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TrxR1 and GPx2 are potently induced by isothiocyanates and selenium, and mutually cooperate to protect Caco-2 cells against free radical-mediated cell death

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ABSTRACT

Currently, there is significant interest in the field of diet–gene interactions and the mechanisms by which food compounds regulate gene expression to modify cancer susceptibility. From a nutrition perspective, two key components potentially exert cancer chemopreventive effects: isothiocyanates (ITCs), present in cruciferous vegetables, and selenium (Se) which, as selenocysteine, is an integral part of selenoproteins. However, the role of these compounds in the expression of key selenoenzymes once the cancer process has been initiated still needs elucidation. Therefore, this investigation examined the effect of two forms of selenium, selenium-methylselenocysteine and sodium selenite, both individually and in combination with two ITCs, sulforaphane or iberin, on the expression of the two selenoenzymes, thioredoxin reductase 1 (TrxR1) and gastrointestinal glutathione peroxidase (GPx2), which are targets of ITCs, in Caco-2 cells. Co-treatment with both ITCs and Se induced expression of TrxR1 and GPx2 more than either compound alone. Moreover, pre-treatment of cells with ITC + Se enhanced cytoprotection against H_2O_2-induced cell death through a ROS-dependent mechanism. Furthermore, a single and double knockdown of TrxR1 and/or GPx2 suggested that both selenoproteins were responsible for protecting against H_2O_2-induced cell death. Together, these data shed new light on the mechanism of interactions between ITC and Se in which translational expression of the enhanced transcripts by the former is dependent on an adequate Se supply, resulting in a cooperative antioxidant protective effect against cell death.

1. Introduction

The association between intakes of fruit and vegetables, and the risk of colon cancer is complex and has been the focus of a large number of epidemiological studies; however, to date the results have been inconsistent [1]. Given the nature of tumour biology and the multiple mechanisms involved in colon carcinogenesis [2], it has been widely recognised that there may be advantages in combining a variety of food constituents since their complementary mechanisms of action may prevent the accumulation of alterations during neoplastic transformation that lead to uncontrolled cell growth and loss of genomic stability. Since sulforaphane (SFN) has been shown to induce two selenoenzymes, thioredoxin reductase 1 (TrxR1) and gastrointestinal glutathione peroxidase (GPx2), both SFN and selenium have been proposed as partners in cancer prevention [3]. However, both selenoenzymes have been shown to be up-regulated during cancer progression [4,5]. In addition, there is solid evidence indicating that the NF-E2-related factor 2 (Nrf2) is involved in colon tumorigenesis [6], and considering that the promoter regions of both selenoproteins contain an antioxidant response element (ARE) that is regulated by Nrf2, it makes them an obvious target of study.

Investigating the consequences of altering the levels of Nrf2 and its associated cytoprotective genes, such as *TrxR1* and *GPx2,* by isothiocyanates (ITCs) and their glucosinolate precursors is important because these natural compounds suppress the initiation of a wide range of carcinogen-induced tumours in rodents and also because they can be present in significant amounts in the human diet [7,8]. ITCs influence the process of carcinogenesis partly by modulation of phase I and II enzymes, induction of apoptosis and cell cycle arrest [9,10]. The fact that SFN metabolites have been detected in all tissues with the highest concentration in the small intestine, colon and prostate [11], has led to the view that they can potentially be used for the prevention of cancer [12,13]. However, further studies are needed since Nrf2 may take on a pro-tumoral identity in cancerous cells.

Selenium (Se) is another dietary compound that has been shown to have cancer-chemopreventive roles. A large body of experimental evidence in animal and cell culture models indicates that this

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essential trace element exerts anticarcinogenic effects by multiple mechanisms, including altered carcinogen metabolism, cell cycle regulation, immune surveillance, cell death programming, cancer cell migration and angiogenesis [14,15]. Some of these functions may be linked to the crucial fact that Se is a component of the amino acid selenocysteine, which is incorporated into 25 genes encoding over 30 mammalian selenoproteins [16]. In addition, there is extensive evidence that monomethylated forms of Se are critical metabolites for the chemopreventive effect of Se [17] and experimental evidence suggests that any precursor that will directly generate a steady stream of methylselenol (CH_3SeH) or its derivative, is more active than selenite or selenomethionine in tumour inhibition [18,19]. However, conflicting findings from clinical trials investigating the possible use of selenium as an anti-carcinogen [20] indicates that a more focused approach is needed to understand the mechanisms of different forms of Se on antioxidant and anticancer activity.

Studies employing different forms of Se and/or ITCs might provide further clues to understand better the potential risks and benefits of altering the level of TrxR1 and GPx2 in a cancer cell model. Therefore, the current study was conducted to understand the direct effect of two ITCs, SFN and iberin, in combination with Se-methylselenocysteine (SeMSC) or sodium selenite and to establish whether a combination of these dietary components exerts additive or synergistic effects on the regulation of TrxR1 and GPx2 in human colon adenocarcinoma Caco-2 cells. Moreover, the role of ITC + Se in protecting cells from H_2O_2-mediated cytotoxicity and the contribution made by TrxR1 and/or GPx2 in promoting the survival of colon cancer cells were also investigated.

2. Material and methods

2.1. Reagents

SFN and iberin was purchased from ENZO Life Sciences (UK). Seleno-methylselenocysteine was obtained from Eburon Organics (Belgium). Complete protease inhibitors and cell proliferation reagent WST-1 were purchased from Roche Applied Science (UK). Rabbit polyclonal primary antibodies to TrxR1, Nrf2, Sam68, goat polyclonal primary antibody to β-actin and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and/or rabbit anti-goat IgG as secondary antibodies were all obtained from Santa Cruz Biotechnology (Santa Cruz, USA). As primary antibody for GPx2, rabbit anti-human antiserum was used [21]. Electrophoresis and Western blotting supplies were supplied by Bio-Rad (UK), and the enhanced chemiluminescence (ECL) kit was purchased from GE Healthcare (Little Chalfont, UK). Nrf2 small interfering RNA (siRNA) was supplied by Applied Systems (UK). TrxR1, GPx2, AllStars Negative Control siRNA and HiPerFect transfection reagent were all obtained from Qiagen (West Sussex, UK). 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) was purchased from Invitrogen, Molecular Probes, USA. Sodium selenite, dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), Bradford reagent, RT-PCR primers and probes used, and all other chemicals were obtained from Sigma-Aldrich (UK), unless otherwise stated.

2.2. Cell culture

The human colon adenocarcinoma cell line, Caco-2, was purchased from the American Type Culture Collection (Middlesex, UK). Cell culture media and supplements were bought from Invitrogen (Paisley, Scotland), except for foetal bovine serum (FBS) which was obtained from Biosera, UK. Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) of L-glutamine (200 mM) and 1% (v/v) antibiotic solution consisting of penicillin (5000 units/ml), streptomycin (5000 μg/ml). The DMEM used in this study is a Se-free medium; however, under normal cell culture conditions FBS is a major source of selenium. The selenium content of the FBS used in this study was 13 μg/l (equivalent to a final concentration of 16 nM Se when 10% of FBS is added to the cell culture medium) as determined by Biosera (UK). Experimental cells were seeded in six-well plates at 6×10^4 cells/well in a final volume of 3 ml and were supplemented with ITCs and/or Se when confluence reached ~70% (usually after 3 days). For time course experiments, all the different groups were treated at the same time and harvested at appropriate intervals. All treatments and controls contained a final DMSO concentration of 0.05% (v/v).

2.3. Cell proliferation assay

The WST-1 cell proliferation assay was used to detect the toxicity of ITC (1–100 μM), selenium (0.2–100 μM) and H_2O_2 (200–800 μM) in Caco-2 cells. The assay was performed in 96-well plates when cells were at approximately 60-70% confluence according to the manufacturer's instruction (Roche Applied Science). The intensity of the coloured compound formed (formazan dye) was then quantified using a micro plate reader (BMG Labtech Ltd, UK). The absorbance was measured at 450 nm, with the reference at 630 nm. Cell proliferation of treated cells was expressed as a percentage of control as follows: (A_{450} nm − A_{630} nm) sample / (A_{450} nm − A_{630} nm) control × 100. The IC_{50} was determined using CalcuSyn software Version 2.0 (Biosoft, Cambridge, UK).

2.4. Real-time PCR

Total RNA was extracted from Caco-2 cells using GenElute™ Total Mammalian RNA kit (Sigma, UK) according to the manufacturer's instruction. First strand cDNA was synthesised with 1 μg of total RNA using qScript cDNA SuperMix (Quanta BioSciences, USA). TrxR1 and GPx2 mRNA quantification was determined by TaqMan using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers and fluorogenic TaqMan probes were designed using Primer Express® Software (Applied Biosystem, UK) based on their human sequences. The probes were labelled with a 5′ reporter dye, FAM (6-carboxyfluoroscein) and 3′ quencher dye, TAMRA (6-carboxytetramethylrhodamine). Real-time PCR reactions were carried out using Precision™ MasterMix (Primer Design, UK) and samples were run following a 10 min hot start at 95 °C, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. The following primer pairs were used for quantitative PCR: TrxR1 forward 5′-CCACTGGTGAAAGACCACGTT-3′, reverse 5′-AGGAGAAAAGATCATCACTGCTGAT-3′, probe 5′-CAGTATTCTT TGTCACCAGGGATGCCCA-3′; GPx2 forward 5′-CACACAGATCTC CTACTCCATCCA-3′, reverse 5′-GGTCCAGCAGTGTCTCCTGAA-3′, probe 5′-CATGCTGCATCCTAAGGCTCCTCAGG-3′; 18S rRNA forward 5′-GGCTCATTAAATCAGTTATGGTTCCT-3′, reverse 5′-GTATTAGCTCTAGA ATTACCACAGTTATCCA-3′, probe 5′-TGGTCGCTCGCTCCTCTCCCA-3′. Data were normalised against an invariant endogenous control, 18S ribosomal RNA. The threshold cycle number (Ct) obtained was converted into fold of relative induction using the ΔΔCt method [22].

2.5. Protein extraction

Total proteins from Caco-2 cells were extracted by washing cells twice with ice-cold PBS and treating them for 5 min with 100 μl of ice-cold cell lysis buffer containing Tris-EDTA buffer pH 8.0 (20 mM Tris, 2 mM EDTA), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40 and mini complete proteinase inhibitor, before the cells were collected by the use of a rubber policeman. Thereafter the lysates were placed in an Eppendorf tube and incubated on ice for 15 min (vortexing the samples 3 times at full speed during incubation) with a final centrifugation step at 13,000 *g* for 10 min to collect the supernatants. Nuclear and cytoplasmic protein extracts were also obtained by

using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein concentrations were determined by the Bradford protein assay using BSA as a standard.

2.5.1. Western blot analysis

Equivalent amounts of protein (40 μg) were mixed with loading buffer and heated at 95 °C for 5 min to be size fractioned in 12% SDS-PAGE prior to transfer to an Immun-Blot polyvinylidene difluoride membrane using a Trans-Blot semi-dry transfer system. The membrane was blocked for 1 h at room temperature with 5% (w/v) non-fat milk in PBST, followed by incubation with primary antibody in the same solution at 4 °C overnight. Membranes were washed with PBST, three times for 10 min each, and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The membranes were then washed three times with PBST for 10 min each, and developed with an ECL system and visualised using a LAS-3000 Fujifilm intelligent dark box. The illuminated bands were detected and the image captured using Image Reader LAS-3000 software. Densitometric analysis of the Western blot was performed using Quantity One® Ver. 4.6.3 basic software (Bio-Rad Laboratories, UK). Results were normalised against β-actin to correct for protein loading, and the protein expression from the different treatments was calculated relative to the control.

2.6. ITC- and/or Se-mediated cytoprotection in Caco-2 cells

The protective effects of ITCs and/or Se against H_2O_2-induced cytotoxicity was investigated. Caco-2 cells (7×10^3 cells/well in a 96-well plate) were grown for 48 h before media was removed and cells treated with 100 μl of DMEM containing the vehicle (DMSO), SFN (6 μM), sodium selenite (50 nM), and a combination of ITC + Se using 12 replicates per treatment. After 24 h, a stock solution of H_2O_2 was diluted with serum-free media, to avoid the possibility that serum components might differentially affect the results [23], to a final concentration of 500 μM before the media was removed from the cells; 6 replicates were treated with 100 μl of this solution and the remaining 6 with an equal amount of the vehicle (water). After 24 h cell proliferation was measured using WST-1 cell proliferation assay.

2.7. Effects of TrxR1, GPx2 and Nrf2 knockdown on H_2O_2-mediated cell death

A single (TrxR1, GPx2, Nrf2) and a double (TrxR1/GPx2) knockdown approach was used to investigate the effect of these proteins on cell protection when cells are pre-treated with SFN + Selenite and challenged with H_2O_2. In summary, on the day of transfection Caco-2 cells were seeded at 1.8×10^4 cells/well of a 96-well plate in 170 μl of 10% FBS DMEM medium (containing L-glutamine and antibiotics). To prepare the transfection complexes of the single and double knockdown treatments, stock solutions of Nrf2, TrxR1 and/or GPx2 siRNA and AllStars negative control siRNA were diluted in 30 μl of plain DMEM, followed by addition of HiPerFect and a 10-min incubation period before being gently added drop-wise to the cells (to give a final siRNA concentration of 50 nM after adding complexes to cells seeded in 170 μl DMEM). In order to guarantee that the single and double knockdown treatments contained an identical final siRNA concentration, individual siRNAs (25 nM) from the former was mixed with AllStars negative control siRNA (25 nM). After 24 h incubation with siRNA, the medium was removed and 100 μl of DMEM containing SFN + selenite added for a further 24 h. The medium was removed again and serum-free media containing 500 μM of H_2O_2 added for 24 h; cell proliferation was measured using the WST-1 cell proliferation assay.

2.8. Reactive oxygen species measurement

To measure the effect of SFN and/or selenite on reactive oxygen species (ROS) production, as well as their effect after knocking down TrxR1 and GPx2, Caco-2 cells were plated at 7×10^5/well in a 24 well plate and cells were either left untransfected or transfected with siRNA, respectively, and treated with SFN + selenite as described in the previous section. After 24 h incubation with SFN + selenite the medium was removed and cells were washed and incubated for 40 min in PBS containing 1 μM of the fluoroprobe CM-H2DCFDA. PBS was discarded and cells were incubated with 500 μM of H_2O_2 diluted in PBS for 1 h. Subsequently, the media was discarded and cells collected, centrifuged, washed and re-suspended in 100 μl PBS. Intracellular ROS production was determined by detection of fluorescent intensity of the oxidised product 2′,7′-dichlorofluorescein in the FL1-A channel with the BD Accuri C6 flow cytometer.

2.9. Assessment of apoptosis and cell death

The effect of siRNA on Caco-2 cells apoptosis was assessed using an Annexin V-FITC apoptosis detection kit, from eBioscience, according to the manufacturer's instructions. Caco-2 cells were trypsinised and collected, including floating apoptotic cells and the cells spontaneously detached during washing procedure. Annexin V-FITC (fluorescein isothiocyanate) was used to stain for the apoptotic cells, and propidium iodide (PI) used to stain the necrotic cells. Flow cytometry for measuring apoptosis was performed with the BD Accuri C6 flow cytometer.

3. Statistical methods

Statistical analyses were carried out using the SPSS 16 statistical program. Results are expressed as means ± SD. Statistical comparisons were made using Student's *t*-test and one-way ANOVA with Dunnett's *post-hoc* test when treatment samples were compared only against the control group and with Bonferroni's *post-hoc* test when comparisons between groups were made. For Western blotting experiments, data are representative of two independent experiments.

4. Results

4.1. Effect of isothiocyanates and selenium on cell survival

Caco-2 cells were exposed to a range of concentrations of ITCs (1–100 μM) and Se (0.2–100 μM) for 24 or 48 h to evaluate cytotoxicity. Concentrations of SFN and iberin above 12 μM reduced cell growth significantly in a time- and dose-dependent manner (Fig. 1A and B). The IC_{50} values for SFN were 31.6 (24 h) and 20.5 μM (48 h), and for iberin were 42.3 (24 h) and 22.7 μM (48 h). These results are in agreement with published data using different cytotoxicity assays [24–26].

Regarding the cell response after Se treatment, Fig. 1C shows an increase of 20–30% in cell growth after treating cells with 0.2–10 μM of selenite for 24 or 48 h. However, decreased cell proliferation was observed when cells were treated with concentrations above 50 μM for 48 h (Fig. 1C). In relation to SeMSC, a similar increase in cell proliferation was observed, but no toxicity was reported with concentrations up to 100 μM (Fig. 1D). This increase in cell number agrees with other data in which increased cell proliferation after Se treatment at concentrations in the nanomolar to micromolar range was detected in Caco-2, HRT18 and HT-29 [27].

Based on these data and taking into consideration other studies, the concentrations used for further experiments were between 3 to 12 μM for ITC and 25 to 200 nM for Se. ITC concentrations in this range may be achieved in human plasma by consumption of ITC-rich vegetables or high glucosinolate broccoli [28,29].

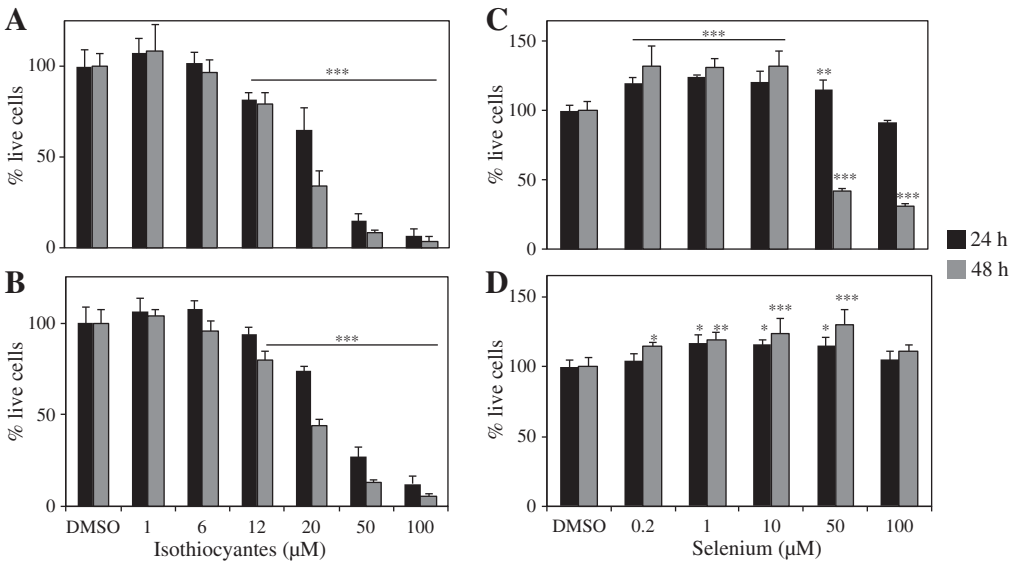

Fig. 1. The effect of (A) sulforaphane, (B) iberin, (C) selenite and (D) SeMSC on Caco-2 cells as measured by cell metabolic assay WST-1. Caco-2 cells were treated for up to 48 h with DMSO (control) or with the indicated concentrations of ITCs or selenium, and cell cytotoxicity was evaluated. Data (optical density) represent the mean ± SD of six replicates expressed as percentage of control. Significant differences from control are indicated (*P<0.05; **P<0.01; ***P<0.001). Lines drawn on the graph indicate that all the bars included within the line are significantly different from their corresponding control group.

4.2. Up-regulation of TrxR1 and GPx2 mRNA by isothiocyanates

TrxR1 and GPx2 are two important redox-modulating enzymes that have been suggested to play an important role during inflammation and cancer [30,31]. The time course effect of ITCs on TrxR1 and GPx2 gene expression after exposing Caco-2 cells to 6 μM of SFN and iberin from 4 to 48 h was investigated to provide information about the regulation of mRNA over time. The results showed a significant increase in expression of TrxR1 and GPx2 after treatment with SFN or iberin in a time- dependent manner compared to control, with a peak induction of 4.5-fold at 12 h for TrxR1 and a peak of ~3.5-fold at 24 h for GPx2 (Fig. 2A and B). The TrxR1 mRNA returned to basal levels after 48 h but only declined slightly for GPx2, which implies that the mRNA turnover rate is different for each transcript. SFN and iberin also significantly induced TrxR1 and GPx2 expression dose-dependently (3, 6, 12 μM) with peak increases of 6.7- and 3.4-fold, respectively, after 12 μM of SFN treatment (Fig. 2C and D).

4.3. Cooperative effects of ITC and Se in the up-regulation of TrxR1 and GPx2

The effect of selenite and SeMSC on levels of TrxR1 and GPx2 mRNA expression, either alone or in combination with SFN and/or iberin, were investigated (Fig. 3A and B). The level of TrxR1 or GPx2 mRNA was not increased by either form of Se within 48 h. However, the combination of iberin and selenite increased TrxR1 expression by 41% after 8 h compared to that of iberin alone (P<0.05), but no significant increase was observed when iberin and SeMSC were

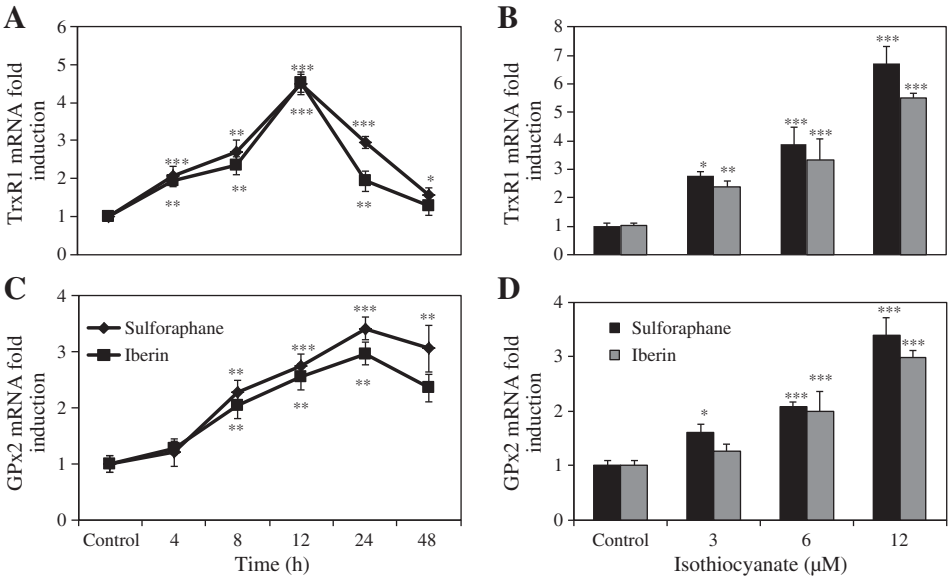

Fig. 2. Time- and dose-dependent effect of ITCs on mRNA expression. Caco-2 cells were exposed to DMSO (control) or 6 μM of sulforaphane or iberin for up to 48 h to evaluate the expression of (A) TrxR1 or (B) GPx2. Also, cells were exposed to DMSO (control) or increasing concentrations of isothiocyanates for (C) 12 h for TrxR1 and (D) 48 h for GPx2. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Significant differences from control are indicated (*P<0.05; **P<0.01; ***P<0.001).

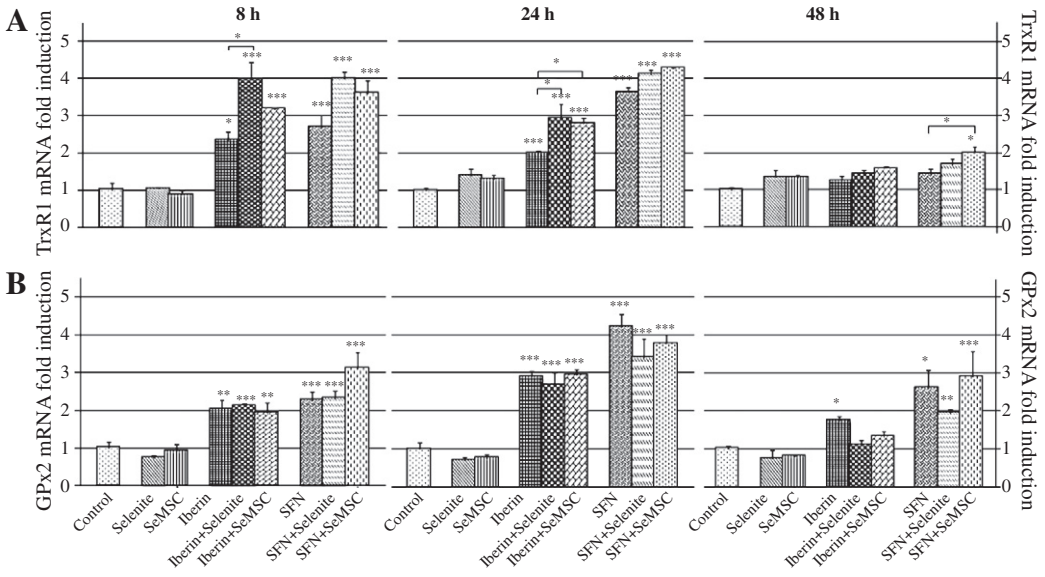

Fig. 3. Effect of ITCs and/or Se supplementation on (A) TrxR1 (B) GPx2 mRNA. Caco-2 cells were exposed to DMSO (control), 6 μM of ITCs and/or 200nM of selenium and were incubated for 8, 24 and 48 h to evaluate gene expression. Results are mean ± SD of triplicate samples normalized against 18S. Significant differences from control are indicated (*P<0.05; **P<0.01; ***P<0.001).

added together. This 4-fold level of induction was similar to that produced by SFN alone after 24 h or SFN + Se at 8 or 24 h. After 24 h an increase of ~29% was observed when iberin was added with either selenite or SeMSC (P<0.05) (Fig. 3A).

Although an additive effect of TrxR1 mRNA was not observed when SFN was added with either form of Se, a comparable trend was seen, especially at 8 h. TrxR1 mRNA expression decreased after 48 h, with only SFN + SeMSC remaining 2-fold higher over the control and still larger than observed in the presence of only SFN (Fig. 3A). The maximum increase of TrxR1 mRNA was detected with SFN + SeMSC at 24 h with a peak of 4.3-fold. This peak was significantly higher than for iberin + SeMSC treated cells (P<0.01).

In a similar manner, GPx2 mRNA was induced ~2-fold after treatment with either ITC (8 h) and was increased to 3- and 4-fold at 24 h for iberin and SFN, respectively, (P<0.001) (Fig. 3B). However, addition of selenium had no significant effect on GPx2 mRNA expression when compared to either ITC alone. To determine whether mRNA induction of these two selenoproteins was translated into protein, Caco-2 cells were treated for 48 h with different concentrations of ITCs and/or Se (Figs. 4A, B and 5A, B) over 8, 24 and 48 h (Figs. 4C and 5C). The resulting proteins were extracted and quantified by Western blot analysis.

ITC treatment did not up-regulate the expression of the two selenoproteins at the protein level as was observed at the mRNA level. The TrxR1 protein induction patterns observed with ITC + Se (Fig. 4) correlated comparatively well with the mRNA level in Fig. 3A. This is different to the GPx2 protein pattern (Fig. 5) which differed clearly from that obtained at the mRNA level (Fig. 3B). However, both selenoproteins showed a similar pattern of induction at the protein level.

The data presented here suggest that Se is the limiting nutrient determining the overall additive effect at the protein level when

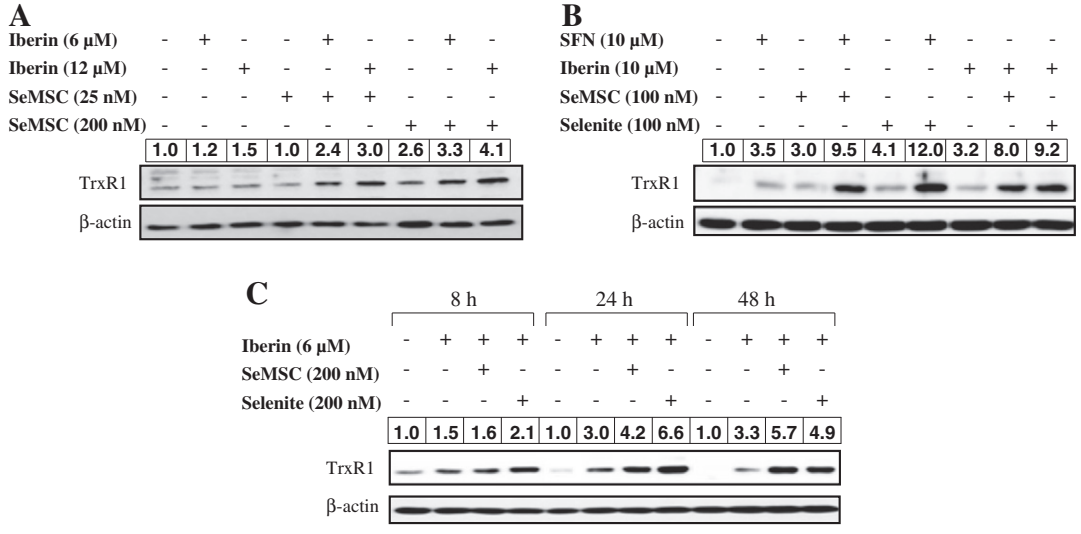

Fig. 4. Differential levels of TrxR1 protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 48 h (A and B) and for 8, 24, and 48 h (C). Whole-cell lysates were harvested and equal amounts of proteins (40 μg) were resolved by SDS/10% PAGE for Western blot analysis. The translated TrxR1 product appeared as a band of approximately 55 kDa and densitometric analysis of these bands were normalized to β-actin signal (42 kDa) and was converted into fold of induction relative to control = 1. Data are representative of two independent experiments.

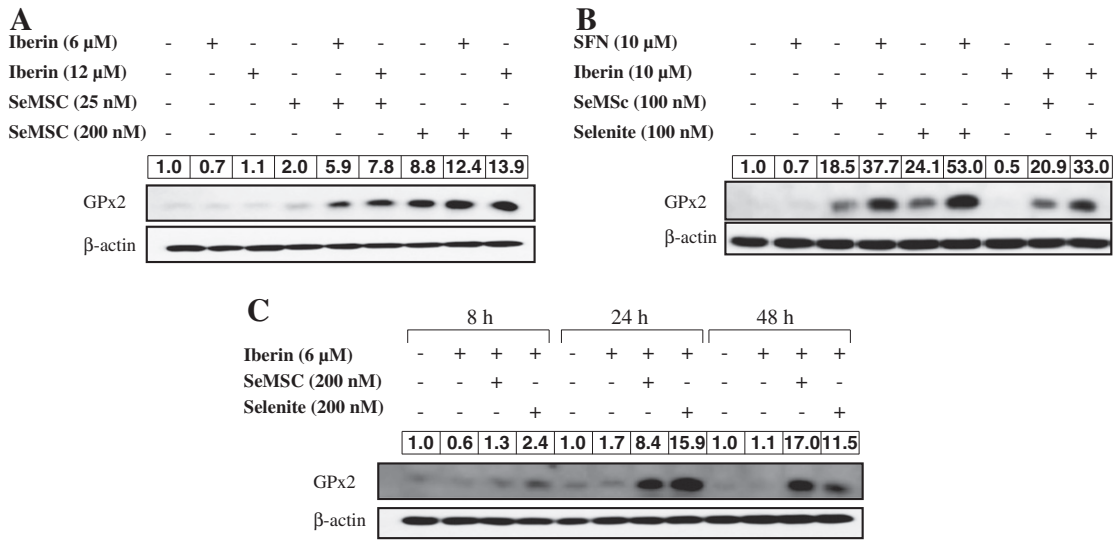

Fig. 5. Differential levels of GPx2 protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 48 h (A and B) and for 8, 24, and 48 h (C). Whole-cell lysates were harvested and equal amounts of proteins (40 μg) were resolved by SDS/10% PAGE for Western blot analysis. The translated GPx2 product appeared as a band of approximately 22 kDa and densitometric analysis of these bands was normalized to β-actin signal (42 kDa) and was converted into fold of induction relative to control = 1. Data are representative of two independent experiments.

cells were supplemented with ITC and/or Se dose-dependently (Figs. 4A and 5A). After comparing ITCs, SFN appeared to be more effective in inducing mRNA, as reflected by a higher protein synthesis (Figs. 4B and 5B).

Regarding the form of Se used, although iberin + selenite led to a maximum peak of TrxR1 protein at 24 h (6.6-fold) (Fig. 4C), the same treatment decreased protein synthesis to 4.9-fold after 48 h, whereas the iberin + SeMSC treatment resulted in a subtle increase from 4.2- (24 h) to 5.7-fold (48 h). In relation to GPx2, it was also observed that Caco-2 cells supplemented with iberin + SeMSC sustained a more prolonged increase in protein expression than did iberin + selenite, increasing from 8.4-fold (24 h) to 17-fold (48 h) for the former (Fig. 5C).

4.4. Nrf2 translational expression pattern in ITC and/or Se treated Caco-2 cells

The underlying mechanisms by which ITC + Se up-regulate further gene expression of intracellular antioxidants need to be addressed. In order to identify whether the mechanisms involved in the induction of antioxidant enzymes when Caco-2 cells are co-treated with ITCs and selenium is also Nrf2 dependent, cytosolic and nuclear protein extracts were studied in Caco-2 cells treated with ITCs + Se to establish the role of this important nuclear factor in the reported cooperative effect of TrxR1 and GPx2.

ITC treatment clearly enhanced Nrf2 in the nucleus (Fig. 6). As expected, exposure to SFN or iberin for 8 h increased nuclear Nrf2 by 3-fold accompanied by a concomitant decrease of Nrf2 in the cytosol (Fig. 6). However, translocation was not influenced by Se.

4.5. ITC and/or Se-mediated cytoprotection in Caco-2 cells

As co-treatment of ITCs and Se resulted in the cooperative induction of TrxR1 and GPx2 in a time- and dose- dependent manner, a study was conducted into whether these increases were correlated with protection of cells against oxidative stress insult. Cells were pre-treated with SFN (6 μM) and/or selenite (50 nM) for 24 h followed by treatment with 500 μM H_2O_2. Sulforaphane and selenite were selected as they induced greater expression of TrxR1 and GPx2 in previous experiments. The cytotoxic effect of H_2O_2 was evaluated by treating Caco-2 cells with increasing concentrations of H_2O_2, ranging from 200 to 800 μM. Doses of H_2O_2 above 200 μM evoked a highly significant decrease in cell proliferation, with an IC_{50} value of 503 μM (Supplementary Fig. 1). This decrease in proliferation was reduced by 35% ($P<0.001$) and 20% ($P<0.001$) after 24 h pre-treatment with selenite and SFN, respectively, (Fig. 7A). Interestingly, co-treatment with SFN + selenite significantly abolished H_2O_2-induced cell damage, an observation which was confirmed using an annexin V/propidium iodide apoptosis assay (Fig. 7C and D). Results were confirmed by staining cells with propidium iodide only (Supplementary Fig. 2). In

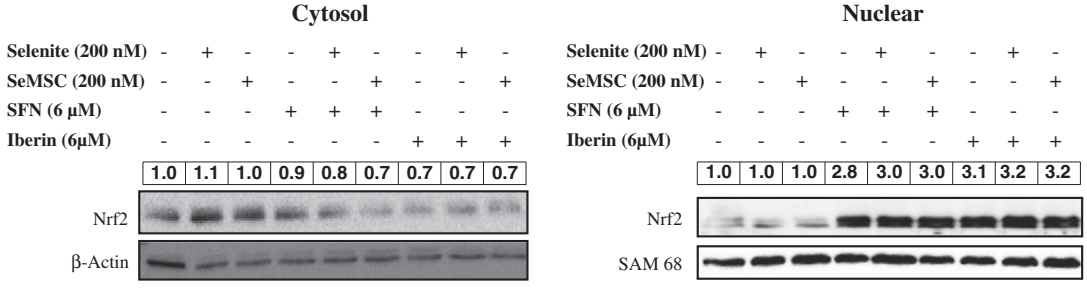

Fig. 6. Representative images of Nrf2 immunoblots with cytosolic and nuclear fractions derived from Caco-2 cells exposed for 8 h with DMSO (control), SFN, SFN + Selenium, iberin or iberin + Selenium. Cellular fractions (30 μg) were resolved by SDS/10% PAGE. Cytosolic and nuclear bands were detected using anti-Nrf2 (C-20) and anti-Nrf2 (H-300) antibodies respectively and bands were seen at 57- (cytosol) and ~110-kDa (nuclear). Densitometric analysis of cytosolic and nuclear bands were normalized to β-actin (42 kDa) and Sam 68 (68 kDa) signals respectively and results were converted into fold of induction relative to control = 1. Data are representative of two independent experiments.

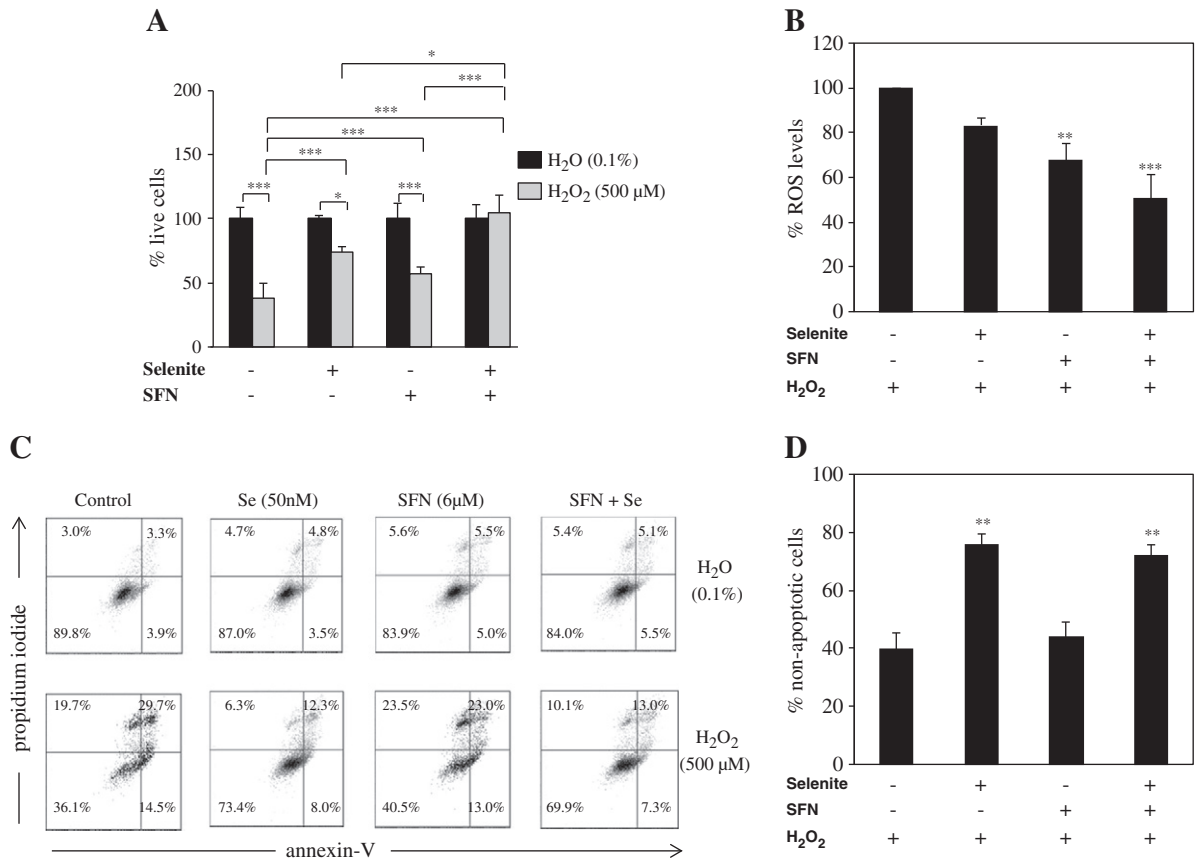

Fig. 7. Effect of ITCs and/or selenium on (A) cell proliferation, (B) ROS and (C, D) cell viability in Caco-2 cells. (A) Caco-2 cells were pre-incubated for 24 h with DMSO, SFN (6 μM) and/or selenite (50 nM) before exposure to vehicle (H_2O) or H_2O_2 (500 μM) in serum-free medium for 24 h. Cell proliferation was measured by WST-1 assay. SFN and/or selenite-mediated cytoprotection are shown in grey bars as a percentage of the H_2O_2 untreated group (black bars). Results are mean ± SD of six replicates. (B) Caco-2 cells were pre-incubated with SFN and Se as specified above, followed by incubation with 1 μM CM-H_2DCFDA for 45 min. Then, cells were washed and incubated with H_2O_2 (500 μM) for 1 h. Cells were then assessed for H_2DCFDA oxidation using flow cytometer. Results are mean ± SD of duplicates. (C) Caco-2 cells were pre-incubated for 24 h with DMSO, SFN (6 μM) and/or selenite (50 nM) before exposure to vehicle (H_2O) or H_2O_2 (500 μM) in serum-free medium for 1 h, following apoptosis detection by flow cytometry analysis of Annexin V/PI staining. (D) Results as in C for two different experiments are shown. Data represent viable Caco-2 cells (Annexin V-negative and propidium iodide-negative). Significant differences from control and between treatments are indicated (*P<0.05; **P<0.01; ***P<0.001).

order to determine whether ITCs and/ or Se-mediated cytoprotection is exerted through regulation of redox equilibrium, the levels of reactive oxygen species (ROS) were assessed. H2DCFDA oxidation was reduced when cells were pre-treated with Se, SFN and SFN + Se by 17%, 32% (P<0.01) and 50% (P<0.001), respectively, (Fig. 7B).

4.6. Effect of TrxR1/GPx2 or Nrf2 knockdown on H_2O_2-induced cell death and ROS levels

To investigate whether *TrxR1/GPx2* and *Nrf2* are the main genes by which ITCs and Se protect against H_2O_2-induced cell death in Caco-2 cells, a single and double knockdown approach was used to suppress the expression of the aforementioned selenoproteins and transcriptional factor. The knockdown efficiency showed an 80% reduction in expression of TrxR1 and GPx2 and 73% reduction in the expression Nrf2 (Fig. 8A–C). Addition of 500 μM of H_2O_2 in control cells transfected with AllStars negative control, without pre-treatment with SFN or Se, decreased cell proliferation by 60% (P<0.001) (Fig. 8D). In contrast, cells transfected with AllStars negative control but after pre-treated with SFN + Selenite, were 100% viable after addition of H_2O_2, confirming the level of protection observed in Fig. 7A. After a single knockdown with either TrxR1- or GPx2-siRNA, prior to a pre-incubation step with SFN + selenite and followed by an oxidative stress challenge with H_2O_2, the levels of protection were reduced by 18% and 27.5% (P<0.01), respectively, (Fig. 8D). Interestingly, when both selenoproteins were knocked down together a 57% reduction in cell proliferation (P<0.001) was observed, equivalent to that obtained in the control group without SFN + Se pre-treatment. In addition, after knock down of Nrf2, a 71% (P<0.001) reduction in cell proliferation was observed which emphasises the importance of this transcriptional factor in promoting induction of other detoxifying enzymes apart from TrxR1 and GPx2. Moreover, the knockdown of Nrf2 in cells without SFN + Se treatment exposed to H_2O_2 (500 or 600 μM) showed a similar susceptibility to the control cells (Supplementary Fig. 3). To investigate whether reduction in cell proliferation is mediated through altered levels of ROS in Caco-2 cells, two of the experimental siRNA knockdowns (TrxR1 and GPx2) were selected to analyse production of ROS using H2DCFDA. A reduction in H2DCFDA oxidation was observed when cells were pre-incubated with SFN + Se without knockdown. In contrast, a significant increase in ROS levels was found after knock down of TrxR1 (156%) and GPx2 (132%) (Fig. 8E). Additionally, the viability of Caco-2 cells was analysed after 48 of siRNA transfection by Annexin V/PI. Cell viability was comparable among the different siRNAs used with that of control cells, indicating that the response observed was not due to siRNA toxicity (Supplementary Fig. 4).

5. Discussion

5.1. Transcriptional and translational regulation of GPx2 and TrxR1 in Caco-2 cells treated with ITCs and/or Se

The present study examined the effect of SFN or iberin, either individually or in combination with an inorganic (selenite) or organic

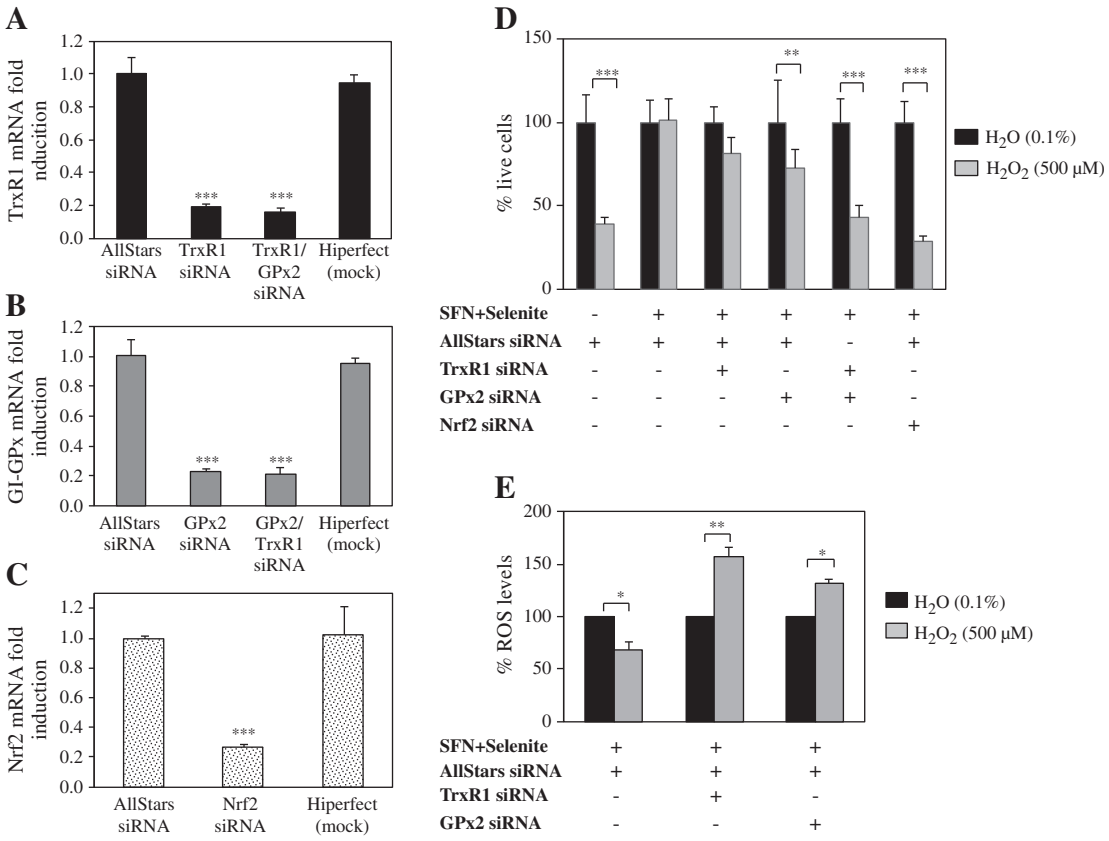

Fig. 8. siRNA knockdown efficiency of (A) TrxR1, (B) GPx2 and (C) Nrf2. (D) Cell proliferation effects of TrxR1 and/or GPx2 and Nrf2 knockdown on Caco-2 cells untreated or pre-treated with SFN + Se followed by incubation with vehicle (H_2O) or H_2O_2, (E) ROS levels effects of TrxR1 or GPx2 knockdown on Caco-2 cells pre-treated with SFN + Se (A-C) Caco-2 cells were exposed to 1) 50 nM of AllStars negative control siRNA (control); 2) 25 nM of TrxR1 or GPx2 siRNA + 25 nM AllStars siRNA (single knockdown); 3) 25 nM of TrxR1 + 25 nM GPx2 siRNA (double knockdown) and 4) 25 nM of Nrf2 + 25 nM AllStars siRNA (single knockdown) for 24 h; Mock-transfected cells were transfected only with HiPerFect, without addition of siRNA. Then, media containing the transfection complex were replaced with culture media and left for an additional 24 h followed by total RNA extraction. Results are mean ± SD of triplicate samples normalized against 18 S rRNA. (D) Caco-2 cells were transfected using the same siRNA treatments described above (1–4) for 24 h before adding DMSO to samples containing only AllStars or SFN (6 μM) + Selenite (50 nM) for 24 h followed by addition of vehicle (black bars) or 500 μM H_2O_2 (grey bars) after 24 h. Cell proliferation was measured after 24 h by the WST-1 method. Results are mean ± SD of six samples. (E) Caco-2 cells were transfected with siRNA TrxR1 or siRNA GPx2 (single knockdown) as specified above for 24 h before Caco-2 cells were pre-incubated with SFN and Se for an additional 24 h, followed by incubation with 1 μM CM-H_2DCFDA for 45 min and incubation with H_2O_2 (500 μM) for 1 h. Cells were then assessed for H_2DCFDA oxidation using flow cytometry. Results are mean ± SD of duplicates. Significant differences are indicated (*P<0.05; **P<0.01; ***P<0.001).

(SeMSC) form of Se, on expression of two selenoproteins that play a major role in controlling redox-regulated processes in colon cells. The cooperation of ITC and selenite in TrxR1 induction has previously been reported in human HepG2 cells and hepatocytes [32,33], and MCF-7 human breast adenocarcinoma cells [34]. Although several studies have documented the apoptotic effect of different ITCs at high concentrations [35,36], no studies had compared different ITCs at relatively low doses in combination with inorganic and organic forms of Se, and modulation of GPx2 under these conditions have not been explored *in vitro*.

By combining ITCs and Se compounds, SFN and selenite were found to be the most effective combination, eliciting large increases in expression of TrxR1 and GPx2 than either compound alone after 24 h treatment. Previous studies have shown that selenium-enriched broccoli, which contains mainly SeMSC did not increase GPx1 activity to the same extent as selenite or selenomethionine, presumably because the SeMSC is metabolised directly to methyl selenol and excreted [37]. However, other workers have reported that SeMSC induced GPx1 to a similar level to selenite in a selenium-deficient Caco-2 cell model [38]. Interestingly, our time-course experiments suggest that SeMSC is able to maintain a more sustained protein up-regulation than selenite after 48 h for both genes.

Activation of ARE facilitates rapid mRNA synthesis which is independent of selenium [39,40], whereas translation into the respective selenoproteins requires Se. Thus, SFN can up-regulate mRNA levels by induction and Se by stabilisation [32,41,42] pathways. However, SFN-induced mRNA can only be translated in the presence of adequate levels of Se. This agrees with the results reported here for TrxR1 and for GPx2, showing that Se is the limiting factor for protein synthesis from increased ITC-induced mRNA. A recent study showed that SFN decreased mTOR-S6K1-S6 signalling and inhibited protein synthesis [43]. The role of mTOR pathway in the expression of TrxR1 and GPx2 requires further investigation.

Expression and activity of GPx2 and TrxR1 are both regulated by Se through the selenocysteine insertion mechanism [44]. However, as observed in this study, selenoprotein expression depends on the form and concentration of Se, and may also depend on the cell-type under study. Conversely, this regulation occurs at different levels for different selenoenzymes, which means that selenoproteins are regulated individually; this might imply a prioritisation of the available Se so that synthesis of some selenoproteins is maintained over that of others. From this phenomenon, called *hierarchy of selenoproteins*, it has been observed that, under conditions of limited selenium supply, the mRNA of GPx2 can even increase and become preferentially

translated when selenium supply is restored [45]. According to its high ranking in the hierarchy, GPx2 mRNA was not influenced by Se, but was increased by ITC independent of Se. This increase was not further augmented when ITC + Se was added. A similar lack of expression was observed for TrxR1 mRNA following Se addition. However, a cooperative effect was observed after adding ITC + Se at the mRNA level.

This variation among selenoproteins has been found to be a result of differences in the selenocysteine insertion sequence (SECIS)-binding protein, SBP2, which is a major determinant in establishing the hierarchy of selenoprotein synthesis and selenoprotein mRNA turnover [46]. When UGA decoding is inefficient, as occurs when Se is limiting, termination occurs at these positions favouring mRNA degradation. This mechanism, termed *nonsense-mediated decay* is also considered to be responsible for the observed induction of TrxR1 and GPx2 in Caco-2 cells after co-addition of ITC + Se; this is considered to be due to a reduced mRNA degradation rate rather than to over-stimulation of the Nrf2-ARE signalling pathway when Se is added.

5.2. TrxR1/GPx2 and Nrf2 knockdown in the cooperation between ITC and Se-mediated cytoprotection

The contribution of oxidative stress towards the aetiology of colon cancer has been widely recognised. It is well known that incidence of colon cancer is at least 30-fold higher than that of the small intestine [47]. In contrast to the latter, the colon is exposed to high levels of superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), nitric oxide (•NO), hydrogen peroxide (H_2O_2) and other powerful mutagenic reactive nitrogen species as a direct result of the presence of fecal bacteria and bile pigments [48–50]. Under normal conditions, these oxidants are scavenged efficiently, but when their production outstrips antioxidant protection mechanisms (e.g., in inflammatory bowel diseases or ageing), their accumulation could give rise to somatic mutations and colon cancer.

Important findings presented here show that, after challenging Caco-2 cells with the endogenous oxidant H_2O_2, simultaneous addition of SFN (6 μM) plus selenite (50 nM) evoked greater cell protection from H_2O_2-induced cell injury than when either compound was added in isolation. However, apart from inducing the two selenoproteins studied here, SFN activates many more genes containing an ARE in their promoter region, such as phase II enzymes, redox-active proteins, GSH-related enzymes and several other novel enzymes recently identified [51]. Therefore, to investigate whether the protection exerted by SFN and selenite after H_2O_2 treatment was driven by TrxR1 and GPx2, Caco-2 cells were transfected with siRNA to generate a single and double knockdown of *TrxR1* and/or *GPx2*. In addition, *Nrf2* was knocked down to identify the role of other Nrf2-dependent/antioxidant genes. Both *TrxR1* and *GPx2* substantially contributed to the preservation of cell integrity after H_2O_2 treatment. The knockdown of *Nrf2* increased the cell death even further, indicating that more *Nrf2* targets, such as catalase or peroxiredoxins [52], might be involved in the protection.

Increasing understanding of the effect of ITCs and Se on TrxR1 and GPx2 expression has many implications. For example, previous studies have supported the opinion that expression of selenoproteins can be mutually exclusive, as shown by the contrasting expression patterns of GPx1 and TrxR1 in both human and mouse cells [53], as well as of GPx2 and GPx3 or selenoprotein P in gastrointestinal cancers [54,55]. However, the results presented here suggest that once these selenoenzymes have been induced by the coordinated action of the studied dietary factors they may act in concert under oxidative stress conditions (regardless of their selenoprotein hierarchy) to maintain or re-establish the aberrant intracellular redox environment present in cancer cells. This has important implications for the treatment of cancer as cells may acquire growth advantage indicated by an enhanced ROS detoxification due to an additional Nrf2 and/or Nrf2-related genes (TrxR1 and GPx2) activation that promoted cell proliferation through decreased apoptosis. The consequences of enhancing ROS detoxification by Nrf2 induction has been recently highlighted by Tuveson and colleagues [56]. In recent years, the role that TrxR1 plays in cancer has become clearer as TrxR1 is highly up-regulated in human tumours and cancer cell lines [57]. In addition, the fact that GPx2 is transiently increased in human colorectal adenomas, with the highest expression in pre-cancerous polyps (adenomas) [55] and reducing amounts in late stage of carcinogenesis, implies that expression of this selenoprotein may depend on the developmental stage of malignant transformation and so may participate in the process of cancer development [58].

Although the protective effect of these selenoproteins in normal cells is well documented [59,60], this study has shown that addition of ITC + Se will up-regulate the expression of the aforementioned genes, providing signals to sustain the cancerous process by protecting Caco-2 cells from H_2O_2-induced cell death. However, results obtained from this research could potentially be translated into normal cells as shown by Campbell et al. [61] who observed oxidative stress protection in normal human endothelial cell line EAhy926 in a synergistic manner in response to SFN and selenite. This dual role suggests that cell response to specific dietary components are likely to differ in non-cancerous and cancerous cells, and will most certainly depend on the cell and/or tissue type, dose and the chemical form in which dietary constituents are administered.

In summary, ITCs and Se in combination provide colon cancer cells with the ability to protect themselves from free radical-mediated cell death effect, which is largely mediated through two selenoproteins, TrxR1 and GPx2. Over-expression of these selenoproteins is achieved by increasing mRNA levels by ITCs at the transcriptional level together with an adequate level of Se supply to guarantee translational expression. Further studies are necessary to investigate the impact of a diet high in cruciferous vegetables and selenium in chemoprevention, and at different stages of cancer development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.07.007.

Abbreviations

ARE	antioxidant responsive element
DMSO	dimethyl sulfoxide
FBS	foetal bovine serum
GPx2	gastrointestinal glutathione peroxidise
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
IB	iberin
ITCs	isothiocyanates
Keap1	the kelch-like ECH-associated protein-1
Nrf2	NF-E2-related factor 2
ROS	reactive oxygen species
Se	selenium
Sec	selenocysteine
SECIS	selenocysteine insertion sequence element
SeMSC	Se-methylselenocysteine
SFN	sulforaphane
siRNA	small inhibitory RNA
TrxR1	thioredoxin reductase-1

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