprevention • sulforaphane • microarray • KLF\textsubscript{4} • AMACR • p21

Epidemiologic studies have provided evidence that a diet rich in cruciferous vegetables may reduce the risk of cancer at several sites (1–6). Particular to crucifers among commonly consumed vegetables and fruits is the presence of glucosinolates, sulfur-containing glycosides. These compounds can degrade during and after consumption to isothiocyanates and indole compounds, both of which were shown to have biological activity consistent with anticarcinogenic properties. Among these is the isothiocyanate sulforaphane [SF,\textsuperscript{5} 1-isothiocyanato-4-(methylsulfinyl) butane; \( \text{CH}_3\text{SO}-(\text{CH}_2)_4\text{N}=-\text{C}=\text{S} \), which is derived primarily from broccoli and contains the corresponding glycoside, 4-methylsulfanylbutyl glucosinolate (GR, glucoraphanin)]. Although the majority of epidemiologic studi

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\textsuperscript{2} Microarray data cited in this study were deposited with the public repository Array Express under Experiment Accession No: E-MEXP-170 (http://www.ebi.ac.uk/arrayexpress/).

\textsuperscript{3} Supplemental Tables 1–4 and Supplemental Figure 1 are available as Online Supporting Material with the online posting of this paper at http://www.nutrition.org.

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\textsuperscript{5} Abbreviations used: AKR, aldo-keto reductase family 1, member C1; AMACR, \( \alpha \)-methylene-CoA racemase; APC, adenomatous polyposis coli; ARE, antioxidant response element; ATF3, activating transcription factor 3; CDX-2, caudal type homeobox transcription factor 2; dChip, DNA-chip analyzer; DMSO, dimethyl sulfoxide; Dnmt1, DNA (cytosine-5-) -methyltransferase 1; FAP, familial adenomatous polyposis; FDR, false discovery rate; GADD45b, growth arrest and DNA-damage-inducible, \( \beta \); GR, glucoraphanin; GSH, glutathione; H0, heme oxygenase 1; Hsp27, heat shock 27kDa protein 1; \( \text{IC}_{50} \), 50\% inhibitory concentration; ITC, isothiocyanate; JNK, c-Jun NH\textsubscript{2}-terminal kinase; KLF, Krüppel-like factor; MCM4, minichromosome maintenance deficient 4; MRP2, multidrug resistance protein 2; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT, methythiazolyldiphenyl-tetrazolium bromide; NQO1, NAD(P)H dehydrogenase, quinone 1 or quinone reductase (QR); NF\textsubscript{κ}B, nuclear factor (erythroid-derived 2)-like 2; p21, cyclin-dependent kinase inhibitor 1A; p57, cyclin-dependent kinase inhibitor 1C; PM, Perfect Match; pNA, p-nitroaniline; SF, sulforaphane; SMAR1, BTG3-associated nuclear protein; TR1, thioredoxin reductase 1.
ies cannot distinguish between consumption of different cruciferous vegetables, some studies have associated a reduction in the risk of cancer specifically with consumption of broccoli (3,6).

After tissue disruption, glucosinolates are hydrolyzed by the plant enzyme myrosinase to yield an unstable aglycone that rearranges to either the isothiocyanate or the nitrile derivative. Cooking crucifers may denature myrosinase, resulting in ingestion of intact glucosinolates, but isothiocyanate (ITC) metabolites are still recovered in the urine (7,8), probably due to microbial activity in the large bowel. Thus, depending upon how broccoli is processed, SF may be absorbed through the stomach, small intestine, or colon. After passive diffusion into epithelial cells, SF is rapidly conjugated with glutathione (GSH), and actively transported into the blood stream. The SF-GSH conjugate is further metabolized via the mercapturic acid pathway, and SF-N-acetylcysteine conjugates are excreted in the urine. The precise nature and concentration of the conjugates that circulate in the plasma have not been fully resolved. However, the concentration of circulating SF metabolites is likely to be on the order of a few μmol/L, and excretion is complete after ~24 h (7,9). Epithelial cells of the gastrointestinal tract will experience both topological exposure of SF of relatively high concentrations, followed by systemic exposure to lower concentrations.

Initial interest in SF was due to its potent ability to induce phase II enzymes, such as quinone reductase (QR) and UDP-glucuronosyl transferase, via antioxidant response element (ARE)-mediated transcription. To what extent the induction of detoxification enzyme in vivo accounts for the anticarcinogenic activity of SF or broccoli is uncertain.

Several recent studies demonstrated that SF can induce cell cycle arrest in a variety of cell types, including prostate (10–12), lymphocyte (10,13), colon (14), and mammary (15). Our own studies with Caco-2 cells confirmed these results; exposure of Caco-2 cells to 50 μmol/L SF resulted in 52% of cells in G2/M phase, compared with 15% G2/M cells in an untreated control (unpublished data). In a similar manner, several studies also reported the ability of SF to induce apoptosis in a range of cell lines (11,13,14,16,17). The mechanisms underlying these physiological processes have not been fully elucidated, although the induction of the c-Jun NH2-terminal kinase (JNK) signal transduction pathway and activation of caspases were implicated in apoptosis (11,18,19).

Although there have been several studies that reported changes in the expression of a single or a small number of genes after exposure of colon cell cultures to SF (14,16,20), we attempted to gain an overview of changes in gene expression in Caco-2 cells via the use of Affymetrix human oligonucleotide arrays, and to investigate whether changes in the expression of a small number of genes of particular interest in colon cell cultures were also observed in surgically resected colon tissue.

MATERIALS AND METHODS

Cell culture and chemicals. The human Caucasian colon adenocarcinoma cell line Caco-2 was obtained from the European Collection of Animal Cell Cultures. The cells were cultured in Eagle’s MEM supplemented with 1% nonessential amino acids, 2 mmol/L glutamine, 1 U/L penicillin, 1 μg streptomycin (Invitrogen), and 10% fetal calf serum, and maintained in 5% CO2 at 37°C. For cell viability and ELISA assays, cells were seeded in 96-well plates. For mRNA to be hybridized onto microarrays, cells were seeded in 6-well plates. For enzyme assays and real-time RT-PCR, cells were seeded in 6-well plates. For mRNA to be hybridized onto microarrays, cells were seeded in 6-well plates.

MTT cell viability assay. The assay is based on the conversion of the yellow meththiazolylphenyl-tetrazolium bromide (MTT) to purple formazan crystals by metabolically active cells and provides a quantitative determination of viable cells (21). Cells were treated with concentrations of SF ranging from 10 to 150 μmol/L in 6 replicate wells for 24 h. Formazan crystals were solubilized in DMSO and absorbance of each sample was measured at 550 nm against a 720-nm reference using a microplate reader. The experiment was repeated twice and the 50% inhibitory concentration (IC50) was calculated with the CalcuSyn software.

Quantitation of apoptosis by CaspACE™ assay. Cells were exposed to vehicle DMSO and 1, 10, 25, and 50 μmol/L SF in triplicate wells for 24 h; 20 μmol/L of the caspase inhibitor Z-VAD-FMK was added to one of the wells at the same time. To confirm the assay, cells were treated for 4 and 72 h with 5 μmol/L camptothecin, which induces apoptosis in Caco-2 cells under these conditions. Caspase-3 activity was assessed using the colorimetric CaspACE Assay System (Promega), which uses a substrate labeled with the chromophore p-nitroaniline (pNA) to measure protease activity. Cleavage of the substrate by caspase-3 produced yellow pNA, which was measured spectrophotometrically at 410 nm. Apoptosis was also measured by the Cell Death Detection ELISA Plus assay (Roche Diagnostics), which uses 1-step sandwich immunosassay to detect and quantify histone-complexed DNA fragments released from cells during apoptosis. Caco-2 cells were treated with 1–70 μmol/L SF for 24 h in triplicate. As a positive inducer, the cells were treated with 5 μmol/L camptothecin for 72 h. Absorbance was measured at 410 nm using 450 nm as a reference wavelength.

RNA extraction and array hybridization. In a single experiment, cells were treated with vehicle DMSO and 1, 5, 25, and 50 μmol/L SF for 24 h, and total RNA from 4 biological replicates of each treatment was isolated using the QIAGEN® RNeasy Mini Kit. The quality of the resulting RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples of each of the replicates were analyzed using Affymetrix Human U133A chips (Affymetrix). Double-stranded cDNA synthesis and generation of biotin-labeled cRNA were performed according to the manufacturer’s protocol. The final cRNA was checked for quality before fragmentation and hybridization onto a total of 20 arrays. Fluorescence intensity for each chip was captured with an Affymetrix HP GeneArray laser confocal scanner (Agilent). Affymetrix Microarray Suite version 5.0 (MAS 5.0, Affymetrix) was used to quantitate each U133A chip (22). The 20 CEL files generated containing the summary intensities for each probe were loaded into the DNA-Chip Analyzer software (dChip), version 1.3 (23), for normalization, generation of expression values, and statistical analysis. For normalization, dChip uses the Invariant Set Normalization method, which chooses a subset of Perfect Match (PM) probes with small within-subset rank difference in the two arrays (baseline and target array), to serve as the basis for fitting a normalization curve. This curve is then used to generate new normalized values for every probe on the chip. After normalization, probe expression levels can be calculated using either the PM-only model or the PM-Mismatch difference model. Previously, comparison between the 2 models showed that applying the PM-only model consistently produced less variable results (24); therefore, it was chosen for the present study. Hierarchical clustering was performed on the genes that were found to be differentially expressed after treatment with 50 μmol/L SF compared with control as well as clustering of the 20 samples.

Real-time RT-PCR. We selected 10 genes that had shown a >2-fold change in expression for further study by RT-PCR in a separate experiment. Two of these [theoredoxin reductase (TR1) and NAD(P)H quinone oxidoreductase (NQO1)] had previously been shown to be affected by SF, whereas the others had been associated with cell cycle suppression and apoptosis [growth arrest and DNA-damage-inducible, β (Gadd45β); Krüppel-like factor (KLF)5; KLF4; minichromosome maintenance deficient 4 (MCM4);
cycin-dependent kinase inhibitor 1C (p57); activating transcription factor 3 (ATF3), fatty acid metabolism associated with carcinogenesis [α-methylacyl-CoA racemase (AMACR)], or transcriptional activation of xenobiotic metabolizing genes [nuclear factor (erythroid-derived 2)-like 2 (Nrf2)]. In addition, we quantified the expression of 4 other genes, cyclin-dependent kinase inhibitor 1A (p21) (p21), caudal type homeo box transcription factor 2 (CDX-2), multidrug resistance protein 2 (MRP2), and DNA (cytosine-5-)-methyltransferase 1 (Dnmt1). These had not met the 2-fold cutoff criterion in the array studies, but had previously been shown to be induced by SF (p21 and MRP2), were associated with KLF4 (CDX-2), or were of particular interest (Dnmt1). Cells were treated with vehicle DMSO and 1, 5, 25, and 50 μmol/L SF for 24 h; total RNA from 3 biological replicates of each treatment was isolated using the QIAGEN RNeasy Mini Kit. Target mRNA was quantified using an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). Primers and probes (Supplemental Table 1) were designed using ABI PRISM Primer Express software v.1.5 (Applied Biosystems). Primers were purchased from Sigma-Genosys and probes from Applied Biosystems. The probes were labeled with a 5′ reporter dye (FAM, 6-carboxyfluorescein) and a 3′ quencher dye (TAMRA, 6-carboxytetramethylrhodamine). RT-PCR reactions were carried out in a microamp optical 96-well plate and real-time RT-PCR was performed as described above.

**Statistical analysis.** Data were expressed as means ± SEM or as means of the fold change ± SEM. For microarray analysis with dChip, 3 criteria were applied to detect differentially expressed genes: 1) a cutoff of 2.0-fold change, 2) absolute difference between the 2 groups means > 100, and 3) P ≤ 0.05 for Welch modified 2-sample t test, adjusted to compensate for multiple testing using false discovery rate (FDR). This analysis was subsequently repeated in R (25). Five genes present in the list generated by dChip were not confirmed by R and are marked accordingly in Supplemental Table 2. Genes showing significant differences in expression were classified into different functional categories, based on Gene Ontology with modifications (26,27). For subsequent analyses of selected genes by RT-PCR, data were analyzed by Student’s t tests using the MINITAB™ statistical software adjusted to compensate for multiple testing using FDR. Differences were considered significant at P ≤ 0.05. Additionally, the expressions of p21, MRP2, CDX2, and Dnmt1 from microarray data in Table 1 were analyzed in the same manner as the RT-PCR data because the fold change of these 4 genes was <2.

**RESULTS**

**SF** is cytotoxic to Caco-2 cells, but does not induce apoptosis at 24 h. SF reduced viability of Caco-2 cells in a

### Table 1

Comparison of changes in gene expression detected by Affymetrix array analysis and real-time RT-PCR after treatment with 50 μmol/L SF

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>Affymetrix</th>
<th>RT-PCR&lt;sup&gt;2,4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioredoxin reductase 1</td>
<td>TR1 NM_003330</td>
<td>3.26 ± 0.33</td>
<td>8.75 ± 0.38</td>
</tr>
<tr>
<td>Growth arrest and DNA-damage-inducible, β</td>
<td>Gadd45b NM_015675</td>
<td>2.68 ± 0.16</td>
<td>120.38 ± 3.94</td>
</tr>
<tr>
<td>NAD(P)H dehydrogenase, quinone 1</td>
<td>NOQ1 AI039874</td>
<td>2.38 ± 0.11</td>
<td>2.49 ± 0.18</td>
</tr>
<tr>
<td>Krüppel-like factor 5 (intestinal)</td>
<td>KLF5 AF132818</td>
<td>2.2 ± 0.12</td>
<td>−1.85 ± 0.15&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Krüppel-like factor 4 (gut)</td>
<td>KLF4 BF514079</td>
<td>2.16 ± 0.07</td>
<td>5.07 ± 0.43</td>
</tr>
<tr>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
<td>Nrf2 NM_006164</td>
<td>2.12 ± 0.06</td>
<td>−1.21 ± 0.05</td>
</tr>
<tr>
<td>Activating transcription factor 3</td>
<td>ATP3 NM_001674</td>
<td>2.1 ± 0.08</td>
<td>15.49 ± 0.23</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>p21 NM_000389</td>
<td>1.45 ± 0.07&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.68 ± 0.29</td>
</tr>
<tr>
<td>ATP-binding cassette, subfamily G (CFTR/MRP), member 2</td>
<td>MRP2 NM_000392</td>
<td>1.23 ± 0.05&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.84 ± 0.06</td>
</tr>
<tr>
<td>Caudal type homeo box transcription factor 2</td>
<td>CDX-2 US5096</td>
<td>−1.26 ± 0.02&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−5.88 ± 0.27</td>
</tr>
<tr>
<td>DNA (cytosine-5-)-methyltransferase 1</td>
<td>Dnmt1 NM_001379</td>
<td>−1.8 ± 0.08&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−1.8 ± 0.18</td>
</tr>
<tr>
<td>Minichromosome maintenance deficient 4 (Saccharomyces cerevisiae)</td>
<td>MCM4 AI859865</td>
<td>−2.05 ± 0.05</td>
<td>−2.5 ± 0.11</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1C</td>
<td>p57 NM_000076</td>
<td>−2.39 ± 0.11</td>
<td>−1.54 ± 0.18</td>
</tr>
<tr>
<td>α-Methylacyl-CoA racemase</td>
<td>AMACR AA88589</td>
<td>−2.43 ± 0.02</td>
<td>−2.12 ± 0.07</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are means ± SEM, n = 4 (Affymetrix) or n = 3 (RT-PCR), estimated in independent experiments; P < 0.05 unless noted. * Not significant, P = 0.056. Positive values represent upregulation; negative values represent downregulation.

<sup>2</sup> Values are gene expression ratios calculated using the PM-only model on normalized arrays in dChip between Caco-2 cells treated with SF and cells treated with equal amounts of vehicle DMSO.

<sup>3</sup> Values are mean gene expression ratios calculated by real-time RT-PCR between Caco-2 cells treated with SF and cells treated with equal amounts of vehicle DMSO.

<sup>4</sup> Data analyzed by Student’s t tests.
dose-dependent manner. The IC_{50} was calculated to be 85 and 83 μmol/L in 2 independent experiments (data not shown). A concentration of 50 μmol/L reduced cell viability by 26% and was therefore chosen as the maximum concentration for further experiments. To determine whether SF induces apoptosis in Caco-2 cells, 2 independent methods of detection were employed on cells treated with concentrations up to 50 μmol/L SF. Caspase-3 activity was not induced in cells treated with SF for 24 h compared with untreated cells. In contrast, Caco-2 cells incubated with camptothecin exhibited apoptosis as early as 4 h after treatment, which was abolished by the addition of the Z-VAD-FMK caspase inhibitor at the time of the treatment (data not shown). Similarly, induction of apoptosis was not detected by immunoassay after SF treatment (data not shown).

**Transcriptome analysis of gene expression after exposure to SF.** Affymetrix U133A oligonucleotide arrays, which contain 22,283 probes corresponding to the best-annotated human genes, were used to detect changes in gene expression in response to treatment with SF and identify potential targets that might mediate the SF effects. When exposed to 50 μmol/L SF, 106 genes exhibited a >2-fold increase in expression, whereas 63 genes exhibited a >2-fold decrease in expression (P < 0.05; Supplemental Table 2). The majority of these genes had not been shown previously to be modulated by SF, and many represent potentially important targets that may help to explain the anticarcinogenic activity of SF. When exposed to 25 and 5 μmol/L, the numbers of genes for which there was a >2-fold change in expression was 14 and 4, respectively (Supplemental Tables 3 and 4). The expression of these genes was also changed at 50 μmol/L. No genes exhibited a >2-fold change in expression when cells were exposed to 1 μmol/L.

Analysis of the subset of the 169 genes that showed a >2-fold up or down change by 50 μmol/L SF by hierarchical clustering revealed that all of the samples belonging to the control and 1 and 5 μmol/L treatment groups appeared to cluster together with a similar expression pattern (Fig. 1), confirming that these concentrations of SF have relatively little effect on gene expression patterns as detected by Affymetrix arrays within the criteria used in this study. In contrast, there were 2 distinct clusters of samples, i.e., those treated with 25 and 50 μmol/L SF, respectively. The extent of changes in the expression of the majority of genes within the 25 μmol/L cluster was intermediate between those in the 50 μmol/L cluster and the [control + low concentration] cluster, indicating relatively little variation among the 4 replicates, and consistent and reproducible dose-dependent changes in expression on exposure to SF with the majority of the 169 genes.

Changes in the expression of 10 of these genes plus 4 additional genes (p21, CDX-2, MRP2, and Dnmt1) were analyzed by RT-PCR in independent experiments (Table 1). In each case, RT-PCR confirmed either a significant increase or decrease in expression (although the precise magnitude of this change may be different), with 2 exceptions. The first exception was the transcription factor Nrf2, for which the induction observed with the use of arrays was not confirmed by RT-PCR and supports previous findings. The second exception was the intestinal KLF5, for which expression was indicated to be upregulated when analyzed by arrays, but reduced when analyzed by RT-PCR. Subsequent studies in independent experiments confirmed the RT-PCR result. In addition, because some studies had reported the induction of p21 by SF, the expression of p21 was investigated in independent experiments by RT-PCR; exposure to 50 μmol/L SF resulted in a 3.5-fold increase in expression, as opposed to the 1.45-fold detected by arrays (Table 1). The pattern of expression identified by microarrays across all of the samples for the genes that were also verified by RT-PCR is shown in Supplemental Figure 1. Similar hierarchical sample clusters were formed as before, indicating dose-dependent expression patterns.

**SF-mediated changes in gene expression in surgically resected tissue.** The MTS tissue viability assay showed that there was a 57.9 ± 1.5% reduction in viability in tissue exposed to 50 μmol/L SF and a 29.1 ± 1.5% reduction when tissue was exposed to 25 μmol/L SF for 2 h, compared with control samples (data not shown). This latter reduction in viability was similar to the 26% reduction in viability that was observed in the MTT assay when Caco-2 cells were exposed to 50 μmol/L SF. Thus, in subsequent experiments, tissue was exposed to 25 μmol/L SF. In addition, tissue was exposed to SF for only 2 h, which is more physiologically appropriate than the 24 h for which cells were exposed to SF. In each of these 3 individuals, the level of CDX-2, KLF4, p21, and KLF5 expression in untreated tissue was lower in cancerous tissue than healthy tissue (Fig. 2a–d). SF had no or little effect on gene expression in healthy tissue from each of the 3 patients. Cancerous tissue from 1 of the 3 patients, patient 1, responded in a manner similar to Caco-2 cells, with an induction in both KLF4 and p21 after exposure to SF, but with little change in expression of KLF5 (Fig. 2a and b). Expression of p21 was restored to the level observed in healthy tissue. There was also an increase in the expression of p21 in cancerous tissue from patient 2, but relatively little change in expression of CDX-2, KLF4, and KLF5 (Fig. 2c). There was little change in gene expression in the tissues of patient 3 (Fig. 2d). Although expression of AMACR was reduced in Caco-2 cells by >2-fold, there was little change when cancerous tissue was exposed to SF in all 3 patients (Fig. 2).
DISCUSSION

Exposure of cells of the gastrointestinal tract to SF results both from topological exposure after consumption of broccoli and subsequent systemic exposure via the bloodstream. Broccoli typically contains ~1 μmol GR/g fresh weight. Thus, if one assumes that myrosinase remains active after mild cooking and 100% of GR is converted to SF, a standard 100-g portion would deliver 100 μmol of SF to the GI tract. The concentration that tissue would be exposed to would depend on subsequent dilution, but local exposure is likely to exceed 50 μmol/L. Alternatively, if a longer period of cooking has denatured myrosinase, intact GR is likely to pass through to the lower tract, where it is converted to SF due to microbial action. Although this may result in lower total conversion of GR into SF, local concentrations may exceed 100 μmol/L. Systemic exposure postabsorption is likely to be considerably lower because SF will be rapidly diluted in the bloodstream, and is further complicated by the formation of GSH-SF conjugates and derivatives that both alter biological activity and are likely to prevent entry of SF into cells. Thus, the range of concentrations of SF that cells were exposed to was chosen to represent the probable concentrations that cells may be exposed to from either systemic exposure (1 and 5 μmol/L) or topological exposure (25 and 50 μmol/L). These levels of exposure are similar to those reported in several recent papers on a variety of cell lines (11,14,16,28). The majority of changes in gene expression were observed only at the higher SF concentrations. These would be found within the GI tract via topological exposure, and also within the bladder where SF and its conjugates accumulate before excretion.

After exposure to SF, the IC50 was 85 and 83 μmol/L in 2 independent experiments. These values were higher than reported previously for Caco-2 cells (55 μmol/L) (16). However, in that earlier study, Caco-2 cells were seeded and left to adhere overnight before treatment, increasing the SF molecules available per cell and thus making them more sensitive to xenobiotics, whereas in this study, Caco-2 cells were seeded and left to grow until they became 80% confluent, ~5 d later, before treatment with SF.

To investigate changes in gene expression in Caco-2 cells exposed to SF, we undertook a study of global gene expression with the use of Affymetrix arrays, and then confirmed the changes in expression of a subset of genes in independent experiments with RT-PCR. In general, the magnitude of the change in expression of this subset of genes was less when quantified with arrays than with RT-PCR (Table 1), consistent with previous studies (29).

As expected, there was upregulation of genes involved in xenobiotic metabolism and changes in the expression of genes involved with cell cycle control and DNA synthesis, discussed in detail below. Of particular interest were changes in the expression of KLF4, which is a transcription factor regulating xenobiotic metabolism (31,32), and downregulation of formyltetrahydrofolate synthase (30), nontargeted select metabolite and is also induced by other dietary antitumorigenic protein and is also induced by other dietary

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**Figure 2** Expression levels of CDX-2, KLF4, p21, KLF5, and AMACR, quantified by real-time RT-PCR and normalized to 18s, after treatment of Caco-2 cells with 50 μmol/L SF for 24 h (a), and after exposure of human colon healthy and cancerous resected tissue from 3 individuals to 25 μmol/L SF for 2 h (b–d). Bars for (a) indicate means ± SEM, n = 3, *P < 0.04; **P < 0.01. Bars for (b–d) indicate a single value of relative quantity for the genes measured.

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__GENES ALTERED BY SULFURAPHANE IN CACO-2 CELLS__

**Table 1** Gene expression changes induced by SF in Caco-2 cells. The change in expression of this subset of genes was less when quantified with arrays than with RT-PCR (Table 1), consistent with previous studies (29).
contraction of SF required to induce apoptosis was reported (16). In contrast, we have no evidence of induction of apoptosis at 24 h, based on both caspase activity and the Cell Death Detection ELISA PLUS assay. Similarly, although previous studies showed that SF can induce caspase-3 activity (15,28,34), we have no evidence of any such activity. Several studies provided evidence for the activation of the JNK signal transduction pathway by SF and other ITCs (18,19,35–37) to be the mechanism of caspase-3 induction and subsequent apoptosis. From our gene array analyses, we identified 2 potential mechanisms that may interfere with JNK/caspase-3 induced apoptosis. First, the expression of GADD45b was shown to lead to downregulation of JNK signaling induced by tumor necrosis factor α (38). Second, a more likely potential mechanism of suppression of apoptosis post-JNK activation is through induction of ATF3, which induces the antiapoptotic factor heat shock 27kD protein 1 (Hsp27) (39). Both ATF3 and Hsp27 were significantly upregulated in our study (Table 1 and Supplementary Table 2). Hsp27 was shown to inhibit apoptosis and caspase activation through negative regulation of the mitochondrial proteins cytochrome c and Smac, and other means (40,41). This mechanism would be consistent with known mechanisms of apoptosis induced by SF and other ITCs (14,19). In addition to these 2 potential antiapoptotic mechanisms, induction of heme oxygenase and p21, as observed in our current study, was also associated with inhibition of apoptosis (42).

As expected, there was upregulation of several genes associated with ARE-mediated transcription, notably NQO1, TR1, aldo-ketoreductase (AKR), and heme oxygenase-1 (HO). NQO1, TR1, and AKR were reported to be induced by SF, in both cell cultures and mammalian tissue (16,43–46). These genes and HO are likely to be upregulated via the activation of the transcription factor Nrf2 following its release from the Kelch-like ECH-associated protein 1 (Keap1) after exposure to SF has altered the cell redox potential (47–49).

Sulforaphane upregulated the expression of several genes of known importance in suppression of the cell cycle. In particular, there was significant upregulation of the cyclin-dependent kinase inhibitor p21, previously reported to have been upregulated in SF-treated prostate cell lines and HT29 colon cells (12,20). Second, there was upregulation of GADD45b, which was shown to interact with p21 in suppressing cell proliferation (50). SF also enhanced the expression of BTG family member 2 (PC3TIS21/BTG2), BTG3-associated nuclear protein (SMAR1), and CDC28 protein kinase 2 (CKSHS2), all of which have been associated with regulation of the cell cycle (51–53). SMAR1 has also been associated with delay in tumor growth (51). Consistent with inhibition of cell cycle, there was a decrease in expression of members of the microchromosome maintenance family (MCM4 and MCM7), all of which are associated with DNA synthesis (54).

To understand how these genes themselves are regulated, it was of interest to observe the induction of the KLF4 transcription factor, in contrast to the reduction in expression of KLF5. KLF4 is expressed in the postmitotic cells lining the villi in the small intestine and in the middle-to-upper gland region in the colon (55,56); it is associated with the region of cell differentiation, whereas KLF5 is expressed in the proliferative cell compartment of the adult gut epithelium (57). Consistent with these respective activities, in Caco-2 cells, we observed an induction of KLF4 with SF, but a reduction in KLF5 (Table 1, Fig. 2a). KLF4 has recently received considerable attention due to its probable role in regulating several processes associated with cell cycle regulation and differentiation (58). Transcriptome profiling of a cell culture line with inducible KLF4 confirmed that expression of KLF4 regulates several genes concerned with inhibition of cell cycle progression and DNA synthesis, including p21 and MCM4 (59). Notably, the inhibition of cell proliferation of cancer cell lines due to expression of KLF4 is not associated with apoptosis (60). KLF4 was reported to be itself regulated via expression of CDX-2 and APC (61,62), as illustrated in Figure 3. Consistent with its potential bioactivity, there is reduced expression of KLF4 in the intestinal tissue of adenomatous polyposis coli (APC) min mice (63), and also within intestinal adenomas of patients with familial adenomatous polyposis (FAP) in whom there is a mutation in APC.

Although SF enhanced KLF4 expression, it reduced expression of CDX-2 by 5.9-fold (Table 1, Fig. 2a), suggesting that SF is mediating the expression of KLF4 independently of CDX-2 and APC. Thus, SF may be able to restore KLF4 expression (and associated downstream genes such as p21) within cells even when the APC-CDX-2 transcriptional pathway is impaired. Because both alleles of APC are mutated in >85% of familial and sporadic colorectal tumors (64), the ability of SF to at least partially restore KLF4 activity may be important. Furthermore, the association between SF and KLF4 and p21 induction links, for the first time, the activity of this dietary anticarcinogen with one of the major pathways of intestinal carcinogenesis, that of APC downregulation or mutation (Fig. 3).

The lower levels of p21 and KLF4 expression in cancerous compared with adjacent healthy surgically resected colon tissue is consistent with an increase in cellular proliferation and with previous studies that reported a reduction in KLF4 expression within intestinal adenomas of FAP patients (65). Although some caution is required in interpretation of these results because gene expression in morphologically normal tissue from cancer patients may have perturbations in gene expression compared with normal subjects (66), the extent of these perturbations is still likely to be less than within proliferating tissue itself. Exposure of tissue to SF supported the results from cell culture, i.e., there was an induction of KLF4 and/or p21, without an induction of CDX-2, in at least some of the individuals. However, these data suggested that p21 could be induced independently of KLF4. Although these...
preliminary ex vivo studies require repeating and expanding, they point toward the potential of SF to perturb expression of KLF4 and p21 in cancerous tissue, indicating that SF may be important not only in preventing initiation of carcinogenesis, but also in reducing cellular proliferation.

Additionally, SF altered expression of the AMACR, a marker upregulated in prostate carcinoma but also recently identified as being highly expressed in colon adenomas and carcinomas compared with normal colon and nonneoplastic polyps (67–70). There was a significant decrease in AMACR expression after exposure of Caco-2 cells to SF. In contrast, SF did not seem to consistently alter the expression in the individuals tested in the present study. However, upregulation in cancerous tissue compared with normal tissue was found in only 1 individual, suggesting that our sample is not representative of colon cancer with high AMACR.

This article is the first report of transcriptome analysis of a cell line exposed to SF; we identified 169 genes that are highly likely to be altered in expression after exposure to SF, and confirmed the changes in expression of a subset of these with RT-PCR. Several of these genes, such as KLF4, CDX-2, Dnmt1, and AMACR, which are involved in diverse biological processes that have been implicated in carcinogenesis, warrant further study.

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LITERATURE CITED


