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Aqueous Extract from Spanish Black Radish (*Raphanus sativus* L. Var. *niger*) Induces Detoxification Enzymes in the HepG2 Human Hepatoma Cell Line

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Spanish black radish (*Raphanus sativus* L. var. *niger*) is a member of the Cruciferae family that also contains broccoli and Brussels sprouts, well-known to contain health-promoting constituents. Spanish black radishes (SBR) contain high concentrations of a glucosinolate unique to the radish family, glucoraphasatin, which represents >65% of the total glucosinolates present in SBR. The metabolites of glucosinolates, such as isothiocyanates, are implicated in health promotion, although it is unclear whether glucosinolates themselves elicit a similar response. The crude aqueous extract from 0.3 to 3 mg of dry SBR material increased the activity of the phase II detoxification enzyme quinone reductase in the human hepatoma HepG2 cell line with a maximal effect at a concentration of 1 mg/mL. Treatment of HepG2 cells with the crude aqueous extract of 1 mg of SBR per mL also significantly induced the expression of mRNA corresponding to the phase I detoxification enzymes: cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 as well as the phase II detoxification enzymes: quinone reductase, heme oxygenase 1, and thioredoxin reductase 1. Previous studies have shown that the myrosinase metabolites of different glucosinolates vary in their ability to induce detoxification enzymes. Here, we show that while glucoraphasatin addition was ineffective, the isothiocyanate metabolite of glucoraphasatin, 4-methylthio-3-butenyl isothiocyanate (MIBITC), significantly induced phase II detoxification enzymes at a concentration of 10 μM. These data demonstrate that the crude aqueous extract of SBR and the isothiocyanate metabolite of glucoraphasatin, MIBITC, are potent inducers of detoxification enzymes in the HepG2 cell line.

KEYWORDS: Spanish black radish; glucoraphasatin; MIBITC; detoxification; quinone reductase; glucosinolate; cruciferous

INTRODUCTION

A diet rich in fruits and vegetables, and in particular cruciferous plants, has been linked to a decrease in risk for many chronic diseases, including cancer (*1*). The chemopreventive properties of cruciferous plants have been linked to high concentrations of a class of phytochemicals referred to as glucosinolates. Upon tissue disruption (such as grinding or chewing), an enzyme also contained within the crucifer commonly called myrosinase (E.C. 3.2.1.147) metabolizes the glucosinolates into numerous breakdown products such as nitriles and isothiocyanates that are hypothesized to be the compounds that help prevent chronic disease. These glucosinolate breakdown products have been shown in both in vivo and in vitro models to induce detoxification enzymes (*2*−*4*) and also to inhibit carcinogenesis in some models (*5*−*7*). In fact, induction of detoxification enzymes has been suggested as the mechanism through which cruciferous vegetable consumption, or the consumption of the glucosinolate breakdown products, reduces cancer risk (*8*, *9*).

Radishes belong to the Cruciferae family and include *Raphanus sativus* L. var. *niger* commonly known as Spanish black radish (SBR). Compared to other crucifers, SBR is a high-yield, non-labor-intensive, pest-resistant crop with a short growing season, which makes it a good whole food source of glucosinolates. In general, SBR has a high total glucosinolate content compared with other cruciferous vegetables (*10*). A particular glucosinolate purified and identified over a decade ago, 4-methylthio-3-butenyl or glucoraphasatin (also known as glucodehydroerucin) (*11*) (**Figure 1**), constitutes the vast majority of the glucosinolates present in SBR (*10*) and all other varieties of radish.

Since radishes are the only dietary source of significant levels of glucoraphasatin, consumption of radishes represents the only significant route of exposure to glucoraphasatin. In terms of this exposure, 141 million pounds of radish was consumed annually in the United States between 2001 and 2003 (*12*); this is in comparison to the most commonly consumed crucifer,

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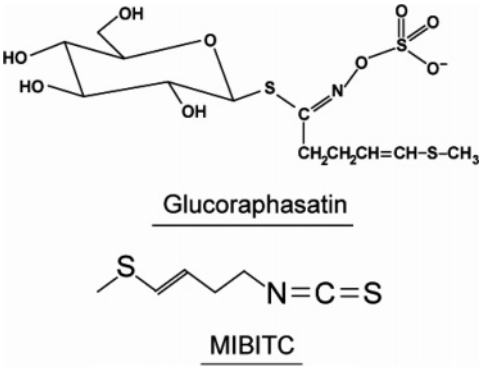

Figure 1. Chemical structures of the primary glucosinolate found in Spanish black radish, glucoraphasatin, and the isothiocyanate metabolite, MIBITC, produced from its myrosinase-mediated metabolism.

broccoli, for which 1.65 billion pounds of broccoli was used in the United States in 1999 (*13*). However, radishes are a far more popular vegetable in other countries, and in fact the daikon, a large white radish that also contains glucoraphasatin as a vast majority of the glucosinolates present, is one of the most commonly consumed vegetables in Japan (*14*, *15*).

Previous studies have demonstrated the antioxidant (*16*−*19*) and antimutagenic properties (*20*) of radishes and the phytochemicals derived from radishes, and recently there have also been published reports of radishes increasing detoxification enzyme activity (*21*, *22*). The high glucosinolate concentration found in SBR suggests the possibility that it may be a potent inducer of detoxification enzymes when individuals are exposed to the food through the diet. Therefore, while consumption of radishes is approximately 10-fold less than broccoli consumption, research demonstrating additional health benefits of radishes could stimulate increased consumption of this cruciferous vegetable in the United States.

The majority of the research on cruciferous vegetables has typically focused on the activity of the most common cruciferous vegetables, such as broccoli and Brussels sprouts, and the specific glucosinolate metabolites derived from these vegetables, such as sulforaphane and indole-3-carbinol (*23*, *24*). Many of the glucosinolate metabolites induce detoxification enzymes, however, differences in their ability to induce phase I and II enzymes have been described (*25*, *26*), and thus one cannot assume that novel isothiocyanates such as the 4-methylthio-3-butenyl isothiocyanate (MIBITC) found in radish would have the same ability to induce detoxification enzymes as the more common isothiocyanates. Therefore, we have examined the effect of SBR extract and the unique glucosinolate and isothiocyanate found in radish on the expression of several phase I and phase II detoxification enzymes.

The unique glucosinolate profile of radishes represents an opportunity to identify foods with unique health-promoting attributes. The objective of these experiments was to determine whether the aqueous extract of a less common, but still readily available, cruciferous vegetable and the primary isothiocyanate, MIBITC, found in radishes are able to induce the expression of detoxification enzymes, neither of which has previously been determined.

MATERIALS AND METHODS

Materials. Plastic ware and general lab supplies used were purchased from Fisher Scientific (Hampton, NH). Chemicals and reagents, including cell culture media, were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Spanish black radishes, *Raphanus sativus* L. var. *niger*, were grown from seeds obtained from Fedco Seeds (Waterville, ME) on the Standard Process farm (Palmyra, WI).

Cell Culture. HepG2 cells at passage 76 were obtained from ATCC (Manassas, VA) and were cultured in Modified Eagle Media (Sigma; St. Louis, MO) supplemented with 1 mM sodium pyruvate (Invitrogen; Carlsbad, CA), 100 μM nonessential amino acid solution (Invitrogen; Carlsbad, CA), 100 units of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (Atlanta Biologicals; Norcross, GA). Cultured cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. HepG2 cells used in the experiments were between passage number 80 and 88.

Preparation of Spanish Black Radish Extracts for Cell Culture Experiments. SBRs were frozen whole and then were freeze-dried. Freeze-dried SBR material was then ground with a coffee grinder, and aqueous extracts were then produced from these dried materials. Five grams of dried material was extracted with 200 mL of water at 25 °C overnight, resulting in complete hydrolysis of all glucosinolates (as determined by high-performance liquid chromatography (HPLC) analysis of glucosinolates). Samples were centrifuged to pellet unextracted material, and the supernatant was filtered through a 0.45 μm filter before being frozen and lyophilized. One gram of freeze-dried SBR produced 423.4 ± 33.7 mg of extracted material, and thus 1 mg of freeze-dried SBR produced 423.4 μg of extracted material. For the cell culture experiments, cells were treated with the amount of extract that corresponded to between 0.1 and 10 mg of freeze-dried SBR plant per mL of treatment media. Thus, cells treated with 1 mg of SBR/mL of media received 423.4 μg of extract/mL of media.

Quinone Reductase Activity. Quinone reductase activity was measured as previously described (*2*). HepG2 cells were cultured in 12-well plates. Confluent cells were treated for 2 days with media containing crude SBR extract, isolated glucoraphasatin or MIBITC, in addition to media containing the same amount of water as a vehicle control. For each independent experiment, three wells of cells were exposed to each treatment, and each well then represented one replicate for the quinone reductase assay. After treatment was completed, media was removed and cells were washed twice with 1 mL of ice-cold phosphate-buffered saline (PBS). Cells were scraped into a cell harvest solution (25 mM Tris-HCl pH 7.4; 125 mM sucrose) and then were sonicated for 10 s. Protein concentration was determined using the Pierce BCA kit, following manufacturer's protocol (Pierce Biotechnology; Rockford, IL). For measurement of quinone reductase activity, 1 μg of protein was added to a 96-well plate and 200 μL of quinone reductase assay solution was added (25 mM Tris-HCl pH 7.4; 60 μg/mL BSA; 0.01% Tween 20; 5 μM flavin adenine dinucleotide; 80 μM 2,6-dichloroindophenol). Absorbance was then monitored at 600 nm over the course of 2 min in the absence and the presence of 30 μM dicumarol (a specific inhibitor of quinone reductase). Quinone reductase activity was calculated as the change in absorbance per minute per microgram of total protein in the absence of dicumarol minus the change in absorbance per minute per microgram of total protein in the presence of dicumarol.

Cell Viability. HepG2 cells were cultured in 96-well plates. Confluent cells were then treated for 2 days with media containing crude SBR extract. Four replicate wells received 100 μL of each treatment media. After 2 days, treatment media was then replaced with fresh, control media before assay was performed as per manufacturer's protocol (Promega CellTiter 96 Non-Radioactive Cell Proliferation Assay; Madison, WI).

Real-Time Quantitative PCR. HepG2 cells were cultured in six-well plates. Confluent cells were treated for 2 days with media containing crude SBR extract, isolated glucoraphasatin or MIBITC, in addition to media containing the same amount of water as a vehicle control. For each independent experiment, three wells of cells were exposed to each treatment. For each sample, total RNA was collected from one well of a six-well plate per manufacturer's protocol using the RNAqueous-4PCR kit (Ambion; Austin, TX). RNA concentrations were determined via a spectrophotometer (NanoDrop; Wilmington, DE). cDNA was generated using 2 μg of total RNA per 20 μL cDNA reaction with the Applied Biosystems High Capacity cDNA Archive Kit following manufacturer's protocol (Foster City, CA). Primer/probe kits for cytochrome P450 (CYP)1A1 (Hs00153120_m1), CYP1A2

(Hs00167927_m1), CYP1B1 (Hs00164383_m1), CYP3A4 (Hs00430021_m1), quinone reductase (Hs00158547_m1), heme oxygenase 1 (Hs00157965), thioredoxin reductase 1 (Hs00182418_m1), and 18s ribosomal RNA (Hs99999901_s1) used for real-time PCR quantitation of RNA were obtained from Applied Biosystems (Foster City, CA). Each treatment was represented by triplicates and each PCR reaction was carried out in duplicate. PCR was performed on an ABI 7300 Sequence Detection System. Each 25 μL reaction contained 1 μL of cDNA, 12.5 μL of 2 X Taqman Universal PCR Mastermix (Applied Biosystems; Foster City, CA), and 1.25 μL of the primer/probe reaction mixture (for the 18s ribosomal RNA reaction, the primer/probe mixture was diluted 1:6 before 1.25 μL was added). All reactions were run with the following parameters: 2 min at 50 °C and then 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fold changes between treatment groups were then determined using the reaction efficiency (*27*) and 18s ribosomal RNA as the reference gene: ratio = $(E_{\text{target}})^{\Delta CP}$target(control sample)/$(E_{\text{ref}})^{\Delta CP}$ref-(control sample).

HPLC Quantitation of Glucosinolate Concentration. Glucosinolate concentrations were measured using a previously described method (*28*, *29*). Two hundred milligrams of dry radish material was extracted two times with 2 mL of boiling 70% methanol. During extraction, samples were incubated at 95 °C and were vortexed every minute. Before the second extraction, 500 μL of benzylglucosinolate (1 mM) was added as an internal standard. After extractions, samples were centrifuged at 1200*g* at 10 °C for 15 min, and the supernatant was transferred to a new tube. Next, 1 mL of the pooled supernatants was transferred to a new tube, and 150 μL of 0.5 M barium acetate/0.5 M lead acetate was added. The sample was vortexed and applied to a column with 100 mg of A-25 Sephadex that had been conditioned with 5 mL of 0.5 M NaOH, 5 mL of 0.5 M pyridine acetate, and 5 mL of water. After the sample was allowed to drip through the column, the column was washed with 3 mL of 0.02 M pyridine acetate and then with 3 mL of water. Then, 500 μL of a 20 U/mL sulfatase solution was allowed to penetrate the column, and the column was incubated overnight at room temperature. The next day, glucosinolates were eluted off the column with 2 mL of water followed by an additional 1 mL of water. Samples were then filtered before 40 μL was injected for HPLC analysis. Samples were analyzed with an Agilent 1100 HPLC (Palo Alto, CA) with a 250 mm Alltech C18 column, 5 μm pore size and 4.6 mm i.d. Desulfoglucosinolates were eluted from the column at a flow rate of 0.8 mL/min with a linear gradient of 0−20% acetonitrile in water up to 53 min and then with an additional 7 min with a linear gradient of 20−0% acetonitrile. The amount of glucoraphasatin was determined using relative response factors (*30*, *31*).

HPLC Quantitation of Total Isothiocyanate Concentration. For the analysis of total ITCs in the extracts used in the cell culture experiments, lyophilized extract was resuspended in water at a concentration of 250 mg of lyophilized extract per mL. Total ITCs were measured as previously described (*32*). Fifty microliters of the extract solution was combined with 450 μL 100 mM potassium phosphate buffer and 500 μL of 8 mM 1,2-benzenedithiol in an HPLC vial. Vials were then capped given a nitrogen headspace and were incubated at 65 °C for 2 h. Ten microliters of the samples was then injected directly onto an RP-C18 column (Vydac, 201TP54, 250 × 4.5 mm id, 10 μm). The mobile phase was 80:20 MeOH:H_2O with a flow rate of 2.0 mL/min. The cyclocondensation product 1,3-benzodithiole-2-thione eluted at approximately 2.7 min with the detection wavelength set at 365 nm. An Agilent 1100 LC (Palo Alto, CA) was used for analysis. Serial dilutions of a 10 μM R-sulforaphane (LKT Laboratories, St. Paul, MN) stock solution were used to create a linear standard; total ITC units were expressed as μmol sulforaphane equivalence/g sample.

Isolation of Pure Glucoraphasatin. Glucoraphasatin (**Figure 1**), the primary glucosinolate in Spanish black radish, was isolated from mature radishes using a previously described method (*18*). Starting with ∼30 g of material, dry Spanish black radish was extracted twice with 500 mL of boiling 70% ethanol for 30 min. Samples were centrifuged to pellet unextracted material, and supernatant was filtered with a 0.45 μm filter. Supernatant was then loaded onto a 25 × 200 mm A-25 Sephadex prepatory column that had been preconditioned with 400 mL of 25 mM sodium acetate pH 5.6. After extract had been allowed to flow through the Sephadex column by gravity, column was washed in succession at 4 mL per minute with 500 mL of 25 mM sodium acetate pH 5.6, with 500 mL of formic acid:isopropanol:water (3:2:5), and then with 500 mL of 25 mM sodium acetate pH 5.6. Glucosinolates were then eluted off of the column in 50 mL fractions with 500 mL of 25 mM K_2SO_4 and then with 350 mL of 50 mM K_2SO_4 at a rate of 4 mL/min. Fractions containing glucoraphasatin, as indicated by HPLC analysis, were freeze-dried and then were pooled into 10 mL of water. Salts were precipitated with 10 mL of ice-cold 100% ethanol and then were pelleted by centrifugation. Supernatant was then lyophilized. Resulting material was resuspended in water at 400 mg/mL before being applied 2 mL at a time to a 25 × 200 mm prepatory column filled with G-10 Sephadex. Glucosinolates were eluted off the G-10 column with water at 2 mL/min, collecting 60 1-min fractions. Fractions 25−45 typically contained the majority of glucosinolates as determined by absorbance at 254 nm. Fractions containing only glucoraphasatin as indicated by HPLC analysis were then pooled together and were lyophilized to produce isolated glucoraphasatin.

Isolation of Pure MIBITC. 4-Methylthio-3-butenyl isothiocyanate (MIBITC) is the isothiocyanate product produced by the myrosinase-mediated metabolism of glucoraphasatin (**Figure 1**). MIBITC was produced through metabolism of isolated glucoraphasatin, obtained according to the method above, with myrosinase (E.C. 3.2.1.147; Sigma; St. Louis, MO). A solution was made consisting of 10 mM glucoraphasatin and 667 mU of myrosinase in 100 mM potassium phosphate pH 6.5, and this solution was incubated at room temperature for 20 min. These reaction conditions have been optimized for ITC production on the basis of previous literature (*33*). After incubation, MIBITC was extracted from this solution with 1 volume of methylene chloride. Methylene chloride was then dried off of the MIBITC with a speed vac, and residue was resuspended in ddH_2O. The concentration of MIBITC in this solution was then measured by gas chromatography according to the following method.

Gas Chromatography (GC) Quantitation of MIBITC. For quantitation of MIBITC in the solutions used in the cell culture experiments, a 1:100 dilution of the solutions was made with ddH_2O. To 1 mL of this dilution, 20 μL of benzyl isothiocyanate (BITC) was added as the internal standard (0.1 g/mL) as well as an additional 480 μL methylene chloride. Samples were vortexed immediately and then were centrifuged to separate solvent and aqueous fractions. Solvent fractions were filtered before analysis using GC/FID as previously described (*34*). Samples were analyzed with an Agilent 6890N GC (Palo Alto, CA) according to the following specifications: injector and detector temperatures were 200 °C and 280 °C, respectively; oven temperature started at 40 °C for 2 min, then increased 10 °C/min from 40 °C to 260 °C, and remained at 260 °C for 10 min; head pressure was 25 psi; and injection volume was 1 μL. An HP-5MS capillary column (J&W Scientific; Folsom, CA) was used for separation (30 m, 0.25 mm i.d., 0.25 μm film thickness). Concentrations of MIBITC were calculated using purified erucin (LKT Laboratories; St. Paul, MN) as a standard since the structure of erucin is very similar to MIBITC (the only difference in structure is that glucoraphasatin has a double bond that is absent in erucin) and since these ITCs elute within 1 min of each other under these chromatographic conditions. The GC was calibrated using standard curves of 0.025−1 mg/mL BITC and erucin in methylene chloride. The identity of MIBITC was confirmed with mass spectrometry.

Statistical Analysis. Each result is presented as mean ± standard deviation. The statistical difference between treatments was determined by analysis in GraphPad Prism (San Diego, CA) by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test with $p < 0.05$ considered statistically different.

RESULTS

Crude Aqueous Extract from Spanish Black Radish Induces Phase I and II Detoxification Enzyme Expression. Like all cruciferous plants, Spanish black radish (SBR) contains several different glucosinolates (**Table 1**; expressed as μmol of glucosinolate per gram of radish dry weight). Unlike many other crucifers, a single glucosinolate, glucoraphasatin, repre-

Table 1. Glucosinolate Content of Spanish Black Radish

glucosinolate	acronym	concentration (μmol/g d.w.)
glucoraphenin	GRE	3.92 ± 1.16
glucoerucin	GER	1.06 ± 0.22
glucoraphasatin	GRH	29.70 ± 5.10
glucobrassicin	GBS	0.97 ± 0.59

sents a majority of glucosinolates present (~65% in the samples that were analyzed; **Figure 2**). However, glucosinolates are only one of the many classes of phytochemicals present in SBR.

Crude aqueous extracts from SBR were prepared by extracting freeze-dried SBR material overnight with water, allowing for complete hydrolysis of glucosinolates (confirmed by HPLC analysis). Measurement of the total ITCs present in the extract indicated that there was 5.76 ± 0.05 nmol of total ITCs per 1 mg (or 5.76 μmol/g) of freeze-dried SBR material. Total ITCs, as opposed to individual ITCs, were measured for the crude extract to account for metabolites of all the glucosinolates present in SBR.

Treatment of the human, hepatoma cell line HepG2 for 2 days with 0.3, 0.6, 1, and 3 mg/mL of dry SBR material resulted in significant induction of quinone reductase activity over cells treated with vehicle (water) alone (**Figure 3A**). This amount of extract corresponded to ~1.5 to ~15.5 μM of total ITCs (**Table 2**). Concentrations of SBR lower than 0.3 mg/mL did not significantly increase quinone reductase activity. Concentrations of SBR of 6 mg/mL and 10 mg/mL decreased cell viability by 45 and 90%, respectively, as measured by MTT activity (**Figure 3B**), which likely explains why these higher concentrations failed to increase quinone reductase activity.

Treatment of the HepG2 cell line for 2 days with 1 mg/mL of SBR significantly increased the amount of mRNA over cells treated with vehicle (water) alone for the phase I detoxification enzymes cytochrome P450 (CYP)1A1, CYP1A2, and CYP1B1 (**Figure 4A**) and for the phase II detoxification enzymes quinone reductase, heme oxygenase 1, and thioredoxin reductase (**Figure 4B**). Treatment with all concentrations of SBR failed to induce mRNA for CYP3A4, another phase I detoxification enzyme that is regulated through a different mechanism than the other CYPs.

Increased Quinone Reductase Activity Persists Even after Radish Extract Is Removed. Quinone reductase activity was determined in cells treated continuously with 1 mg/mL SBR for 11 days (continuous; **Figure 4**) and in cells which have been treated with 1 mg/mL SBR for 4 days before being switched to control media for an additional 7 days (washout). Cells that were continuously exposed to media containing SBR exhibited increasing quinone reductase activity throughout the entire 11

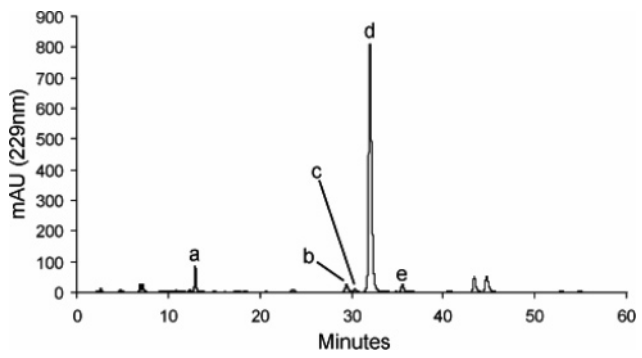

Figure 2. HPLC chromatogram of desulfoglucosinolates from Spanish black radish. Typical HPLC chromatogram of desulfoglucosinolates in Spanish black radish. Peaks: **a**, glucoraphenin; **b**, benzylglucosinolate (internal standard); **c**, glucoerucin; **d**, glucoraphasatin; **e**, glucobrassicin.

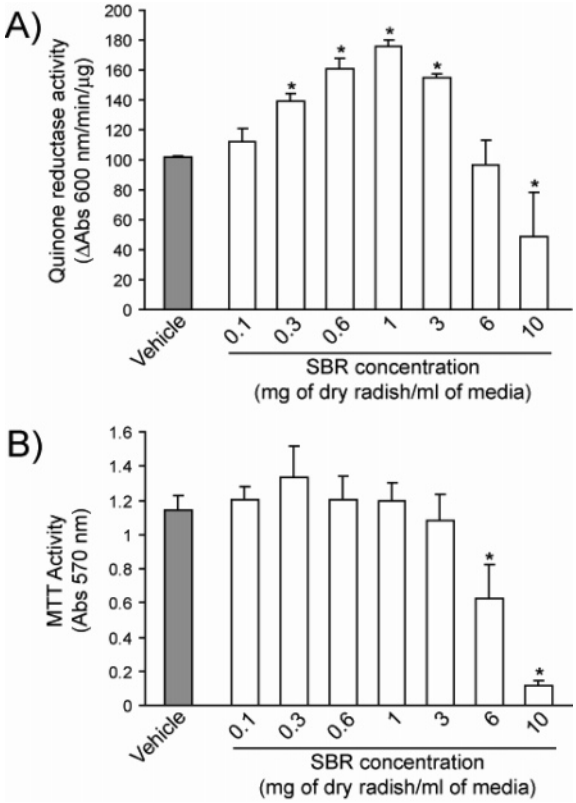

Figure 3. The effect of aqueous extract from Spanish black radish on quinone reductase activity and cell viability. HepG2 cells were treated for 2 days with the amount of aqueous extract corresponding to 0.1, 0.3, 0.6, 1, 3, 6, and 10 mg of dry whole Spanish black radish per mL of media. Data are representative of three independent experiments, using independent cell cultures, all of which produced similar results. (**A**) Quinone reductase data represent the average of three samples ± standard deviation. * = significantly different from vehicle (ANOVA, Tukey's post-hoc). (**B**) Cell viability data represent the average of four samples ± standard deviation. * = significantly different from vehicle (ANOVA, Tukey's post-hoc).

Table 2. Nanomoles of Total ITC per mL of Treatment Media

treatment concn (mg/mL)	nmol ITC per mL (μM)
10	57.59
6	34.55
3	17.28
1	5.76
0.6	3.46
0.3	1.73
0.1	0.58

days of treatment, and activity in these cells was significantly higher than in cells treated with vehicle (water) alone from day 2 to 11 (**Figure 5**). Cells that had the SBR removed on day 4 (washout) exhibited significantly higher quinone reductase activity than cells treated with vehicle (water) alone on treatment day 6, 2 days after being switched into control media. However, these cells had significantly less quinone reductase activity than cells treated continuously with SBR on treatment days 6−11. In fact, by treatment day 8 the quinone reductase activity in washout cells was no longer significantly greater than in vehicle-treated cells.

MIBITC, the Isothiocyanate Metabolite of Glucoraphasatin Can Induce Quinone Reductase Activity. Pure glucoraphasatin was isolated from freeze-dried SBR material using open column chromatography. MIBITC was produced from that

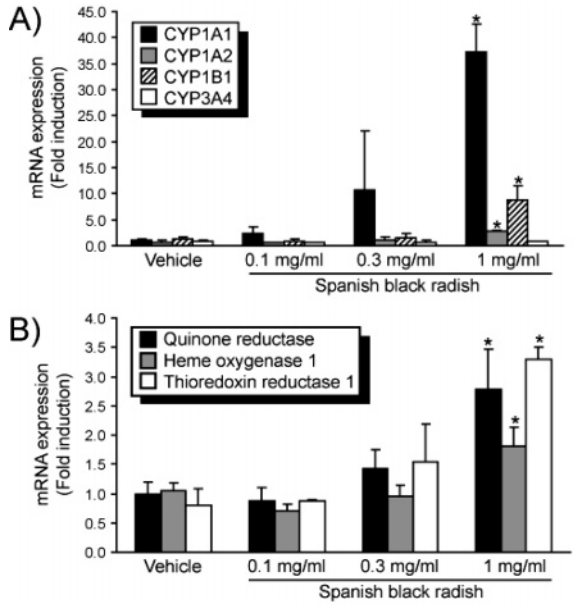

Figure 4. The effect of aqueous extract from Spanish black radish on the mRNA expression of phase I and II detoxification enzymes. HepG2 cells were treated with the amount of aqueous extract corresponding to 0.1, 0.3, and 1 mg of dry whole Spanish black radish per mL of media for 2 days. Data are representative of three independent experiments, using independent cell cultures, all of which produced similar results. Data represent mRNA expression as fold induction over vehicle for three samples ± standard deviation. * = significantly different from vehicle (ANOVA, Tukey's post-hoc). (**A**) Fold induction of phase I detoxification genes. (**B**) Fold induction of phase II detoxification genes.

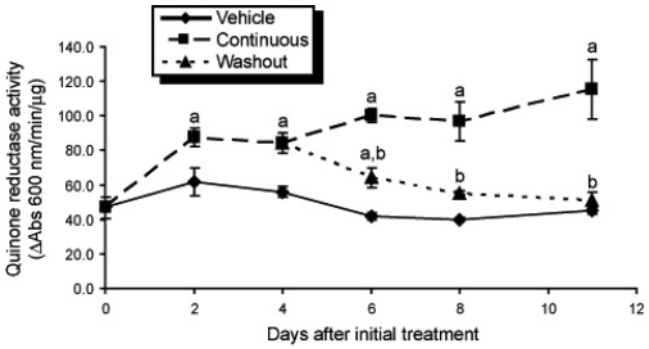

Figure 5. Measurement of quinone reductase activity in HepG2 cells treated with aqueous Spanish black radish extract up to a week after removal of the extract. HepG2 cells were treated as described in the Results. Cells were either continuously treated with SBR extract (continuous, squares and dashed line) or for only the first 4 days of treatment (washout, triangles and dotted line). Data represent the average of three samples ± standard deviation. **a** = significantly different from control (ANOVA, Tukey's post-hoc), **b** = significantly different from continuous (ANOVA, Tukey's post-hoc). Data are representative of three independent experiments, using independent cell cultures, all of which produced similar results.

material by metabolism with pure myrosinase enzyme, as described in Materials and Methods. Treatment of HepG2 cells for 2 days with 1−30 μM glucoraphasatin had no affect on quinone reductase activity (**Figure 6**). However, treatment with the same concentrations of MIBITC increased quinone reductase activity in the cells with significant induction over vehicle (water) control and glucoraphasatin-treated cells at all concentrations above 4 μM. MIBITC-mediated induction of quinone reductase activity was similar to that seen with sulforaphane,

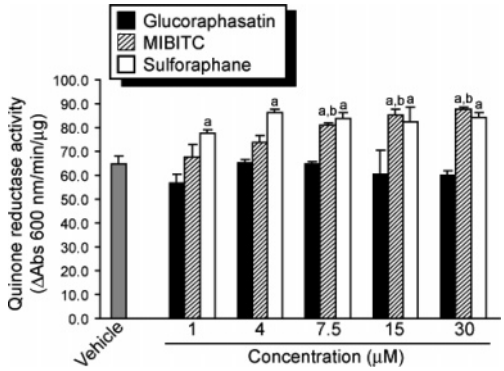

Figure 6. Effect of glucoraphasatin and MIBITC on quinone reductase activity. HepG2 cells treated for 2 days with media containing vehicle (water) or the indicated concentrations of glucoraphasatin (black bars), MIBITC (striped bars), or sulforaphane (white bars). Data represent the average of three samples ± standard deviation. **a** = significantly different from cells treated with vehicle (water) alone (ANOVA, Tukey's post-hoc), **b** = significantly different from glucoraphasatin at the same concentration (ANOVA, Tukey's post-hoc). Data are representative of three independent experiments, using independent cell cultures, all of which produced similar results.

which was run in parallel. Sulforaphane-mediated induction of quinone reductase activity in these experiments was very similar to previously reported values from another laboratory (*2*). No concentrations of glucoraphasatin, MIBITC, or sulforaphane up to 30 μM significantly affected cell viability as assayed by MTT activity (data not shown).

On the basis of the results from the dose−response experiment (**Figure 6**), another set of HepG2 cells was treated for 2 days with vehicle (water) control or 10 μM concentrations of either glucoraphasatin (GRH), MIBITC, or sulforaphane (SUL). Treatment with 10 μM glucoraphasatin did not result in significant changes in the expression of any of the seven detoxification enzymes measured (**Figure 7**). Treatment with 10 μM sulforaphane resulted in significant induction of mRNA corresponding to CYP1B1, quinone reductase, thioredoxin reductase, and heme oxygenase 1 as well as a significant reduction in the amount of mRNA corresponding to CYP1A1 and CYP1A2. Treatment with 10 μM MIBITC resulted in induction of quinone reductase and thioredoxin reductase that was significantly greater than both vehicle and glucoraphasatin and significantly reduced the expression of CYP1A2 from both vehicle and glucoraphasatin-treated levels.

DISCUSSION

Cruciferous plants, and their phytochemicals, have been shown to induce detoxification enzyme activity (*2*−*7*). The majority of the research in this area, however, has focused on only a fraction of the compounds present in the many cruciferous varieties. In this paper, we focus on a unique crucifer, Spanish black radish (SBR), and glucoraphasatin, a glucosinolate only present at significant levels in members of the radish family. To better reflect the response from consumption, initial experiments were performed using crude aqueous extracts of SBR which contained the complex mixture of water-soluble components present in the plant. These experiments were followed by further characterization of specific chemical constituents, glucoraphasatin and MIBITC, and of how these constituents relate to biological action.

The initial experiments examining the biological activity of a crude SBR extract allowed for the contribution of other

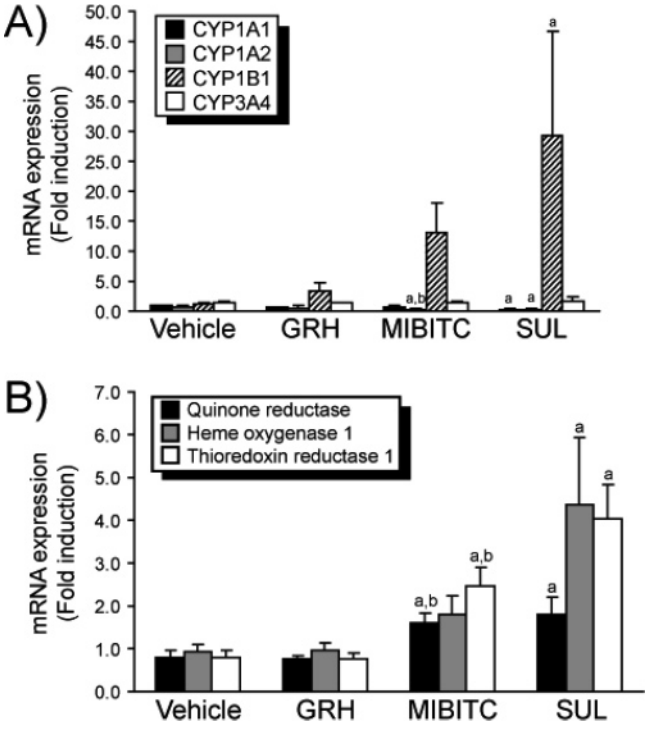

Figure 7. Effect of glucoraphasatin and MIBITC on phase I and phase II detoxification enzyme mRNA expression. HepG2 cells treated for 2 days with media containing vehicle (water) or 10 μM glucoraphasatin (GRH), 10 μM MIBITC, or 10 μM sulforaphane (SUL). Data are representative of three independent experiments, using independent cell cultures, all of which produced similar results. Data represent mRNA expression as fold induction over vehicle for three samples ± standard deviation. **a** = significantly different from vehicle (ANOVA, Tukey's post-hoc), **b** = significantly different from GRH (ANOVA, Tukey's post-hoc). (**A**) Fold induction of phase I detoxification genes. (**B**) Fold induction of phase II detoxification genes.

phytochemicals present in SBR toward the induction of detoxification enzymes in either a positive or negative manner. Despite any contribution of other phytochemicals present in the crude SBR extract, the concentration range of total ITCs present in the crude extract (**Table 2**, **Figure 3A**) that produced significant induction of quinone reductase activity was similar to the concentrations of pure sulforaphane and MIBITC that significantly induced quinone reductase activity (**Figure 6**).

However, there was a discrepancy between the amount of glucosinolates present in freeze-dried SBR (>30 nmol/mg) and the amount of total ITCs present after overnight hydrolysis (5.76 nmol/mg). HPLC analysis demonstrated that all glucosinolates were metabolized after overnight hydrolysis; therefore, incomplete myrosinase metabolism of glucosinolates cannot explain this discrepancy. Other possible explanations include the possibility that the glucosinolates from SBR were metabolized to non-ITC products and the possibility that the ITCs produced were further metabolized.

The myrosinase metabolites of different glucosinolates vary in their ability to affect the expression of phase I and phase II detoxification enzymes (*25*, *26*). It was therefore important to measure the effect of aqueous SBR extract and MIBITC on the expression of enzymes in both pathways. The aqueous extract from SBR at 1 mg/mL was able to significantly increase the mRNA expression of the phase I and II detoxification enzyme quinone reductase (**Figure 4**), while pure MIBITC only resulted in significant increases in the expression of phase II detoxification enzymes (**Figure 7A**, **7B**).

Regulation of many phase I and II enzymes occurs through well-documented mechanisms, including the aryl hydrocarbon receptor (AhR) (*35*) and the NF-E2-related factor 2 (Nrf2) (*36*) pathways. Induction of CYP1A1, CYP1A2, CYP1B1, and quinone reductase is at least partially controlled through activation of the AhR by exogenous ligands (*37*−*40*), all of which are induced by crude SBR extract. The other phase I enzyme, CYP3A4, examined here is not induced through activation of the AhR, and in fact CYP3A4 was not induced by crude SBR extract. These data suggest that crude SBR extract may be activating the AhR in HepG2 cells. Induction of quinone reductase, heme oxygenase 1, and thioredoxin reductase 1 is at least partially controlled by Nrf2 (*41*−*43*), and thus these data also indicate that crude SBR extract and MIBITC may be activating Nrf2. However, in both cases more research is needed to confirm that these pathways are being activated by the phytochemicals within SBR.

CYP1B1 differed from the other phase I detoxification enzymes in that it was significantly induced by treatment with 10 μM sulforaphane and was upregulated (although not significantly) by 10 μM MIBITC. CYP1B1 is expressed at very low amounts in HepG2 cells relative to the other CYPs, and thus the relevance of this response is questionable. Expression of CYP1B1 has been shown to be controlled by the AhR similar to CYP1A1 and CYP1A2 (*37*). This would explain how the crude SBR extract induced CYP1B1 as well as the other CYPs (**Figure 4A**), however, it would not explain why CYP1B1 was induced by MIBITC and SUL (**Figure 7A**) while the other CYPs were not. More recently, it has been demonstrated that CYP1B1 expression can also be modulated through a cAMP-dependent mechanism (*44*), and therefore there may be a mechanistic reason why CYP1B1 is regulated differently than the other CYPs. Yet, more experiments are needed to demonstrate that sulforaphane and MIBITC are acting through this alternative mechanism to induce CYP1B1.

The induction of phase I detoxification enzymes causes concern because of the formation of reactive intermediates from chemicals which could lead to increased toxicity, a situation for which there are many examples (*45*). However, the coordinated upregulation of both phase I and II enzymes could provide the best protection against toxic insult because both phases are needed for the most efficient elimination of many toxic compounds. Yet, even an extensive study of the effects of radish extract on the expression of numerous detoxification enzymes in multiple cell lines would be unlikely to predict the overall benefit/detriment of the relationship between phase I and II enzymes in an in vivo model. For example, the issue of bioavailability would not be addressed.

In a previous study, Keck et al. demonstrated that Fischer 344 rats fed a diet that consisted of 20% broccoli, which had a glucoraphanin concentration of 11.0 μmol/g, for 5 days had elevated quinone reductase activity in the liver and colon (*46*). Since MIBITC demonstrated a similar potency as sulforaphane in terms of induction of quinone reductase activity (**Figure 6**), it is possible that consumption of SBR containing an equivalent amount of glucoraphasatin would produce a similar induction of detoxification enzymes in vivo.

There are many reasons why SBR is an attractive whole food source of bioactive glucosinolates in terms of human dietary consumption. The high concentration of what we have demonstrated in this paper to be biologically active components in SBR suggests that the consumption of a much lower amount of SBR, in comparison with broccoli or other crucifers, would result in the same biological effect. However, crude SBR extracts

produced a different effect on detoxification enzyme expression than isolated MIBITC. Thus, the benefit derived from a diet containing crucifers may be more complex than simply understanding the induction of detoxification enzymes by these individual metabolites. The persistence of the induction of detoxification enzymes (**Figure 5**) after exposure to SBR extract is also important in terms of dietary exposure, especially if SBR is not consumed on a regular basis. Additionally, from a production standpoint, compared to other crucifers, SBR is a high-yield, low-labor-intensive, and pest-resistant crop with a short growing season.

Dietary exposure to radishes, because at least in part of the biological activity of MIBITC, may increase the expression of both phase I and II detoxification enzymes, although additional in vivo studies must be performed to confirm this. This has significant consequences for populations such as in Japan (*14*, *47*) where consumption of radishes is very common but also represents an opportunity for populations where radish consumption is low to increase their detoxification enzyme expression by means of dietary intervention.

ABBREVIATIONS

CYP, cytochrome P450; ITC, isothiocyanate; MIBITC, 4-methylthio-3-butenyl isothiocyanate; GRH, glucoraphasatin; SBR, Spanish black radish.

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