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Synergy between sulforaphane and selenium in protection against oxidative damage in colonic CCD841 cells

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Abbreviations:

ITCs; isothiocyanates

Nrf2; nuclear factor-erythroid 2-related factor 2

Sam68; Src-associated in mitosis 68 kDa

Se; Selenium

SFN; Sulforaphane

TrxR-1; thioredoxin reductase
Abstract

Dietary isothiocyanates (ITCs) are potent inducers of the NF-E2-related factor-2 (Nrf2) pathway. Sulforaphane (SFN), a representative ITC, has previously been shown to upregulate antioxidant enzymes such as selenium (Se) dependent thioredoxin reductase-1 (TrxR-1) in many tumour cell lines. In the present study, we hypothesized that a combination of SFN and Se would have a synergistic effect on the upregulation of TrxR-1 and the protection against oxidative damage in the normal colonic cell line CCD841. Treatment of cells with SFN and Se significantly induced TrxR-1 expression. Pre-treatment of cells with SFN protects against H$_2$O$_2$-induced cell death; this protection was enhanced by co-treatment with Se. The siRNA knockdown of either TrxR-1 or Nrf2 reduced the protection afforded by SFN and Se co-treatment; TrxR-1 and Nrf2 knockdown reduced cell viabilities to 66.5 and 51.1% respectively, down from 82.4% in transfection negative controls. This suggests that both TrxR-1 and Nrf2 are important in SFN-mediated protection against free radical-induced cell death. Moreover, flow cytometric analysis showed that TrxR-1 and Nrf2 were involved in SFN-mediated protection against H$_2$O$_2$-induced apoptosis. In summary, SFN activates the Nrf2 signaling pathway and protects against H$_2$O$_2$-mediated oxidative damage in normal colonic cells. Combined SFN and Se treatment resulted in a synergistic upregulation of TrxR-1 that in part contributed to the enhanced protection against free radical-mediated cell death provided by the co-treatment.

Keywords: Cruciferous vegetable; Sulforaphane; Selenium; Thioredoxin reductase; Nrf2; Colon cancer
1. Introduction

Some early epidemiological studies suggest that intake of cruciferous vegetables is inversely correlated with the morbidity of various cancers including those of the lung, bladder and colon [1, 2]. However, the results of other epidemiological studies are inconsistent and inconclusive [3, 4]. Since cruciferous vegetables are rich sources of glucosinolates, it is inevitable that their chemoprotective activity is attributed to the isothiocyanates (ITCs). ITCs, derived from the glucosinolates in cruciferous vegetables, have in themselves significant cancer chemopreventive potential [5]. Among all the ITCs, sulforaphane (SFN), which is derived from glucoraphanin - commonly found in broccoli and cauliflower - has been the most intensively studied ITC in relation to cancer prevention. Administration of crucifers or ITCs to experimental animals has been shown to inhibit the development of colonic aberrant crypt foci [6] and to reduce the incidence and multiplicity of chemical-induced tumors, including those of the bladder and colon [7, 8]. ITCs are potent inducers of phase II enzymes, which are involved in detoxifying potential endogenous and exogenous carcinogens [9, 10]. Importantly, ITCs have been shown to exert antioxidant effects via the regulation of NF-E2-related factor-2 (Nrf2)-antioxidant responsive element (ARE) pathways [11]. Nrf2 regulates a major cellular defence mechanism, and its activation is important in cancer prevention [12]. However, overexpression of Nrf2 in cancer cells protects them against the cytotoxic effects of anticancer therapies, thus promoting chemo-resistance [13, 14]. Thioredoxin reductase 1 (TrxR-1) is an Nrf2-driven antioxidant enzyme, and it has been shown to play a dual role in cancer [15]. We have previously shown that TrxR-1 plays an important role in the protection against free radical-mediated cell death in
cultured normal and tumour cells [16, 17]. Moreover, the induction of TrxR-1 and glutathione peroxidase-2 (GPx2) by SFN is synergistically enhanced by selenium (Se) co-treatment in colon cancer Caco-2 cells [18].

The mechanisms by which ITCs act in cancer prevention may involve multiple targeted effects, including the induction of phase II antioxidant enzymes, cell cycle arrest, and apoptosis [19, 20]. Other potential targets include kinases, transcriptional factors, transporters and receptors [21-24]. Since both Nrf2 and TrxR-1 can play dual roles in cancer [25-28], the benefits or risks of Nrf2 activation or TrxR-1 induction may depend upon the nature of the cells (normal vs. tumor). Therefore, it is important to investigate the effects of ITCs on normal cells. We hypothesized that a combination of SFN and Se would have a synergistic effect on the upregulation of TrxR-1 and on the protection against oxidative damage in the normal colonic cell line CCD841. Recently, we demonstrated that SFN promoted cancer cell proliferation, migration and angiogenesis at low concentrations (<2.5µM), whilst demonstrating opposite effects at high concentrations (>10µM) [29].

Activation of Nrf2 signalling and TrxR-1 in normal cells may be beneficial, and this effect is associated with the chemoprotective activity of SFN. In the present study, we have demonstrated that pre-treatment of cells with SFN and Se protects against free radical-mediated cell death in normal colon epithelial CCD841 cells.
2. Methods and materials

2.1. Materials

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane, purity 98%) was purchased from Alexis Biochemicals (Exeter, EXETER UK). Sodium selenite (purity 98%), dimethylsulfoxide (DMSO), thioredoxin reductase, hydrogen peroxide and Bradford reagent were all purchased from Sigma (Sigma-Aldrich. Dorset, UK). Complete protease inhibitors were obtained from Roche Applied Science (West Sussex UK). Rabbit polyclonal primary antibodies to Nrf2 and TrxR-1, goat polyclonal primary antibody to β-actin, rabbit polyclonal primary antibody to the RNA-binding protein, Sam68, HRP-conjugated goat anti-rabbit, and rabbit anti-goat IgG were all purchased from Santa Cruz Biotechnology (Santa Cruz, Germany). The siRNAs for Nrf2 (Cat No. SI03246950, target sequence, 5′-CCCATTGATGTTTCTGATCTA-3′), TrxR-1 (Cat No. SI00050876, target sequence, 5′-CTGCAAGACTCTCGAAATTAT-3′), and AllStars Negative Control siRNA (AS) were all purchased from Qiagen (West Sussex, UK). The Annexin V-FITC apoptosis detection kit was purchased from eBioscience (Hatfield, Hertfordshire, UK). Electrophoresis and Western blotting supplies were obtained from Bio-Rad (Hertfordshire, UK), and the chemiluminescence kit was from GE Healthcare (Little Chalfont, Bucks UK).

2.2. Cell culture

CCD841 cells were cultured in DMEM supplemented with fetal bovine serum (10%), 2mM glutamine, penicillin (100 U/ml) and streptomycin (100µg/ml) under 5% CO₂ in air at 37°C.
When the cells achieved 70-80% confluence, they were exposed to various concentrations of SFN and/or Se for different times with DMSO (0.1%) as control.

2.3. Cell viability and apoptosis assays

The cell proliferation MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was employed to determine the toxicity of SFN (1-160µM) and H$_2$O$_2$ (100-1600µM) towards cultured CCD841 cells. Cells were seeded on 96-well plates at a density of 1.0×10$^4$ per well in DMEM with 10% FCS. When cells were at approximately 70–80% confluence, they were exposed to SFN at various concentrations for different times, using DMSO (0.1%) only as control. After all treatments, the medium was removed and then MTT (5mg/ml) was added, and incubated with the cells at 37°C for 1 h to allow the MTT to be metabolized. Then supernatant was removed and the produced formazan crystals were dissolved in DMSO (100µl per well). The final absorbance in the wells was recorded using a microplate reader (BMG Labtech Ltd, Aylesbury, Bucks, UK), at a wavelength of 550-570nm and a reference wavelength of 650-670nm.

For apoptosis analysis, CCD841 cells were seeded on 12-well plates at a density of 5.0×10$^4$ cells per well and incubated at 37°C for 48 h. After treatment with 2.5µM SFN and/or 0.1µM Se for 24 h, cells were exposed to 100-150µM H$_2$O$_2$ for 24 h. Cells were then trypsinized and collected by centrifugation at 180 g for 5 min at room temperature. The pellet was washed with cold PBS before being re-suspended in binding buffer at a cell density adjusted to 2.0-5.0×10$^5$/ml according to the instructions from the Annexin V-FITC apoptosis detection kit (eBiosciences, UK). Annexin V-FITC (fluorescein isothiocyanate) was used to stain for the apoptotic cells and propidium iodide (PI) used to stain the necrotic
cells. For each sample, 10,000 events were collected and the data were analysed using the FlowJo software (Tree Star Inc. Ashland, OR, USA).

2.4. Knockdown of Nrf2 and TrxR-1 by siRNA

CCD841 cells were seeded on 12-well plates in DMEM with 10% FCS. After 24 h, the cells were transfected with siRNA targeting Nrf2 or TrxR-1. Briefly, the cell medium was replaced with 1000µl 12% FCS medium, then 20nM siRNA and 6µl HiPerFect transfection reagent were combined in 100µl medium (without serum and antibiotics) and incubated at room temperature for 10 min, and then gently added drop-wise to the cells. AllStars Negative Control siRNA was used as a negative control (this siRNA has no homology to any known mammalian gene). After 24 h incubation with siRNA, SFN and Se were added in fresh medium for a further 24 h, then the effects of H$_2$O$_2$ (150µM, 24 h) on cell viability and apoptosis were measured using flow cytometric analysis.

2.5. Protein extraction and immunoblotting of Nrf2 and TrxR-1

To extract total protein, CCD841 cells were washed twice with ice-cold PBS, then harvested by scraping in 20mM Tris-HCl (pH 8), 150mM NaCl, 2mM EDTA, 10% glycerol, 1% Nonidet P-40 (NP-40) containing mini-complete proteinase inhibitor and 1mM PMSF, in an ice bath for 20 min to lyse cells. Then the lysate was centrifuged at 12,000 g for 15 min at 4°C and the protein-containing supernatant was collected. To extract nuclear protein, the Nuclear Extract Kit (Active Motif, Rixensart, Belgium) was used, following the manufacturer’s instructions. Protein concentrations were determined by the Brilliant Blue G dye-binding assay of Bradford, using BSA as a standard.
Protein extracts were heated at 95°C for 5 min in loading buffer and then loaded onto 10% SDS-polyacrylamide gels together with a molecular weight marker. After routine electrophoresis and transfer to PVDF membranes, membranes were blocked with 5% fat free milk in Phosphate Buffered Saline Tween-20 (PBST) (0.05% Tween 20) for 1 h at room temperature, and then with specific primary antibodies against Nrf2 or TrxR-1 (diluted in 5% milk in PBST) overnight at 4°C. Membranes were then washed four times for 40 min with PBST, then incubated with secondary antibodies (diluted in 5% milk in PBST) for 1 h at room temperature. After four further washes for 40 min with PBST, antibody binding was detected using an ECL kit (GE Healthcare), and the density of each band was measured with the FluorChem Imager (Alpha Innotech, San Leandro, CA), or the Li-Cor Odyssey Imager (Li-Cor Biotechnology UK Ltd, Cambridge, UK).

2.6. Statistics

Data are represented as the means ± SD. The differences between the groups were examined using the one-way ANOVA/LSD test, or Student’s t-test. A p value <0.05 was considered statistically significant. IC₅₀ values of SFN and H₂O₂ were determined using the CalcuSyn software (Biosoft, Cambridge, UK).
3. Results

3.1. Effect of SFN on cell growth.

SFN has been shown to promote the growth of some tumor cell lines at low concentrations, but to be toxic to the same cells at higher concentrations through the induction of stress-related cell cycle arrest and apoptosis [29-31]. CCD841 cells were cultured in 96-well plates (seeding 5.0×10^3 cells per well) and treated with SFN for 24 or 48 h once they reached 70-80% confluence. In this study, 2.5 and 5µM SFN moderately stimulated the growth of CCD841 cells. 24 h treatment with 2.5 and 5µM SFN increased cell viability by 13 and 15% respectively versus control, while 48 h treatment with the same concentrations did so by 25 and 11% respectively (Fig. 1). Treatment with higher concentrations of SFN (20-160µM) significantly reduced cell viability. SFN had IC_{50} values of 30.0µM (24 h) and 40.4µM (48 h) for CCD841 cells. In contrast, the IC_{50} values of SFN for Caco-2 were 47.1µM (24 h) and 50.6µM (48 h) as reported previously [18], suggesting that normal colonic cells are more susceptible to SFN-induced cell death.

3.2. Effect of SFN on nuclear accumulation of Nrf2

Untreated CCD841 cells exhibited very low Nrf2 levels in both the cytoplasm and the nucleus, consistent with the degradation of Nrf2 by proteasomes in a Keap1-dependent manner under homeostasis [32]. However, upon SFN treatment (1.25-40µM for 4 h), a significant increase of Nrf2 in the nucleus was observed, suggesting the rapid liberation of Nrf2 from Keap1-coupled suppression and its subsequent nuclear translocation (Fig. 2A)
3.3. Effect of SFN and/or Se on TrxR-1 expression

SFN induced TrxR-1 expression in a dose-dependent manner in CCD841 cells. These data are consistent with previous publications on tumor cell lines such as colon cancer Caco-2 and breast cancer MCF-7 cells [34, 35]. The effect of SFN and/or Se on TrxR-1 protein expression in CCD841 was determined using Western blot analysis. A dose-dependent response was observed in cells exposed to 2.5-20μM SFN (with 0.1% DMSO only as control) (Fig. 3A). Co-treatment with SFN (2.5μM) and Se (0.1μM) produced a synergistic effect especially after 24 and 48 h. 2.5μM SFN alone induced TrxR-1 1.8-fold, and Se (0.1μM) alone induced it 1.4-fold, whereas the combination of 2.5μM SFN and Se (0.1μM) induced it 3.3-fold. (Fig. 3B). Moreover, co-treatment with 10μM SFN and Se (0.1μM) also produced a synergistic effect, especially after 24 and 48 h. 10μM SFN alone induced TrxR-1 3.7- and 2.6-fold at 24 and 48 h respectively, Se (0.1 μM) alone induced it 1.9- and 1.5-fold at 24 and 48 h respectively, whereas the combination of 10μM SFN and Se (0.1μM) induced it 4.3- and 4.0-fold at 24 and 48 h respectively (Fig. 3C).
3.4. Protective effect of SFN and/or Se against \( H_2O_2 \)-induced cell death

Hydrogen peroxide is known to activate the mitochondrial apoptotic pathway, to decrease Nrf2 expression, and to increase reactive oxygen species (ROS) levels, leading to cell death [36]. CCD841 cells were cultured in 96-well plates (seeding 5.0×10^3 cells per well) and when they reached 70-80% confluence they were treated with a concentration series (0-1600µM) of \( H_2O_2 \) for 24 h. The IC\(_{50}\) value of \( H_2O_2 \) for CCD841 cells was 64.1µM. 100µM \( H_2O_2 \) treatment decreased cell viability to 11.4% of the control (Fig. 4). Pre-treatment with SFN at 2.5 or 5µM for 24 h significantly protected against the reduction in cell viability induced by 100µM \( H_2O_2 \). Following 2.5 and 5µM SFN pre-treatment, the 100µM \( H_2O_2 \) treatment only reduced cell viabilities to 18.9 and 21.6% respectively. When the cells were pre-treated with 0.1 and 0.2µM Se for 24 h, the 100µM \( H_2O_2 \) treatment only reduced cell viabilities to 36 and 35% respectively. For cells that were co-treated with 2.5µM SFN and 0.1µM Se, or with 5µM SFN and 0.2µM Se, subsequent 100µM \( H_2O_2 \) treatment only reduced cell viabilities to 61.8 or 56.1% respectively. Moreover, in a separate experiment using siRNA to knockdown TrxR-1 or Nrf2, the protection afforded by pre-treatment with SFN (2.5µM) and Se (0.1µM) against the induction of apoptosis by 150µM \( H_2O_2 \) treatment was reduced such that the proportion of viable cells as indicated by Annexin V/PI staining was reduced from 82.4% in transfection negative controls, to 66 or 51% in TrxR-1 or Nrf2 knockdowns, respectively (Fig. 5A). This suggests that Nrf2 and TrxR-1 play important roles in SFN-mediated protection against \( H_2O_2 \)-induced cell death in normal colonic cells. \( H_2O_2 \) caused a concomitant rise in early (single positive) and late stage (double positive) apoptotic cells as indicated by Annexin V/PI staining. \( H_2O_2 \) induced a 59.9% proportion of
apoptotic cells; co-treatment with SFN and Se reduced the proportion of apoptotic cells to 13.0% (Fig. 5A). The siRNA knockdown of either TrxR-1 or Nrf2 abrogated the protection afforded by SFN and Se co-treatment, and increased the apoptotic cell population to 25.4 or 44.9% respectively, suggesting that Nrf2 signaling is important in the protection against free radical-mediated apoptosis in normal colonic cells.
4. Discussion

Oxidative stress is one of the most critical factors implicated in many gastrointestinal diseases, including inflammatory bowel disease and colon cancer [37]. Many selenoproteins including TrxR-1 are involved in cellular homeostasis and protecting normal and tumor cells against oxidative stress [38]. Fruits and vegetables are rich in various antioxidants. Increasing the consumption of fruits and vegetables may inhibit certain cancers [39].

Although the results from many epidemiological studies are inconsistent and inconclusive, one exception is the Netherlands Cohort Study on Diet and Cancer, in which women (but not men) who had a high intake of cruciferous vegetables were shown to have a reduced risk of colon cancer [3]. Cruciferous vegetables are rich sources of glucosinolates, which can be broken down to ITCs under the action of myrosinases when the plant tissue is damaged or cooked. Several studies have demonstrated that dietary ITCs possess significant cancer chemopreventive potential [24]. However, ITCs have been shown to exert both chemopreventive and oncogenic activities. Overexpression of Nrf2 and/or TrxR-1 in cancer cells might be undesirable; high constitutive levels of Nrf2 occur in many tumors and can promote chemoresistance [13]. On the other hand, the induction of Nrf2 and TrxR-1 by ITCs in normal cells could be beneficial in cancer prevention [29]. There are over 1000 genes driven by Nrf2, many of which possess antioxidant or chemopreventive potential [40, 41].

Apart from TrxR-1, other enzymes such as glutathione transferases (GSTs), quinone reductase (QR) and heme oxygenase (HO-1) might also be involved in chemoprevention [42, 43]. GSTs are key enzymes in the metabolism of ITCs in cells. A recent comprehensive meta-analysis demonstrated an increased cancer risk in Caucasian populations conferred by GSTM1 and GSTT1 null genotypes [44]. Conversely, results from another study reveal statistically significant protective effects of crucifer consumption against colorectal
neoplasms that is stronger among individuals with a single null GSTT1 genotype [45]. To better understand the mechanisms behind the role of Nrf2 in the chemoprevention of colorectal cancer, more studies, especially into the genetic aspects of responses to ITCs, are required.

The upregulation of antioxidant enzymes by ITCs is one of the most important factors in chemoprevention. TrxR-1 is an important Se-dependent enzyme involved in the regulation of cell redox [46]. Similarly to Nrf2 activation, TrxR-1 induction may protect against carcinogenesis in normal cells, but TrxR-1 overexpression has been reported in a large number of human tumors [15]. A very recent study suggested that both TrxR-1 and 15kDa selenoprotein (Sep15) participate in interfering regulatory pathways in colon cancer cells [38]. The relationship between Se and cancer is complex; an optimal intake may promote health [47]. In general, individuals who have low serum Se levels may benefit from Se supplementation, but those with high serum Se levels are at increased risk for other diseases [48]. The cancer-preventive properties of Se in colon cancer are believed to be mediated by both selenoproteins and low molecular weight selenocompounds [49].

Although TrxR-1 has been suggested as a novel target for cancer therapy [50], the function of TrxR-1 in tumor cell growth, migration and invasion warrants further *in vitro* and *in vivo* studies.

In the present study, we have demonstrated that SFN can activate the Nrf2 signaling pathway and interact with Se in the upregulation of TrxR-1 in normal colonic cells. Co-treatment of colonic cells with SFN and Se resulted in a synergistic induction of TrxR-1 expression, and provided a greater protective effect against hydrogen peroxide-induced cell death than treatments with either component individually. An optimal combination of Se and
SFN may be able to achieve the same level of gene expression using relative less concentration of each compound than when they are used alone. A limitation of this study is that the synergy was identified in *in vitro* cell cultures. Further *in vivo* studies could consider positive interactions between bioactives and nutrients to test if they result in greater protection against oxidative stress and stronger chemopreventive activities. It would be interesting to identify more synergistic or antagonistic interactions between food components and whole foods, to help inform healthy dietary recommendations. An optimal combination of different bioactive phytochemicals, vitamins and minerals may be able to upregulate chemoprotective enzymes, reduce oxidative stress and improve gut health. In conclusion, combined SFN and Se treatment synergistically upregulated TrxR-1, which plays a significant role in maintaining intracellular redox homeostasis and contributed to the SFN-induced protection against free radical-mediated oxidative damage in normal colonic cells.
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There are no conflicts of interest for any of the authors.
References


Figure legends

**Fig. 1. Effect of SFN on CCD841 cell growth.**

Cells at 70–80% confluence were treated with SFN (0-160μM) in cell culture medium for 24-48 h. Control cells were treated with DMSO (0.1%) only. Cell viability was determined by the MTT cell proliferation assay. Each data point represents the means ± SD (n=5). *, P<0.05; **P<0.01.

**Fig. 2 Effect of SFN on the translocation of Nrf2 into the nucleus.**

CCD841 cells were exposed to SFN (with DMSO (0.1%) only as control). Nuclear protein fractions were isolated as described in Methods and materials. Nrf2 was detected and quantified by Western blot analysis. Nrf2 band densities were normalized against Sam68 (68 kDa), and results were expressed as fold induction relative to controls. Data are expressed as means ± SD (n=3). (A) Dose-response, SFN (0-40μM) for 4 h. (B) Time course, SFN (10μM) for 0-48 h. *, P<0.05; **P<0.01.

**Fig. 3. Effect of SFN on TrxR-1 protein expression.**

CCD841 cells were exposed to SFN (2.5-40μM) for 24 h (DMSO (0.1%) only was used as a control) (A). Synergistic effect of SFN with Se: dose response (B), and time response (C). Folds of change were determined by Western blot analysis, from the average TrxR-1 band densities (normalized to those of β-actin). Data are expressed as means ± SD (n=3). *, P<0.05; **P<0.01.
Fig. 4. Effect of co-treatment with SFN and Se on H\textsubscript{2}O\textsubscript{2}-induced cell death.

CCD841 cells were cultured in 96-well plates (seeding 7.0 × 10\textsuperscript{3} cells per well) and when they reached 70-80% confluence, were pre-treated with SFN (2.5 or 5\(\mu\)M) (or DMSO (0.1%) only as control) and/or Se (0.1 or 0.2\(\mu\)M) for 24 h, and were then exposed to H\textsubscript{2}O\textsubscript{2} (100\(\mu\)M) in serum-free medium for further 24 h. Cell viability was measured by the MTT assay. *, P<0.05; **P<0.01.

Fig. 5. Effect of siRNA knockdown of TrxR-1 and Nrf2 on the protection against H\textsubscript{2}O\textsubscript{2}-induced cell death mediated by SFN and Se co-treatment.

CCD841 cells were pre-treated with SFN (2.5\(\mu\)M) and Se (0.1\(\mu\)M) for 24 h, then siRNA (20nM) knockdown of TrxR-1 or Nrf2 (with AllStars Negative Control siRNA (AS) as negative control) was performed. Then the cells were exposed to H\textsubscript{2}O\textsubscript{2} (100\(\mu\)M) for 24 h. The cells were then stained with Annexin V and PI, and flow cytometric analysis was carried out. The H\textsubscript{2}O\textsubscript{2}-treated cells have a higher percentage of apoptotic cells (Annexin V positive), as indicated by the percentage of gated cells (B). SFN and Se pre-treatment afforded significant protection against H\textsubscript{2}O\textsubscript{2}; siRNA against TrxR-1 or Nrf2 abrogated this protection. Early and late apoptotic data (red bars) are expressed as means ± SD (n=3). *P<0.05; **P<0.01 in comparison to the AS control.
Fig. 1.
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