Synergy between sulforaphane and selenium in the induction of thioredoxin reductase 1 requires both transcriptional and translational modulation

Jinsong Zhang1,2, Vanda Švehlíková1, Yongping Bao1,4, A. Forbes Howie5, Geoffrey J. Beckett2 and Gary Williamson6

1Nutrition Division, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK, 2University of Science and Technology of China, Hefei, 230052, P. R. China and 3University Department of Clinical Biochemistry, The Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, Scotland, UK

4To whom correspondence should be addressed
Email: yongping.bao@bbsrc.ac.uk

Thioredoxin reductases (TrxRs) catalyse the NADPH-dependent reduction of thioredoxin and play an important role in multiple cellular events related to carcinogenesis including cell proliferation, apoptosis and cell signaling. We have used human hepatoma HepG2 cells to examine the regulation of TrxRs by isothiocyanate (sulforaphane) and selenium (Se). We show that TrxR1 mRNA, but not TrxR2 mRNA, is induced up to 4-fold by sulforaphane, and this increase was abolished by actinomycin D, a transcription inhibitor. Se, in the form of sodium selenite, induced TrxR1 at the translational level, as shown by an increase in protein (2.1-fold) and activity (4.8-fold), but not mRNA. In combination, sulforaphane and Se synergistically induced TrxR1 protein (5.5-fold), activity (13-fold) and mRNA (6.5-fold). Although Se does not induce TrxR1 mRNA, Se can delay the degradation of sulforaphane-induced TrxR1 mRNA. Modulation of TrxR1 mRNA by sulforaphane was glutathione and protein kinase C-dependent, as 1-buthionine-S,R-sulfoximine (a specific inhibitor of glutathione synthesis), and the protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine, significantly reduced the induction. The combination of sulforaphane and Se also efficiently protected HepG2 cells from paraquat-induced cell death, whereas selenium-only and Se-only treatments showed very little if any protective effect. These results demonstrate that synergy can result from a combination of induction at the levels of transcription and translation.

Introduction

Enzymes involved in regulation of cell redox function play a key role in maintaining homeostasis. Numerous studies have shown induction of mRNA encoding redox-modulating and carcinogen-metabolizing enzymes by drugs, xenobiotics and food components (1,2). Other studies have demonstrated that selenium (Se) treatment increases the activity of most selenoproteins, many of which are involved in redox reactions, and include phospholipid hydroperoxide glutathione peroxidase (PHGpx), glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) (3,4). Mammalian TrxRs contain a conserved -Cys-Val-Asn-Val-Gly-Cys-redox catalytic site and a selenocysteine (SeCys) residue in the conserved C-terminal sequence -Cys-SeCys- (where SeCys is in the active site) and that truncated TrxR is inactive (5). TrxR, in conjunction with its substrate thioredoxin (Trx), forms a redox system, which has multiple functions and also acts on a number of other substrates including lipoc acid, lipid hydroperoxides, vitamin K₃ and ascorbyl free radicals (6). TrxR activity, which promotes the reduction of Trx, modulates oxidative stress. The oxidation of Trx also affects cell-signaling pathways such as nuclear factor NF-κB, protein tyrosine phosphatase and antioxidant enzymes (6). There are two confirmed forms of mammalian TrxRs, TrxR1 and TrxR2. TrxR1 was found in all subcellular locations, whereas TrxR2 was found predominantly in mitochondria (7-9).

Isothiocyanates such as sulforaphane (SFN) inhibit phase I enzymes such as cytochrome P450 (10), and induce phase II detoxification enzymes such as quinone reductase, glutathione transferases and UDP-glucuronosyl transferases (11-14). SFN also increases the expression of cyclins A and B1, and proapoptotic protein bax, which result in cell-cycle arrest and apoptosis in HT-29 human colon cancer cells (15). These properties of SFN have led to the view that it is not only an important cancer chemopreventive agent capable of inhibiting multiple steps of the carcinogenic process, but also a potentially useful cancer therapeutic drug (16). Se is an essential trace element for humans and its deficiency is associated with the incidence of many types of cancers. A double-blind, placebo-controlled study involving a total of 1312 patients with follow-up of over 6 years demonstrated that oral administration of 200 μg Se/day reduced the incidence of lung, colorectal and prostate cancers (17). Although the exact mechanism for its anticarcinogenic activity still remains to be elucidated, it is widely believed that multiple pathways are involved, including the formation of Se metabolites that act directly to inhibit cancer cells and the formation of critical selenoproteins (18). The chemopreventive activity of Se has been ascribed to the ability of the glutathione peroxidase family to remove DNA-damaging H₂O₂ and lipid hydroperoxides (18). However, animal studies have not shown a link between alteration in glutathione peroxidase activities and the prevention of carcinogenesis (19). Other selenoproteins are also shown to reach a maximum level in tissues at normal dietary intakes of Se and, in fact, excessive Se intake may even result in decreased activity (21). Recent reports showed that high-Se broccoli protects against chemically induced colon cancer in rats and decrease intestinal tumorigenesis in mice (22,23). The present study is an investigation of the combined effect of a phytochemical, SFN and a mineral, Se on TrxR1 induction and how this synergy protects cells against oxidative damage.

Abbreviations: ARE, antioxidant response element; AREs, AU-rich elements; BSO, buthionine sulfoximine; CHX, cycloheximide; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GPx, glutathione peroxidase; H₇, 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine; HUVECs, human umbilical-vein endothelial cells; LDH, lactate dehydrogenase; PHGpx, phospholipid hydroperoxide glutathione peroxidase; PKC, protein kinase C; Se, selenium; SeCys, selenocysteine; SFN, sulforaphane; Trx, thioredoxin; TrxR, thioredoxin reductase.

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Materials and methods

Chemicals and reagents
TrxR (EC 1.6.4.5) from Escherichia coli, Trx from E. coli, cycloheximide (CHX), guanidine hydrochloride, sodium selenite, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), digitonin, dimethylsulfoxide (DMSO), buthionine sulfoximine (BSO) consisting of 100 µM guanidine hydrolyfonal-2-methyl-piperazin (HT) and 3-[N-(dimethyl-amino) propyl]-3-iodolyl]-4-[3-iodolyl] maleimide (GF109203X) were purchased from Sigma (UK). Actinomycin D was from Fluka (UK). SFN, (4-methylsulfinylbutyl isothiocyanate), was purchased from ICN Bio- medicals (UK).

Cell culture and treatments
Human HepG2 cells were cultured in MEM supplemented with FBS (10%), penicillin (100 U/ml) and streptomycin (100 µg/ml) under 5% CO2 in air at 37°C. For enzyme assays, cells were seeded in 10-cm dishes. For mRNA and cytotoxicity assays, cells were seeded in 6 well plates. Cells were allowed to reach ~80% confluence at the time of Se and SFN supplementation. SFN was dissolved in DMSO. All treatments and controls contained a final 0.1% of DMSO (v/v). For BSO treatment, HepG2 cells were pre-treated with BSO (0.5 and 2.5 mM) for 21 h, followed by medium containing the same concentration of BSO plus 12 µM SFN for a further 7.5 h incubation. RNA was then isolated for TrxR mRNA quantification. To measure the effects of Se and BSO on SFN-mediated TrxR activity, HepG2 cells were exposed to 120 nM Se or BSO (0, 0.5 and 2.5 mM), or were co-treated with Se and BSO (0, 0.5 and 2.5 mM) for 24 h. Normal MEM media with 10% FBS contained ≤12.8 nM of Se.

Cell extract preparation
Adherent cells were washed twice by ice-cold PBS, and harvested by scraping in 0.1 M Tris–HCl, pH 7.4. Cell pellets were homogenized using a QiAshreder column. RNA was eluted from a binding cation. To measure the concentration of BSO plus 12 µM Se for a further 7.5 h incubation. RNA was then isolated for TrxR mRNA quantification. To measure the effects of Se and BSO on SFN-mediated TrxR activity, HepG2 cells were exposed to 120 nM Se or BSO (0, 0.5 and 2.5 mM), or were co-treated with Se and BSO (0, 0.5 and 2.5 mM) for 24 h. Normal MEM media with 10% FBS contained ≤12.8 nM of Se.

TrxR activity
TrxR activity (including both TrxR1 and TrxR2) was measured spectrophotometrically using insulin as a substrate (25). A stock reaction mixture was made by mixing 200 µl of 1.0 M, HEPES buffer (pH 7.6), 40 µl of NADPH (40 mg/ml), 40 µl of 0.2 M EDTA and 500 µl of insulin (10 mg/ml). To each test tube, 40 µl of the reaction mixture, 10 µl of 60 µM Trx, 20 µl sample, and water were added to a final volume of 120 µl. The samples were incubated at 37°C for 20 min. The reaction was stopped by addition of 500 µl of 0.4 M EDTA/6 M guanidine hydrochloride in 0.2 M Tris–HCl (pH 8.0), and the absorbance at 412 nm was recorded. A non-enzyme reaction control was a sample of cell extract containing all components except Trx. The absorbance of the control was subtracted from the absorbance of the sample. TrxR activity was calculated based on the standard curve prepared using pure TrxR (48 U/mg protein, one unit is defined as the amount of enzyme causing an increase in absorbance of 0.12 at 412 nm when measured in a coupled assay with E. coli TRX and DTNB per min per milliliter at pH 7.0 at 25°C). One unit increases in absorbance of 5.14 at 412 nm when insulin was the substrate per min per milliliter at pH 8.0 at 37°C.

TrxR1 protein
TrxR1 protein level was measured by an in-house RIA assay as described previously (26). Briefly, the tracer was [125I]-labeled human placental TrxR1, prepared using Bolton-Hunter reagent (Amersham International). Standards were prepared using purified placental human TrxR1 diluted in fetal bovine serum (Gibco, Paisley, Scotland, UK). Standard or sample (100 µl) was added with 125Ilabeled TrxR1 tracer (10,000 d.p.m.; 50 pg/tube) and primary antibody (100 µl). After an overnight incubation at 4°C, second-antibody, donkey anti-rabbit reagent (100 µl) was added. After a further 1 h at room temperature with shaking, wash solution (0.05% Brij solution) was added to each tube, followed by centrifugation for 30 min at 1800 g at 4°C. The supernatant was decanted and the precipitate was washed with a further 1.5 ml of wash solution. The 125Ilabeled radioactive in the precipitate was counted in a multiwell γ-radiation counter, and results were interpolated using the LKB 1224-RIACalc RIA evaluation program.

RNA isolation
Total RNA was extracted from cells using a Qiagen RNeasy mini kit according to the protocol described by the manufacturer (Qiagen, UK) after homogenization using a QiAshred column. RNA was eluted from a binding column with RNase-free water and RNase inhibitor was immediately added (20 µl/preparation) and stored at ~70°C. The concentration and purity of the RNA were determined by measurement of the absorbance at 260 and 280 nm. A ratio of >1.8 of A260/A280 indicated suitable purity.

mRNA quantification
TrxR1 and TrxR2 mRNA were determined by real-time RT–PCR (TaqMan assay) using the ABI prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems). Primers and the fluorogenic TaqMan probes were designed using Primer Express Software according to human TrxR1 and TrxR2 sequences. Primer/probe sets were homology-searched to ensure that they were specific for TrxR1 and TrxR2 mRNA transcripts using a NCBI BLAST search. Primer/probe set for TrxR1 (Genbank accession number AF208018) is: forward primer: 5’-CCACTGCTGTAAGACACCTT-3’. Reverse primer: 5’-AGGAGAAAAATCATCAGACTGAT-3’. Probe: 5’-CATTACCTTGTTGCAACGGAGTCCCA-3’. For TrxR2 (Genbank accession number AF106697): forward primer: 5’-GACACCCCGAGGAAGA-3’. Reverse primer: 5’-CAACCTTCTCCATTTACACTTCTCTC-3’. Probe: 5’-CTTGGGCTACATTGGTACCCATCCCA-3’.

The probes were labeled with a 5’ reporter dye, FAM (6-carboxyfluorescein) and 3’ quencher dye, TAMRA (6-carboxy tetramethylrhodamine). RT–PCR reactions were carried out in a 96 well plate using TaqMan one-step RT–PCR master mix Reagent kit (Applied Biosystems) in a total volume of 25 µl/well consisting of 100 nM probe, 200–300 nM forward and reverse primers and 10 ng of total RNA. TaqMan RT–PCR conditions: 48°C 30 min, 95°C 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analysed with TaqMan software. Reactions were carried out in triplicate. TaqMan threshold cycle number (Ct) was normalized into fold of relative induction using the ΔCt method, fold of induction = 2^(-ΔΔCt) - (Omit treatment).

Standard curves were constructed using 1, 5, 10, 20 and 40 ng of total RNA in triplicate. The results for TrxR1 was Y = −3.672X + 31.766 (where 3.672 is the slope, X is the log starting amount of RNA (ng), and 31.766 is Y intercept), and for TrxR2 was Y = −3.257X + 29.158. Linear correlation coefficients (r2) for TrxR1 and TrxR2 were 0.994 and 0.993, respectively.

Cytotoxicity evaluation
Cytotoxicity was measured by lactate dehydrogenase (LDH) release using a Cytotox 96 Assay kit (Promega, Madison, WI). This assay quantitatively measures the activity of LDH, a stable cytosolic enzyme that is released upon cell lysis. Cells were pre-treated with Se in combination with SFN for 72 h, then the medium was changed into that containing the same concentrations of Se and SFN, but also with 7 mM paraquat for a further 24 h. The cytotoxicity assay was performed according to the manufacturer’s instructions. Cytotoxicity (%) was calculated using the following formula:

\[ \text{Cytotoxicity} \% = \frac{100 \times (A_{950} \text{ of treated cells})}{(A_{950} \text{ of maximal lysis} - A_{950} \text{ of control cells})} \]

Statistical analysis
Statistical calculations were done using the Student’s t test. A P value < 0.05 was considered statistically significant.

Results

TrxR1 mRNA, activity and protein
TrxR1 has been detected in many cell types and is also present in human plasma (27), but little is known concerning the regulation of TrxR1 expression by dietary phytochemicals. In human HepG2 cells, TrxR1 mRNA, but not TrxR2 mRNA, was found to be up-regulated by SFN. TrxR1 mRNA (maximum 4-fold induction), specific activity (maximum 2.8-fold) and immunodetectable protein (2.2-fold) were up-regulated in a dose-dependent manner by SFN. Se increased specific activity (maximum 6.1-fold induction) and immunodetectable protein (maximum 2.3-fold) and was also dose-dependent (Tables I and II). However, in marked contrast to SFN, Se (30–480 nM) did not increase mRNA. When added in combination, Se and SFN together induced mRNA by 60% more as compared with SFN alone (6.5 versus 4-fold). However, specific activity was increased synergistically by adding the two inducers together (12 µM SFN and 120 nM Se), leading to a maximum induction of 13.2-fold, which compared with an induction of 2.8 by SFN (12 µM) and 4.8-fold by Se (120 nM). These inductions were also reflected as the changes in immunodetectable protein, i.e. SFN (12 µM) and Se (120 nM) can induce TrxR1 protein...
Table I. Effects of SFN and Se on TrxR1 mRNA and activity in HepG2 cells (24h)

<table>
<thead>
<tr>
<th></th>
<th>mRNA induction (fold)</th>
<th>Activity (U/mg protein)</th>
<th>Activity induction (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.18</td>
<td>0.12 ± 0.01</td>
<td>1</td>
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<tr>
<td>SFN (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.36 ± 0.11</td>
<td>0.14 ± 0.01</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>2.61 ± 0.05</td>
<td>0.18 ± 0.01</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>4.00 ± 0.21</td>
<td>0.16 ± 0.02</td>
<td>1.3</td>
</tr>
<tr>
<td>Se (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.00 ± 0.04</td>
<td>0.24 ± 0.02</td>
<td>2.0</td>
</tr>
<tr>
<td>120</td>
<td>0.89 ± 0.09</td>
<td>0.32 ± 0.03</td>
<td>2.7</td>
</tr>
<tr>
<td>480</td>
<td>1.28 ± 0.21</td>
<td>0.34 ± 0.02</td>
<td>2.8</td>
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<tr>
<td>SFN (µM) + Se (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 + 120</td>
<td>2.69 ± 0.22</td>
<td>0.57 ± 0.04</td>
<td>4.8</td>
</tr>
<tr>
<td>6 + 30</td>
<td>3.65 ± 0.32</td>
<td>0.54 ± 0.05</td>
<td>4.5</td>
</tr>
<tr>
<td>6 + 120</td>
<td>3.76 ± 0.08</td>
<td>0.60 ± 0.03</td>
<td>5.0</td>
</tr>
<tr>
<td>6 + 480</td>
<td>4.19 ± 0.08</td>
<td>0.70 ± 0.03</td>
<td>5.8</td>
</tr>
<tr>
<td>12 + 120</td>
<td>6.50 ± 0.07</td>
<td>0.56 ± 0.05</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with control. Data are mean ± SD of triplicate determinations.

Table II. Effects of SFN and Se on TrxR1 Protein and activity in HepG2 cells (72 h)

<table>
<thead>
<tr>
<th></th>
<th>Activity (U/mg protein)</th>
<th>Activity induction (fold)</th>
<th>Protein (ng/mg protein)</th>
<th>Protein induction (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.17 ± 0.01</td>
<td>1</td>
<td>307</td>
<td>1</td>
</tr>
<tr>
<td>SFN (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.38 ± 0.02</td>
<td>2.2</td>
<td>419</td>
<td>1.4</td>
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<tr>
<td>6</td>
<td>0.28 ± 0.01</td>
<td>1.7</td>
<td>513</td>
<td>1.7</td>
</tr>
<tr>
<td>12</td>
<td>0.47 ± 0.02</td>
<td>2.8</td>
<td>680</td>
<td>2.2</td>
</tr>
<tr>
<td>Se (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.70 ± 0.08</td>
<td>4.1</td>
<td>500</td>
<td>1.6</td>
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<tr>
<td>120</td>
<td>0.82 ± 0.04</td>
<td>4.8</td>
<td>647</td>
<td>2.1</td>
</tr>
<tr>
<td>480</td>
<td>0.90 ± 0.08</td>
<td>5.3</td>
<td>635</td>
<td>2.1</td>
</tr>
<tr>
<td>2000</td>
<td>1.03 ± 0.06</td>
<td>6.1</td>
<td>719</td>
<td>2.3</td>
</tr>
<tr>
<td>SFN (µM) + Se (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 + 120</td>
<td>1.44 ± 0.09</td>
<td>8.5</td>
<td>893</td>
<td>2.9</td>
</tr>
<tr>
<td>6 + 30</td>
<td>1.51 ± 0.06</td>
<td>8.9</td>
<td>1234</td>
<td>4.0</td>
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<tr>
<td>6 + 120</td>
<td>1.65 ± 0.12</td>
<td>9.7</td>
<td>1419</td>
<td>4.6</td>
</tr>
<tr>
<td>6 + 480</td>
<td>2.04 ± 0.07</td>
<td>12.0</td>
<td>1607</td>
<td>5.2</td>
</tr>
<tr>
<td>6 + 2000</td>
<td>1.77 ± 0.14</td>
<td>10.4</td>
<td>1419</td>
<td>4.6</td>
</tr>
<tr>
<td>12 + 120</td>
<td>2.25 ± 0.15</td>
<td>13.2</td>
<td>1700</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with control. Data for activity are mean ± SD of triplicate determinations, and for protein is the average of duplicate determinations (variations are <10%).

~2.2- and 2.1-fold, respectively, in combination, the induction was maximum 5.5-fold (Table II).

Time course of TrxR1 mRNA and activity

Time courses of induction were performed to examine the mechanism further and to provide information on mRNA stability. The amount of TrxR1 mRNA was not induced by Se treatment within 72 h, but was significantly induced by SFN with a maximum at ~8 h, declining thereafter almost to baseline after 72 h. Se and SFN together showed an increase in the amount of mRNA at all the time points measured; again the amount declined after 8 h but was still significantly above baseline after 72 h (Figure 1A). This suggests that although Se does not induce mRNA directly, it could stabilize TrxR1 mRNA that had been induced by SFN. Hence, we further...
investigated TrxR1 mRNA at 72 h to see the effect of different doses of Se on TrxR1 mRNA induced by SFN. Again, Se at all levels used did not induce TrxR1 mRNA, but maintained TrxR1 mRNA at higher levels (Figure 1C). HepG2 cells were treated with SFN, Se or a combination of both SFN and Se for 24 h. There was no induction of TrxR2 mRNA expression in all of the treatment (Figure 2). Apart from TrxR2, other selenoproteins such as gastrointestinal GPx (GI-GPx) and PHGPx mRNA are also not induced by SFN treatment in HepG2 cells, although Se induced the GPx and PHGPx activities as expected (data not shown).

**Mechanism of induction of TrxR1 mRNA by SFN**

Isoquinolinesulfonamide H7 and 3-[N-(dimethyl-amino)propyl-3-indolyl]-4-[3-indolyl] maleimide (GF109203X) are two widely used protein kinase C (PKC) inhibitors, that inhibit distinct cAMP and cGMP-dependent PKC isotypes (30,31). As shown in Figure 3A, H7 treatment at concentrations of 30 and 50 µM reduced amounts of TrxR1 mRNA to ~35–50% of control level, and to ~10–25% of the SFN-treated level. However, treatments with another selective inhibitor of PKC, GF109203X, did not inhibit TrxR1 mRNA of both control and SFN treatments. Treatment with the transcription inhibitor, actinomycin D at 5 µg/ml, reduced TrxR1 mRNA levels to ~20% of control level, and to 10% of the SFN-treated level (Figure 3B). Pre-treatment of cells with actinomycin D for 1 h abolished the induction of TrxR1 mRNA by SFN. Treatment with the translation inhibitor, CHX (40 µg/ml), which may disturb PKC expression (32), reduced the amount of TrxR1 mRNA to ~20% of control level, and pre-treatment of cells with CHX for 1 h reduced the induction of TrxR1 mRNA by SFN to 50% of the SFN-treated level (Figure 3B). These results show that the regulation of TrxR1 mRNA by SFN involves cAMP and cGMP-dependent PKC, and also requires active transcription and translation for induction.

BSO treatment depletes intracellular GSH (32). For SFN metabolism, the formation of an intracellular SFN-SG conjugate is a crucial step in facilitating uptake of SFN into cells (33,34). BSO induced TrxR1 mRNA but the effect was only 1.5–2-fold at 2.5 mM BSO (Figure 4A). SFN induced TrxR1 mRNA as expected by 4-fold, but this induction was repressed by BSO. These results suggest that the induction of TrxR1 by SFN is in relation to the depletion of intracellular GSH as BSO alone also induces TrxR1, albeit less effectively. Moreover, the depletion of intracellular GSH by BSO abolishes much of the SFN expression of TrxR1 (Figure 4A), implying that high intracellular GSH is required in order to get sufficient amounts of SFN into the cell to activate the induction pathways. In support of this, there is a synergistic effect between BSO and Se on TrxR specific activity (3.6-fold, Figure 4B), although the extent of synergy was lower than that between Se and SFN (5-fold, Table I, P < 0.05). This again demonstrates that temporary depletion of intracellular GSH is essential for both induction of TrxR1 and for observed synergy.

**Protection of HepG2 against paraquat by SFN and Se**

Paraquat is a cytotoxic reagent, which acts via a free radical-dependent mechanism (35). As shown in Figure 5, Se at 30...
Synergistic induction of thioredoxin reductase 1

Effect of BSO on SFN-mediated TrxR1 mRNA induction and TrxR activity. (A) HepG2 cells were pre-treated with BSO for 21 h, followed by changing to a medium containing the same concentration of BSO plus 12 µM SFN, for a further 7.5 h incubation. Data are mean ± SD of triplicate determinations: *P < 0.05 when compared with control; #P < 0.05 when compared to SFN-only. (B) HepG2 cells were exposed to 120 nM Se or BSO, or Se + BSO for 24 h. Data are mean ± SD of triplicate determinations. *P < 0.05 when compared with control, #P < 0.05 when compared with Se-only.

Discussion

TrxRs are the only family of enzymes known to reduce Trx-S2 to Trx-(SH)2, thus regulating Trx activity. Exogenous Trx-(SH)2 can attenuate oxidative damage in a variety of cells (36,37). In addition to reducing Trx-S2, TrxR1 has been shown to have a number of other important cellular antioxidant functions including reduction of selenite to selenide (38), reduction of the ascorbyl radical, and direct scavenging of lipid peroxides and peroxynitrite using SeCys as a cofactor (39,40). Over-expression of Trx facilitates protection against a wide variety of oxidative stresses including cytokines, UV irradiation and ischemic injury (41). On the other hand, cells with lower TrxR activity are more prone to oxidative damage, for example, bovine aortic endothelial cells with 25-fold lower TrxR activity compared with human endothelial cells are more sensitive to t-BuOOH (26).

This is the first report of synergy between inducers of TrxR1 expression, and the effect can be attributed mainly to the induction of TrxR1 mRNA by SFN and subsequent increase in protein level and specific enzyme activity by Se. Se supplies adequate SeCys for incorporation of Se into the mature enzyme, i.e. keeping the translation step active. This may contribute to the delaying TrxR1 mRNA degradation. It also could be that Se enhanced the production of mRNA in response to SFN and the mechanism requires further investigation. In agreement with our results, Se at 30–2000 nM did not significantly induce TrxR1 mRNA in HepG2 cells in the short term (42). In the same study, Se increased TrxR1 protein and activity in MCF-7, HT-29 and A-549 cancer cells, although prolonged exposure (5 days) of these cells to Se at 1000 nM increased TrxR1 mRNA by 1.7–4.5-fold (42), presumably by mRNA stabilization. Depletion of intracellular GSH by SFN, to form the SFN–SG conjugate (16,33,34), is a key step in modulation of TrxR1 mRNA expression. BSO also induced TrxR activity synergistically with Se, although the effect was not as pronounced as with SFN, demonstrating that intracellular GSH is important for the observed synergy. A specific PKC isoform may also be involved in the induction of TrxR; however, the response appears to dependent on cell type, for example, activation of PKC with phorbol esters cause a down-regulation of TrxR in human umbilical vein endothelial cells (HUVECs) (43), but an up-regulation in mouse skin (44) and human thyrocytes (45). We showed in this study that H7, which inhibits cAMP-dependent and cGMP-dependent PKC isotypes may play a role in TrxR1 induction. However, another PKC isoform,
which was inhibited by GF109203X in HUVECs after PMA treatment did not appear to play a role.

Recently, Arné et al. have characterized the core promoter of human TrxR1 and identified functional AU-rich elements (AREs) (46), which may be responsible for the stabilization of mRNA and fast response to various signals (47). Although the TrxR1 promoter lacked TATA boxes, an increased GC content with functional Sp1 sites is a feature of a typical ‘housekeeping’ type of gene. TrxR1 is up-regulated by many exogenous agents, including epidermal growth factor, H₂O₂ and 1-chloro-2,4-dinitrobenzene treatment of epidermal carcinoma A431 cells (6), and calcium ionophore and phorbil 12-myristate 13-acetate treatment of thyrocytes and HUVECs (43,45). From the promoter analysis, TrxR1 regulation involves Sp1/Sp3, and this transcriptional activation appears to be mediated by PKC (48). There is also a putative antioxidant response element (ARE, TGAC NNNN GCA) in the promoter region (−54 to −43) (46). Regulation via ARE involves the NF-E2-related factor (Nrf2), an essential component of an ARE-binding transcriptional complex. PKC-directed phosphorylation of Nrf2 may be a critical event for the nuclear translocation of the ARE-binding transcriptional factor (49). Nrf2 has also been demonstrated as an important transcription factor in phase II enzyme induction including glutathione transferase, quinone reductase and Trx expression (50,51). Recently, Talalay et al. have demonstrated that disruption of Keap1–Nrf2 complex by inducer SFN leads to Nrf2 migrating to the nucleus where it binds to the ARE regions of phase II genes and accelerates their transcription (52). SFN regulation of TrxR1 may also involve Sp1, which only has DNA binding activity in a reduced form, and so the Sp1 and Oct-1 interactions, and Sp1/Sp3 ratio, may be further levels of regulation. It is interesting to note that the TrxR2 does not contain both AREs (29) and antioxidant response element (ARE). Up to 5k of TrxR2 5′ region has been analysed using the AliBaba2.1, a program for predicting transcription factor binding sites in an unknown DNA sequence (www.gene-regulation.com), no ARE was noted that the TrxR2 does not contain both AREs (29) and antioxidant response element (ARE). Up to 5k of TrxR2 5′ region has been analysed using the AliBaba2.1, a program for predicting transcription factor binding sites in an unknown DNA sequence (www.gene-regulation.com), no ARE was found. The lack of ARE in TrxR2 gene may be a possible answer that SFN could not elevate TrxR2 expression. Further work is needed using promoter construction to identify the role of both AREs and ARE in controlling the expression of TrxRs and this may help to understand the different responses of these two enzymes to SFN and Se. The increased TrxR1 expression by SFN and Se treatment was positively correlated with the protective effects against paraquat attack in HepG2 cells. Moreover, it was observed that TrxR activity continued to increase with the availability of high doses of Se in several tumor cell lines in vitro (42) and there was a transitional increase of TrxR activity at supranutritional dose of Se in vivo (53), which led to a new hypothesis proposed by Ganther that TrxR would be more directly related to the anticancer mechanism of Se (18). However, this was recently challenged in view of the fact that TrxR1 did not further increase at supranutritional dose in rat (54). The data in this paper show that TrxR1 is not significantly increased by supranutritional doses of Se (480–2000 nM) in comparison with moderate doses (30–120 nM) in human HepG2 cells. This is similar to the observation in HUVECs where optimal TrxR1 expression is achieved using 50 nM Se (43).

In conclusion, we demonstrated that SFN and Se could synergistically up-regulate TrxR1 expression in human HepG2 cells and protect them against oxidative damage. As both SFN and Se have been used in human cancer prevention trials it would be interesting to examine the combinational effects of SFN and Se in normal human cells and this would help in the understanding of the importance of TrxR1 and the anticancer mechanisms of both SFN and Se.

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