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Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats

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Abstract

Sulforaphane (SF), a glucosinolate-derived isothiocyanate found in cruciferous vegetables, is considered an anticarcinogenic component in broccoli. Sulforaphane induces a battery of detoxification enzymes, including quinone reductase (QR). Induction is thought to be mediated through a common regulatory region termed the antioxidant response element (ARE). To test the hypothesis that the antioxidant selenoprotein thioredoxin reductase (TR) may be induced as part of this coordinated host-defense response to dietary anticarcinogenic compounds, TR activity was measured in livers of rats pair-fed diets containing SF and/or broccoli (n = 6/group). At the doses used, neither SF nor broccoli alone significantly elevated TR activity, whereas treatments containing both broccoli and SF caused a significant increase in TR activity. Glutathione peroxidase (GSH-Px), a second selenium-dependant enzyme with antioxidant activity, was downregulated in rats fed both SF and broccoli, compared to the control diet.

A second experiment, using mouse hepatoma Hepa1c1c7 cells, tested whether an interaction exists between selenium (Se) and SF in TR inducibility, since Se is known to induce TR activity. Selenium (2.5 μM) plus SF (2.0 μM) caused significantly greater TR activity than either treatment alone. All treatments with added Se or SF caused significantly greater TR activities than no Se or SF treatment. Glutathione peroxidase activity was elevated by Se, but not by SF. These data suggest that TR, known to be regulated by Se, is also upregulated as part of a host response to the dietary anticarcinogen SF, a trait not shared by another Se-dependent enzyme, GSH-Px. Published by Elsevier Inc. All rights reserved.

Keywords: Thioredoxin reductase; Sulforaphane; Selenium; Glutathione peroxidase

1. Introduction

Mammalian TR is a 110 kDa homodimeric selenoprotein that catalyzes the NADPH-dependent reduction of thioredoxin. Electrons are transferred from NADPH via FAD to a conserved redox site. Each monomer is comprised of a prosthetic FAD group, a redox active disulfide site and a pentultimate selenocysteine amino acid residue [1].

Mammalian TR is an antioxidant that it is able to reduce various substrates including lipoic acid, lipid hydroperoxides, NK-lysin, vitamin K_3, dehydroascorbic acid, ascorbyl free radical, and the tumor suppressor protein p53 [for review see 2]. It is hypothesized that the presence of the selenocysteine redox site in mammalian TR allows for this broad reducing substrate specificity [2].

Thioredoxin provides reducing equivalents for ribonucleotide reductase, which is essential for DNA synthesis [3]. Also, it regulates the action of several gene transcription factors including NF-κB [4], AP-1 [5] and the glucocorticoid receptor [6] through a redox mechanism by providing reducing equivalents to facilitate binding to DNA [7]. Thioredoxin must be in a reduced form to be active and TR is the primary means of reduction. Ultimately, all of the functions of thioredoxin are tied to the presence of TR.

In animals, TR activity is regulated by dietary Se, and

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activity drops to less than 5% of normal values in selenium deficient rat livers [8,9]. In cell culture, TR activity is increased in a dose dependent manner by 1-10 μM supplemental Se, and falls to below 5% of controls when cultures are depleted of Se [10].

Both TR activity and protein levels are induced by t-butylhydroquinone (BHQ) in cultured cortical astrocytes [11]. Because BHQ is known to upregulate a number of detoxification enzymes via the ARE, these researchers suggested that TR may be regulated in part by the ARE which is known to mediate the induction of QR and other enzymes such as γ-glutamylcysteine synthetase and glutathione S-transferase [for review see 12]. The objective of the present study was to determine if the antioxidant selenoprotein TR can be induced by SF, a compound from broccoli known to induce detoxification enzymes via the ARE [12].

2. Materials and methods

2.1. Materials

Solvents used for extraction and purification were of reagent-grade, and solvents used for gas chromatography (GC) or high performance liquid chromatography (HPLC) analysis were of HPLC-grade. All solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Sulforaphane was purified from broccoli seed by the method of Matusheski et al., [13]. Purity was determined to be >99% by GC analysis. Unless otherwise noted, all other materials used in this study were obtained from Sigma (St. Louis, MO).

2.2. Broccoli processing

Broccoli (*Brassica oleracea* var. Italica cv. Majestic) plants were grown at the University of Illinois, Urbana, IL. At harvest, heads were cut from the mature vegetables, and held on ice for <4 hr. Florets (2 inches long) were cut, plunged into liquid nitrogen, and stored at 80°C until being freeze-dried. Freeze-dried broccoli samples were finely ground in a water-cooled Tekmar A-10 analytic mill (IKA® Labortechnik, Staufen, Germany) for 15 s and stored at 20°C until used.

Control diets (AIN 76-B40) were formulated to contain the same amount of fiber as the 20% broccoli diets. Experimental diets containing broccoli (20%) were adjusted to balance nutrients and maintain a constant caloric value. Nutritive values for broccoli were from the USDA Nutrient Database for standard reference (Release 13, Nov. 1999). Purified SF was premixed with corn oil for 15 min before mixing with the rest of the diet. All experimental diets were mixed the same day and stored in the dark for fewer than 10 days at 4°C in airtight plastic bags. Diets containing isothiocyanates may be stored for at least 2 weeks and remain stable [14].

2.3. Animals and housing

Animal usage was approved by the University of Illinois Animal Care and Use Committee. Male F344 rats (Harlan, Indianapolis, IN), 4 weeks old (40–60 g), were housed individually, in shoebox cages with corncob bedding in a controlled environment with a 12-hr light/dark cycle under uniform temperature and humidity. The animals were acclimated over six days; during the first two days they received rat chow followed by four or more days of powdered semi-purified control diet (AIN76 B-40, ICN Biochemicals, Cleveland, OH). Animals were allowed *ad libitum* access to food and water throughout the acclimation period.

2.4. Experimental design

The experiment used a completely randomized design. Animals were stratified by weight and randomly allotted to one of six dietary treatments. Treatment duration and doses were based on previous work [15].

2.5. Experimental diets

Experimental diets were provided for 5 days; all diets were provided fresh daily. During the 5-day experimental period, groups of 5 rats received one of 6 diets: 1) control diet (modified AIN 76 B40); 2) 20% broccoli, 3) 0.32 mmol SF/kg diet, 4) 5.16 mmol SF/kg diet, 5) 20% broccoli + 0.16 mmol SF/kg diet, or 6) 20% broccoli + 5 mmol SF/kg diet. The broccoli used in diets contained 11 μmol glucoraphanin/g dry weight broccoli or 2.2 mmol SF/kg diet. Glucoraphanin is hydrolyzed by the plant enzyme myrosinase to form SF. Based on preliminary work, under laboratory conditions the conversion of glucoraphanin to SF was estimated to be 6%.[1] Therefore, rats consuming 20% broccoli diets would be supplied with approximately 0.16 mmol SF/kg diet. Food intake was recorded daily and animals were pair-fed to the mean of the group with the lowest average food intake on the previous day.

2.6. Tissue collection and preparation

Rats were anesthetized with ketamine (87 mg/ml)/xylazine (13 mg/ml) dosed at 0.1 ml/100 g BW, and killed by cervical dislocation. Perfused livers were blotted dry, weighed, cut into 1 g pieces, and snap-frozen in liquid nitrogen. Samples were stored at −80°C. Defrosted livers were homogenized in four volumes of buffer (0.15 M KCl, 0.25 M K_2HPO_4/KH_2PO_4, pH 7.25) and centrifuged (10,000 × g for 20 min). The supernatants were centrifuged at 105,000 × g for 1 hr and the cytosolic fraction was collected, snap-frozen in liquid nitrogen and stored at −80°C.

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2.8. Cell culture

Hepa1c1c7 mouse hepatoma cells were obtained from American Tissue Culture Collection seeded at 1×10^6 cells/flask and grown at 37°C in 95% ambient air and 5% CO_2 in 75 cm^2 culture flasks. Cells were grown for 24 hr in control medium (α-modified MEM) with 26.2 mM sodium bicarbonate and 10% fetal bovine serum). Experimental media were then introduced to produce a 2X2 factorial design: 1) 2.5 μM Se as sodium selenite, 2) 2.0 μM SF, 3) 2.5 μM Se + 2.0 μM SF, 4) control medium. A 0.1% solution of DMSO was used as a carrier for SF. After 24 hr, cells were trypsinized, centrifuged at 5,000 RPM, washed with PBS, centrifuged at 5,000 RPM, lysed in 0.05% deoxycholate solution and stored at −80°C until further analysis.

2.9. Biochemical analysis

Quinone reductase activity was assessed spectrophotometrically (Hitachi Instruments, Inc., Conroe, TX) using 2,6-dichlorophenolindophenol (DPIP) as the substrate by the method of Ernster [15] as modified by Benson et al. [16]. Specific activity is reported as nmol DPIP reduced per minute per mg cytosolic protein.

Thioredoxin reductase activity was determined spectrophotometrically by the method of Holmgren and Bjornstedt [18] as modified by Hill et al. [9] following the reduction of 5,5-dithiobis[2-nitrobenzoic acid] (DTNB) giving two molecules of TNB (that have a maximum absorbance at 412 nm) in the presence of NADPH. Enzymatic TR activity was determined by subtracting the time-dependent increase in absorbance at 412 nm in the presence of the TR activity inhibitor aurothioglucose (20 μM) from total TR activity. One unit of activity is described as 1μmol TNB formed per minute per mg protein.

Glutathione peroxidase activity was determined using the coupled enzyme method of Paglia and Valentine [19] using H_2O_2 as the substrate and NADPH as the source of reducing equivalents. Protein concentrations were measured by the BioRad assay (Hercules, CA) on a μQuant microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Western blots of rat liver cytosolic TR and GSH-Px were performed using NuPage (Carlsbad, CA) protocols and reagents: Bis-Tris, 12% polyacrylamide gels, MOPS running buffer and NuPage sample buffer. Proteins from gels were transferred to PVDF membranes at 125 millivolts for 90 min and blocked overnight in a 5% powdered milk and a 1% Tween solution in PBS. Membranes were incubated with polyclonal TR and GSH-Px antibodies (1000X dilution with PBS and 1% Tween 20) for 1 hr, washed 3x for 5 min with PBS-Tween and incubated for 45 min with anti-rabbit IgG, horseradish peroxidase conjugated secondary antibody (5000× dilution with PBS-Tween 20; Amersham, Buckinghamshire, England) and visualized by ECL-plus (Amersham) on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Western blots using our TR antibody resulted in doublet bands, possibly from antibody recognition of newly synthesized mitochondrial TR before localization into the mitochondria.

2.10. Selenium analysis

Selenium content of diets was determined by hydride generation atomic absorption spectrometry according to a previously described procedure [20]. Briefly, samples were digested in nitric acid with magnesium nitrate present as an aide to prevent Se volatilization. Samples were then ashed in a muffle furnace and resuspended in 6M hydrochloric acid. Selenium determination was by a Perkin-Elmer 5100 AAS with a continuous flow hydride generator. Quality control was maintained by running bovine liver standard (NBS standard 1577b). The detection limit for Se analyzed by this method was 0.001 μg Se/ml of wet ashed sample. All samples were analyzed in triplicate.

2.11. Statistical analysis

Data from animal experiments were analyzed by one-way ANOVA. When an overall treatment effect was found, individual means were compared by Tukeys Studentized Range Test. Pearson correlation coefficients were used to determine correlations between enzyme activity and urinary sulforaphane mercapturic acid. Data from cell culture were analyzed by two-way ANOVA as a 2X2 factorial design. All procedures were performed using PC/SAS [21].

3. Results

3.1. Animal experiment

Food intake for the highest treatment group, [20% broccoli + 5mmol SF/kg diet] was significantly lower than intake for the group receiving control diet. However, it was not different from any other diet containing 20% broccoli. Weight gain was not different among groups (data not shown).

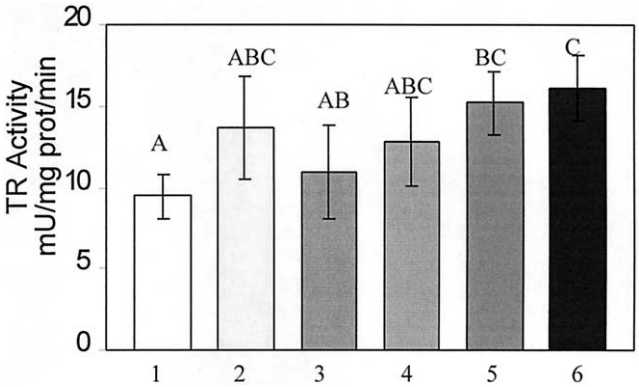

Fig. 1. Effect of diets enriched in purified sulforaphane, 20% broccoli, or purified sulforaphane + 20% broccoli on rat hepatic thioredoxin reductase activity. 1) AIN 76 B40 control diet; 2) 20% broccoli diet; 3) 0.32 mmol SF/kg diet; 4) 5.16 mmol SF/kg diet; 5) 20% broccoli + 0.16 mmol SF/kg diet; 6) 20% broccoli + 5 mmol SF/kg diet. Values are means (n = 6) ± SD. Differently lettered bars denote significantly different treatment means ($P < 0.05$).

Diets containing both 20% broccoli and purified SF increased hepatic TR activity compared to the control diet ($P < 0.05$, Fig. 1). In contrast, hepatic GSH-Px activity was significantly depressed in animals fed broccoli plus purified SF compared to rats receiving control diets ($P < 0.05$, Fig. 2). Western blot analysis revealed that TR and GSH-Px protein concentrations varied in response to diets in a pattern similar to TR and GSH-Px activities (Figs. 3a and b). Thioredoxin reductase protein concentrations were highest in the livers of rats receiving broccoli plus purified SF. Conversely, these same tissues exhibited the lowest GSH-Px concentrations.

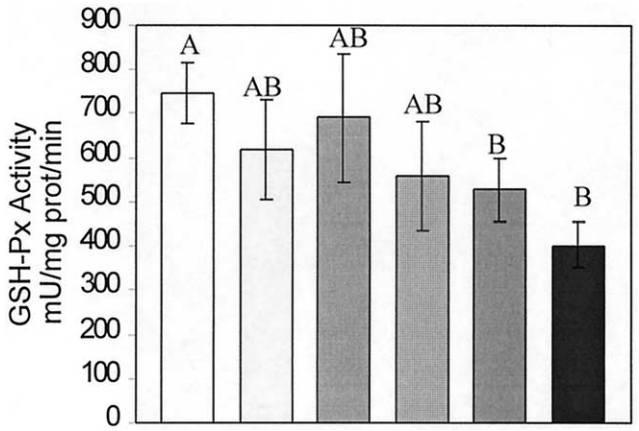

Fig. 2. Effect of diets enriched in purified sulforaphane, 20% broccoli, or purified sulforaphane + 20% broccoli on rat hepatic glutathione peroxidase activity. 1) AIN 76 B40 control diet; 2) 20% broccoli diet; 3) 0.32 mmol SF/kg diet; 4) 5.16 mmol SF/kg diet; 5) 20% broccoli + 0.16 mmol SF/kg diet; 6) 20% broccoli + 5 mmol SF/kg diet. Values are means (n = 6) ± SD. Differently lettered bars denote significantly different treatment means ($P < 0.05$).

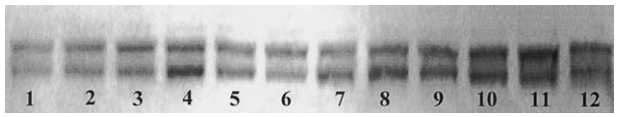

Fig. 3a. Effect of diets enriched in purified sulforaphane, 20% broccoli, or purified sulforaphane + 20% broccoli on rat hepatic thioredoxin reductase protein determined by western blot analysis of hepatic cytosol. Lanes 1, 2: AIN 76 B40 diet; lanes 3, 4: 20% Majestic broccoli diet; lanes 5, 6: 0.32 mmol SF/kg diet; lanes 7, 8: 5.16 mmol SF/kg diet; lanes 9, 10: 20% Majestic broccoli diet + 0.16 mmol SF/kg diet; lanes 11, 12: 20% majestic broccoli diet + 5 mmol SF/kg diet.

3.2. Cell culture

In a preliminary experiment, SF caused an increase in TR activity in Hepa1c1c7 cells, with the highest dose tested (2.0 μM SF) causing significantly greater TR activity than that seen in untreated cultures (5.2 ± 0.4 compared to 3.9 ± 0.6 mU/mg prot/min, $P < 0.05$). A second experiment tested the interaction between Se and SF on TR inducibility. Individually Se and SF treatments both significantly induced TR activity ($P < 0.05$, Fig. 4). While the combined Se and SF treatment caused a greater induction of TR than either treatment alone, ANOVA revealed no significant interaction ($P = 0.14$). Glutathione peroxidase activity was increased by Se treatment ($P < 0.05$) but was not affected by SF (Fig. 5). Conversely, SF significantly induced QR activity, while Se had no effect at this dose (Fig. 6).

4. Discussion

The present study demonstrates that in livers of rats fed adequate Se, TR activity and protein can be increased by dietary SF + broccoli. Conversely, we found that hepatic GSH-Px activity and protein were decreased in rats by dietary SF + broccoli. The animals employed in this study were also used in a study relating dietary SF to the induction of hepatic and colonic QR.[2] In that study, hepatic QR induction was found to correlate closely with urinary SF mercapturate ($r = 0.73$, $P = 0.0006$). Therefore, using data from the aforementioned study, we evaluated the association between urinary metabolites of SF and both TR and GSH-Px activities. We found that TR activity is significantly associated with urinary content of the SF conjugate (r

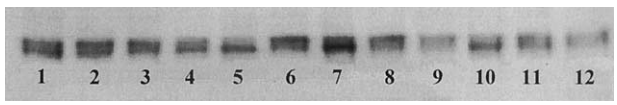

Fig. 3b. Effect of diets enriched in purified sulforaphane, 20% broccoli, or purified sulforaphane + 20% broccoli on rat hepatic glutathione peroxidase protein determined by western blot analysis of hepatic cytosol. Lanes 1, 2: AIN 76 B40 diet; lanes 3, 4: 20% Majestic broccoli diet; lanes 5, 6: 0.32 mmol SF/kg diet; lanes 7, 8: 5.16 mmol SF/kg diet; lanes 9, 10: 20% Majestic broccoli diet + 0.16 mmol SF/kg diet; lanes 11, 12: 20% majestic broccoli diet + 5 mmol SF/kg diet.

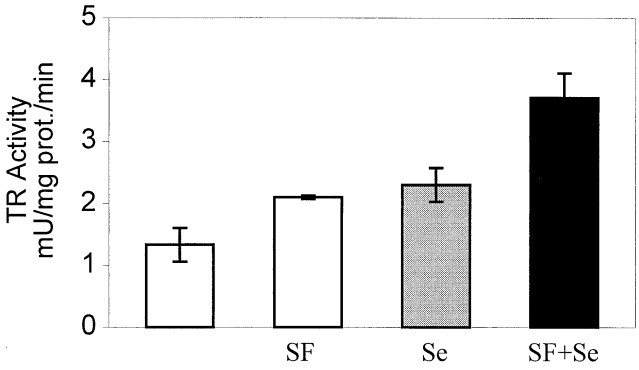

Fig. 4. The effect of purified sulforaphane and/or sodium selenite on Hepa1c1c7 mouse hepatoma cell thioredoxin reductase activity. Cells were exposed to:control αMEM media, 2.0 μM SF, 2.5 μM Se, or 2.0 μM SF + 2.5 μM Se. Values are means (n = 3) ± SD. Two-way ANOVA showed a significant treatment effect for selenite ($p < 0.001$) and SF ($p < 0.0002$), but no interaction ($p < 0.12$).

= 0.66, P = 0.003) while GSH-Px is significantly negatively associated with the urinary content of the SF conjugate (r = −0.69, P = 0.002).

In Hepa1c1c7 cells TR activity was induced by both Se and SF and this induction was greater when SF and Se were combined (Fig. 4). In contrast, GSH-Px was only increased by Se, whereas QR activity was only increased by SF. The additive, but not interactive, effect of Se and SF on TR activity of Hepa1c1c7 cells suggests that Se and SF act through separate mechanisms. Thioredoxin reductase activity is known to increase with supplemental Se *in vitro* [10] and *in vivo* [8] and is decreased *in vivo* in Se deficiency [9,22]. The selenoproteins GSH-Px and 5′ deiodinase are similarly regulated by Se [for review see 23].

Selenium deficiency has been reported to decrease TR mRNA in rat liver, possibly the result of increased nonspecific degradation of inactive TR mRNA [22]. It is possible

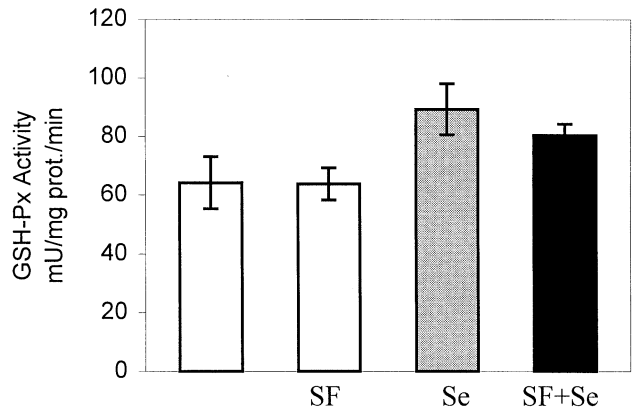

Fig. 5. The effect of purified sulforaphane and/or sodium selenite on Hepa1c1c7 mouse hepatoma cell glutathione peroxidase activity. Cells were exposed to:control αMEM media, 2.0 μM SF, 2.5 μM Se, or 2.0 μM SF + 2.5 μM Se. Values are means (n = 3) ± SD. Two-way ANOVA showed a significant treatment effect for selenite ($p < 0.009$), but not for SF (<0.29), and no interaction ($p < 0.33$).

that supplemental Se induces TR activity by increasing mRNA stability, while SF upregulates transcription of TR mRNA. More work is needed to determine the exact mechanism of this interaction. That GSH-Px was upregulated by Se while in the same cells, QR was unaffected is to be expected, since QR is not Se dependent.

Our finding that SF upregulates TR protein production is supported by western blotting that showed that animals treated with the highest dose of dietary SF tended to have the highest content of TR protein. All animals in the study were fed similar concentrations of dietary Se (~1 mg Se/kg), making it unlikely that Se was responsible for the activity and protein changes observed in TR and GSH-Px. However, a possible explanation for the SF-dependent decrease in GSH-Px is that enhanced expression of TR increased the demand for Se, such that GSH-Px expression became limited due to Se being sequestered for TR synthesis. This idea is consistent with earlier findings; Hill and colleagues demonstrated that TR in rats is more conserved than GSH-Px during Se deficiency [9]. Although the minimum Se requirement for the rat is 0.1 mg/kg, similar to the amount present in all of the diets used in this study, hepatic GSH-Px activity is known to fluctuate greatly when animals are fed diets containing Se concentrations between 0 and 0.1 mg/kg [22,23]. It has been postulated that GSH-Px may serve as a Se "buffer" or storage protein that provides Se to other pools [25]. It is possible that GSH-Px functioned in this capacity in the present study and provided Se for newly expressed TR.

The induction of TR by an electrophilic compound like SF, known to upregulate phase II detoxification enzymes, is not unprecedented. Both TR activity and protein content are increased in cultured mouse cortical astrocyte cells exposed to t-butylhydroquinone [11]. The authors postulated that the increase in TR activity and protein may be modulated through the ARE, as is the case for phase II enzymes such as QR and γ-glutamylcysteine synthetase. Our finding that TR upregulation correlated with urinary SF-M in a similar fashion to QR upregulation supports this hypothesis.

Examination of the TR promoter sequence [26] (accession number AF 247671) has revealed two possible ARE, on the non-coding strand from bp −57 to −49 and on the coding strand from bp −45 to −36. In the original report, several different transcription binding sites were identified by electrophoretic mobility shift assay (EMSA) but the presence of an ARE was not reported [26]. The region containing the putative AREs (−85 to −15) was reported to not have specificity for any transcription factors. Nuclear protein from A549 cells was used in the EMSA and it is possible that the A549 cells did not express active ARE binding transcription factors such as Nrf2, because the cells were probably not exposed to compounds triggering ARE transcription factor synthesis and/or binding. Thus, it is likely that the concentration of active ARE-specific transcription factors was too low to reveal binding at the putative ARE.

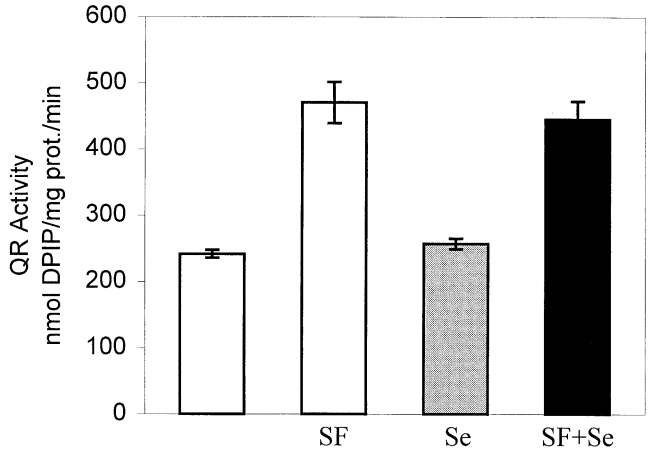

Fig. 6. The effect of purified sulforaphane and/or sodium selenite on Hepa1c1c7 mouse hepatoma cell quinone reductase activity. Cells were exposed to:control αMEM media, 2.0 μM SF, 2.5 μM Se, or 2.0 μM SF + 2.5 μM Se. Values are means (n = 3) ± SD. Two-way ANOVA showed a significant treatment effect for SF ($p < 0.0001$), but not for selenite (<0.69), and no interaction ($p < 0.14$).

The putative ARE, 5′-TGACAAAGC-3′ (−57 to −49, non-coding strand) contains both the complete consensus ARE sequence (5′-TGACnnnGC-3′) and the core ARE sequence (5′-TGAC-3′) which is most important for transcription factor binding [27,28]. It is the exact sequence of the GST-A2 ARE consensus (5′-TGACAAAGC-3′) [29]. The GST-A2 ARE is reported to be activated by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate [29] which compound has also been reported to induce TR, providing further evidence that an ARE may mediate TR expression [30]. The putative ARE 5′-TGACTCTGGC-3′(−45 to −36, coding strand) also contains the core 5′-TGAC-3′ sequence and has GGC in place of the GC that is part of the consensus sequence (5′-TGACnnnGC-3′).

Recently it was reported that thioredoxin, the primary substrate for reducing equivalents from TR, has a functional ARE that responds to Nrf2 transcription factors [31]. Thioredoxin reductase functions as a bridge for reducing equivalents going from NADPH to thioredoxin. Because thioredoxin is the primary substrate, it is logical that an increase in thioredoxin would necessitate a concerted increase of TR and concerted control could be accomplished if both genes were controlled by an ARE.

The inducibility of TR by SF may have unique implications for broccoli. Broccoli can accumulate large amounts of Se if grown in seleniferous soils [24]. Selenium added to the diet as high-Se broccoli has been demonstrated to be beneficial in the reduction of DNA adducts [32], aberrant colon crypts [33] and mammary tumors [34]. The primary bioactive glucosinolate breakdown product derived from broccoli is considered to be SF [35]. Sulforaphane has also been shown to reduce the formation of aberrant colon crypts [36] and has been implicated as a bioactive ingredient responsible increasing phase II enzyme expression in a number of studies [12].

Thioredoxin reductase seems to be at the crossroads of these two entirely different mechanisms of chemoprevention. Broccoli high in SF and Se may increase both phase II enzymes and increase TR activity. Previous studies using high-Se broccoli for chemoprevention have been efficacious in cancer models [24,34]. This benefit is undoubtedly an effect of Se and not SF, since these studies used very low levels of broccoli (0.3% of the diet) with a very high Se concentration (~750 mg Se/kg broccoli). To induce phase II enzymes, broccoli is typically included in rat diets at approximately 10–20%. However, it remains to be determined what role, if any, TR plays in anticarcinogenesis. As an antioxidant it may be beneficial; but as an activator of thioredoxin it may actually promote cell growth.

Notes

1. Keck, A.S., Qiao, Q., and Jeffery, E.H. *in preparation.*
2. Keck, A.S., Qiao, Q., and Jeffery, E.H. *in preparation.*

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