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Sulforaphane and erucin increase MRP1 and MRP2 in human carcinoma cell lines

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Abstract

Multidrug resistance (MDR) transporters have been termed the Phase III detoxification system because they not only export endogenous metabolites but provide protection from xenobiotic insult by actively secreting foreign compounds and their metabolites from tissues. However, MDR overexpression in tumors can lead to drug resistance, a major obstacle in the treatment of many cancers, including lung cancer. Isothiocyanates from cruciferous vegetables, such as sulforaphane (SF) and erucin (ER), are known to enhance the expression of Phase II detoxification enzymes. Here we evaluated the ability of SF and ER to modulate MDR mRNA and protein expressions, as well as transporter activity. The expression of P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1) and multidrug resistance protein 2 (MRP2) in liver (HepG2), colon (Caco-2) and lung (A549) cancer cells treated with ER or SF was analyzed by Western blotting. Neither SF nor ER affected P-gp expression in any of the cell lines tested. Both SF and ER increased the protein levels of MRP1 and MRP2 in HepG2 cells and of MRP2 in Caco-2 cells in a dose-dependent manner. In A549 lung cancer cells, SF increased MRP1 and MRP2 mRNA and protein levels; ER caused a similar yet smaller increase in MRP1 and MRP2 mRNA. In addition, SF and ER increased MRP1-dependent efflux of 5-carboxyfluorescein diacetate in A549 cells, although again the effect of SF was substantially greater than that of ER. The implication of these findings is that dietary components that modulate detoxification systems should be studied carefully before being recommended for use during chemotherapy, as these compounds may have additional influences on the disposition of chemotherapeutic drugs.

Keywords: Sulforaphane; Erucin; Multidrug resistance

1. Introduction

Increased consumption of fruits and vegetables has been repeatedly linked to reduced risk for many cancers, including prostate, breast, lung and colon cancers [1]. Of the fruits and vegetables studied, cruciferous vegetables are often found to offer substantially greater chemoprotective benefits than other plant foods [2,3]. A great deal of research regarding crucifers has focused on the isothiocyanate sulforaphane [isothiocyanato-4-(methylsulfinyl)-butane or SF]. SF is a hydrolysis product of glucoraphanin, the primary glucosinolate found in broccoli. Best known for the up-regulation of several Phase II detoxification enzymes, SF has been shown to augment the cell's defense systems against carcinogenesis through multiple pathways, including inhibition of the cell cycle and induction of apoptosis [4,5]. The isothiocyanate erucin [1-isothiocyanato-4-(methylthio)-butane or ER] is the reduced analog of SF. It is formed both from hydrolysis of glucoerucin (the predominant glucosinolate in arugula) and reduction of SF in vivo. Approximately 12% of an SF dose was recovered as the *N*-acetyl conjugate of ER in the urine of rats administered a single intraperitoneal dose of SF (50 mg/kg); 67% of an ER dose was recovered as SF conjugate [6]. ER has a potency similar to that of SF for a number of endpoints and has been reported to increase the gene expression of NAD(P)H quinone oxidoreductase (QR) and glutathione-*S*-transferase (GST) at concentrations similar to those necessary to cause the same level of induction by SF [7]. Despite this, few studies have investigated the bioactivity of ER.

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Enhancing detoxification is important with regard to protection against some carcinogens (by enhancing their elimination). However, whether drug–nutrient interactions, such as those between isothiocyanates and the metabolism of chemotherapeutics and other drugs are generally beneficial during cancer chemotherapy has seldom been addressed. Relatively little research exists regarding the effects of dietary bioactive compounds on drug therapies, particularly chemotherapy. Most drug–nutrient interactions are considered to act by the modulation of Phase I cytochrome *P*450 enzymes, particularly cytochrome *P*4503A, which is responsible for the metabolism of a majority of drugs [8]. Recently, studies of drug–nutrient interactions have been extended to evaluate drug efflux systems belonging to a family of ATP-binding cassette membrane transport proteins termed multidrug resistance (MDR) proteins.

MDR is a major obstacle to the success of cancer chemotherapy. In MDR, tumor cells become resistant to a wide variety of structurally and functionally unrelated cytotoxic agents due to the fact that multiple drugs share an efflux system, which has undergone up-regulation. MDR transporters are able to transport not only unmetabolized drugs and endogenous substrates but also products of Phase I and Phase II drug metabolism and, for this reason, have been termed the Phase III detoxification system [9]. Whereas more than 10 MDR transporters have been discovered to date, P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1) and multidrug resistance protein 2 (MRP2), in order of discovery, are the most well characterized. Overexpression of P-gp and MRP1 appears to play a major role in the development of resistance, although this varies with cell type and the specific drugs concerned. Recent data indicate that Phase III detoxification transporters may be up-regulated in concert with Phase I and/or Phase II enzymes [10–13]. Thus, the system that improves defense against toxic compounds and aids in cancer prevention is the same as that found to be up-regulated in a variety of hematological and solid tumors expressing clinical drug resistance.

The 170-kDa P-gp encoded by the *mdr1* gene is overexpressed in various tumor cells and endogenously expressed in adrenal tissues, kidney, lung, liver, jejunum, rectum and colon [14]. The distribution of P-gp on the apical membrane in the epithelia of excretory organs and the ability of the protein to transport a wide range of lipophilic substrates support a role for P-gp in detoxification. Acute exposure to capsaicin (found in chili peppers), curcumin (turmeric) and resveratrol (grapes) has been shown to inhibit P-gp-dependent efflux and to increase substrate accumulation in KC-C2 cells [15]. In the same study, SF, indole-3-carbinol (cabbage) and diallyl sulfide (garlic) had no effect on substrate accumulation. However, earlier studies indicated that indole-3-carbinol may reverse P-gp protein overexpression in mouse liver to afford protection from MDR in vivo [16]. Clearly, data are needed to determine whether SF and ER differentially affect P-gp across cell types.

MRP1 was originally identified in resistant lung cancer cells, although it is expressed throughout the tissues of the body at high levels in the lung, testis and kidney and at lower levels in the liver and intestine [17]. MRP1 is localized to the basolateral membrane, where it transports both unmodified compounds and glutathione, glucuronide and sulfate conjugates to the blood for excretion in the urine [18]. In addition, MRP1 has been shown to act in concert with several GST isoforms, providing coordinated protection against toxic insult. These data suggested that dietary components causing the up-regulation of Phase II detoxification enzymes, such as isothiocyanates, might also cause the up-regulation of Phase III efflux proteins.

In contrast to either P-gp or MRP1, MRP2 response to isothiocyanates has been the subject of several studies. SF increased MRP2 mRNA and protein expressions in primary human and rat hepatocytes coordinately with the induction of the detoxification enzymes QR and GST [19]. Microarray analysis of Caco-2 cells treated with SF showed an increase in both mrp2 and *nqo1* (the gene for QR) expressions [20]. More recently, SF and ER have been found to up-regulate mrp2 transcription concomitantly with QR and UGT1A1 in human colon Caco-2 cells [21]. Taken together, these studies strongly suggest that SF and ER alter the expression of mrp2 in multiple cell types, including transformed cell lines and primary cells.

The present study aims to determine whether P-gp and MRP1 are similarly up-regulated by SF and ER, using three human cell lines. As transporter distribution varies widely among tissue types, cancer cell lines originating from three different tissues were chosen for the study, including: (a) HepG2 cells, which are known to express all three transporters; (b) Caco-2 cells, which do not express MRP1 but have been found to respond to SF and ER by an increase in MRP2 mRNA [18]; and (c) A549 lung carcinoma cells, which, of the several lung cell lines tested, express the lowest MRP1 levels, suggesting their suitability for induction studies. Here we report that MRP1, but not P-gp, is induced in the presence of SF and ER, increasing mRNA, protein and activities in A549 human lung cells.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and all consumables were purchased from Fisher Scientific (Pittsburgh, PA). 5-Carboxyfluorescein diacetate (5-CFDA) and probenecid were purchased from Molecular Probes (Eugene, OR). MK-571 was purchased from Alexis Biochemical (San Diego, CA). SF and ER were obtained from LKT Laboratories (St. Paul, MN).

2.2. Cell culture

Three transformed human cell lines (A549 non small cell lung carcinoma, HepG2 hepatocellular carcinoma and Caco-

2 colorectal adenocarcinoma cells) were purchased from American Type Culture Collection (Manassas, VA). A549 cells were maintained in Ham's modified F12K minimum essential medium containing 10% fetal bovine serum. HepG2 cells were maintained in α-modified Eagle's minimum essential medium containing 10% fetal bovine serum. Caco-2 cells were maintained in Eagle's minimum essential medium containing 20% fetal bovine serum. Cells were routinely subcultured prior to reaching 80% confluence. Treatments (SF or ER) were added in a fresh culture medium at 48 h and again at 24 h prior to harvest. This treatment period of 48 h was chosen based on studies showing an increase in MRP2 mRNA at this time point [18]. SF and ER were dissolved in dimethyl sulfoxide (DMSO), and a final concentration of 0.1% DMSO was maintained in all controls and treatments.

2.3. Western blot analysis

Cells were plated in 150-cm^2 polystyrene flasks at a density of 1×10^6 cells/flask. Twenty-four hours later, the medium was removed and replaced with a medium containing 0–20 μM SF or ER. Cells were harvested by the addition of 200 μl of lysis buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate and 0.1% deoxycholate) containing a mammalian protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined using the BioRad Protein Assay Reagent (BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions. Total protein (10 μg) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene fluoride) membranes. Membranes were blocked in a buffer containing 5% nonfat milk, 1% Tween-20, 1 M Tris–HCl and 0.5 M NaCl for 1 h at room temperature with agitation. Membranes were probed with monoclonal antibodies to P-gp (C494; Calbiochem), MRP1 (MRPm6; Kamiya Biomedical, Seattle, WA) or MRP2 (M2III-6; Kamiya Biomedical) for 1 h at room temperature with agitation followed by a polyclonal horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA). Cadherin (Santa Cruz Biotech) was detected as a loading control for all blots. Membranes were developed using the ECL Plus system (GE Healthcare Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

2.4. Real-time polymerase chain reaction (PCR)

A549 lung cells were plated out in six-well dishes at a density of 1×10^5 cells/well. Twenty-four hours later, the medium was removed and replaced with a treatment medium containing 0–20 μM SF or ER. Cells were harvested and total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. cDNA was generated using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green MasterMix system (Applied Biosystems) according to the manufacturer's instructions. Primers were as follows: MRP1, 5′-GAAGGCCATCGGACTCTTCA-3′ (forward) and 5′-cagcgcggacacatggt-3′ (reverse); MRP2, 5′-TGCAGCCTCCATAACCATGAG-3′ (forward) and 5′-GATGCCTGCCATTGGACCTA-3′ (reverse); 18*S*, 5′-GATCCATTGGAGGGCAAGTCT-3′ (forward) and 5′-AACTGCAGCAACTTTAATATACGCTATT-5′ (reverse). Real-time PCR was performed using a *TaqMan* ABI 7900 real-time PCR machine (Applied Biosystems).

2.5. Cell viability assay

To evaluate cell viability, A549, HepG2 or Caco-2 cells were seeded into 96-well dishes at a density of 1×10^4 cells/well. After 18 h, the medium was removed and replaced with a medium containing 0–40 μM SF or ER. After a further 48 h, the CellTiter 96 Aqueous One cell proliferation kit (Promega) was used according to the manufacturer's instructions. Absorbance was measured at 490 nm using a μQuant microplate reader (BioTek, Winooski, VT). The CellTiter 96 Aqueous One solution reagent contains tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium or MTS] and an electron coupling reagent (phenazine ethosulfate). MTS is reduced by NADPH/NADH from living cells into a colored formazan product (measured at 490 nm) that is soluble in tissue culture medium. Therefore, absorbance at 490 nm is proportional to the number of metabolically active/living cells in culture. Although this proliferation assay is commonly used to measure cell viability in vitro, conclusions cannot be drawn as to the cause of any cell death (cytotoxicity or apoptosis) based on this assay.

2.6. Efflux assay

5-CFDA is a nonpolar nonfluorescent compound that diffuses freely into cells, where it is cleaved by esterases to the fluorescent 5-carboxy-2′–7′-dichlorofluorescein, which is a substrate for efflux by MRP1 [22]. A549 cells were incubated with a medium containing 20 μM 5-CFDA for 60 min at 37°C. Cells were washed twice with cold phosphate-buffered saline (PBS). Cells were then incubated in a medium with or without 20 μg/ml MK-571, a specific inhibitor of MRP1, for 15 min at 37°C. Cells were detached and centrifuged at 500×*g* for 5 min at 4°C. Cell pellets were suspended in 1 ml of ice-cold PBS and immediately placed on ice. Fluorescence retained within the cells was analyzed by flow cytometry within 1 h, using an ABI LSRII flow cytometer (Applied Biosystems). Samples were excited at 488 nm using an argon laser, and fluorescent emission was detected at 530 nm. Data from 10,000 cells were acquired based on forward scatter and side scatter. MRP1-mediated efflux was determined as mean fluorescence in the presence of the MRP1-specific inhibitor MK-571 less mean fluorescence in the absence of MK-571.

2.7. Statistical analysis

Statistical analysis was performed through analysis of variance with Fisher's protected least standard difference (LSD) (α=.05) using Statistical Analysis Systems software (SAS, Cary, NC).

3. Results

To ensure that doses were not toxic to the cells, we evaluated the cytotoxicity of SF and ER in all three cell lines, using the MTS cell proliferation assay as a measure of cell viability. At 20 and 40 μM, SF caused a significant decrease in cell viability in all three cell lines (Fig. 1A). This is in agreement with published data showing that treatment of HT-29 colon cells with SF concentrations of >10 μM caused a significant decrease in cell viability [23]. In contrast, ER caused no loss in cell viability even at the highest concentration tested (40 μM) in Caco-2 cells and caused loss only at 40 μM in A549 and HepG2 cells (Fig. 1B). Cell counts in A549 cells confirmed the observed decrease in cell number at high SF and ER concentrations (data not shown). At very low concentrations (1–5 μM), SF caused a significant increase in cell number in all cell types evaluated. ER had a similar effect on A549 and HepG2 cells at concentrations from 1 to 10 μM (Fig. 1B).

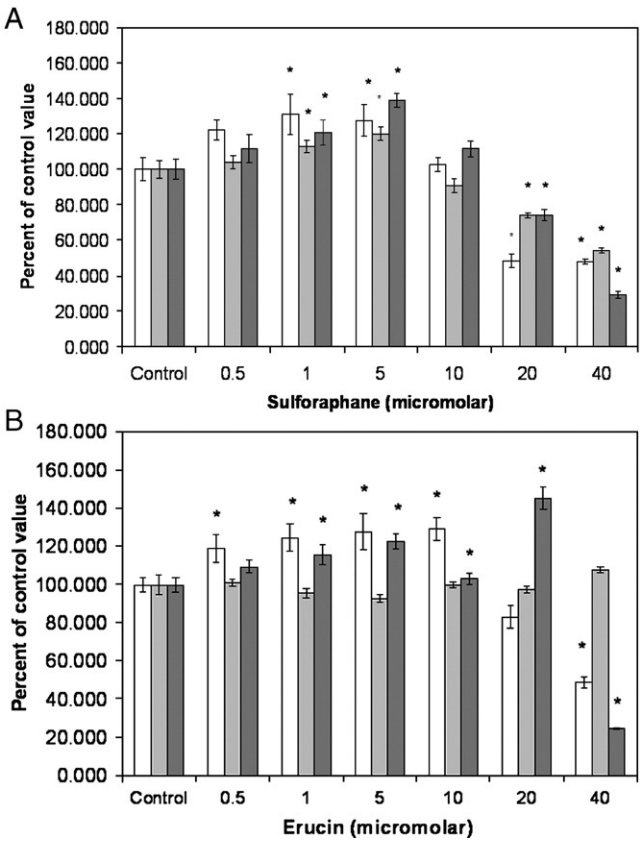

Fig. 1. The effect of (A) SF or (B) ER treatment for 48 h on the cell viability of A549 (open bars), Caco-2 (gray bars) and HepG2 (dark gray bars) cells as measured by the MTS assay. Data are presented as mean±S.E. (n=8); values that are significantly different from untreated controls are indicated by an asterisk (P<.05).

The effect of SF and ER on the expression of MRP1, MRP2 and P-gp was evaluated in A549 (lung), Caco-2 (colon) and HepG2 (liver) cells. SF increased MRP1 protein levels at concentrations of 5–20 μM in HepG2 and A549 cells (Fig. 2A). The increase in MRP1 by SF was greatest in A549 cells, showing a dose-dependent increase in MRP1 protein level compared to untreated control cells (Fig. 2A and D). ER was less potent, and an increase in MRP1 protein levels was not observed in HepG2 cells until a concentration of 10 μM had been reached (Fig. 2A). Furthermore, in A549 cells, ER did not cause a significant change in MRP1 protein level at any of the doses studied (Fig. 2A and D). MRP1 protein expression was below the level of detection in Caco-2 cells, and treatment with SF or ER did not cause any increase in protein expression (data not shown). Studies have suggested that MRP1 may not be associated with the cell membrane in Caco-2 cells, which may alter our ability to measure the protein through Western blotting [14]. MRP2 protein levels were increased by SF and ER in HepG2 cells at concentrations as low as 0.5 μM (Fig. 2B). A similar response was observed in Caco-2 cells, but the lowest concentration to cause a visible increase was 1.0 μM (Fig. 2B). A549 cells exhibited a small increase in MRP2 expression in response to SF treatment, but no response to ER (Fig. 2B). The 48-h exposure to SF or ER had no effect on P-gp protein levels in any of the cell lines tested (Fig. 2C).

Using real-time reverse transcription–PCR, the levels of MRP1 and MRP2 mRNA in A549 cells were determined following SF and ER treatment (Fig. 3). SF caused a significant elevation in MRP1 and MRP2 mRNA levels at concentrations of ≥5 μM (Fig. 3). As with MRP1 protein expression, SF treatment caused a dose-dependent increase in MRP1 mRNA levels up to eightfold and a similar increase in MRP2 mRNA levels up to 10-fold, compared to levels in untreated control cells (Fig. 3). Treatment of A549 cells with ER also resulted in a significant increase in MRP1 and MRP2 mRNA levels, although ER was not as potent as SF. Concentrations of 10 and 20 μM resulted in a 50% increase in MRP1 mRNA levels and a doubling of MRP2 mRNA levels (Fig. 3).

To determine whether the observed increases in MRP1 protein levels in A549 cells would result in an increase in MRP1 transporter activity, cells were loaded with 5-CFDA and the disappearance of the fluorescent efflux substrate 7′-CF at 15 min was evaluated by flow cell cytometry. SF treatment of A549 cells at concentrations of 2–10 μM resulted in significant increases in MRP1-dependent efflux (Fig. 4A). ER treatment of A549 cells

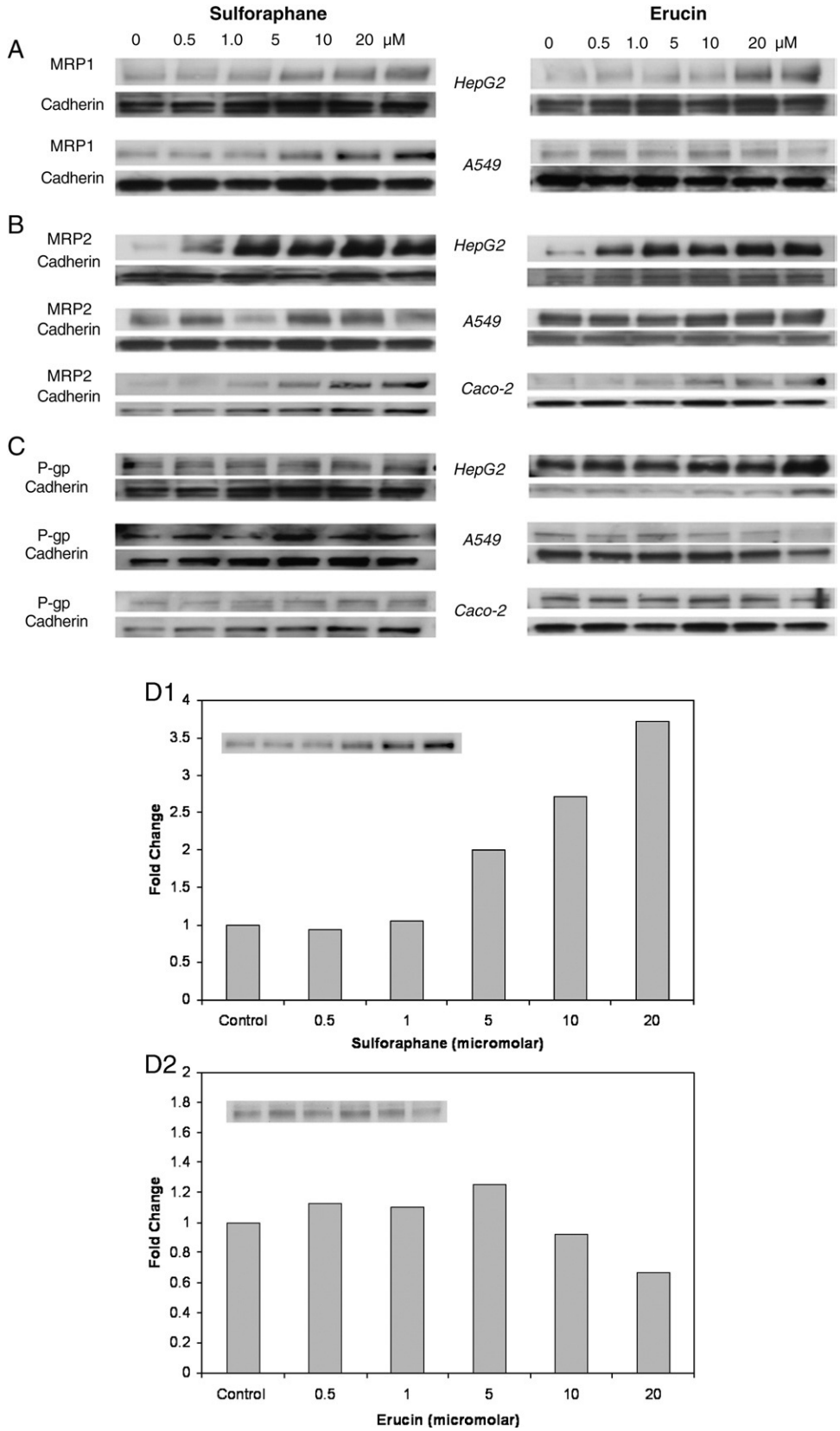

Fig. 2. The effect of SF (left panels) and ER (right panels) treatment on (A) MRP1, (B) MRP2 and (C) P-gp protein levels. Cells were treated for 48 h. Blots are representative of two independent experiments. Equal protein loading was confirmed by cadherin detection. (D) Densitometric analysis for data from A549 cells showing SF (top panel) and ER (bottom panel) effects on MRP1.

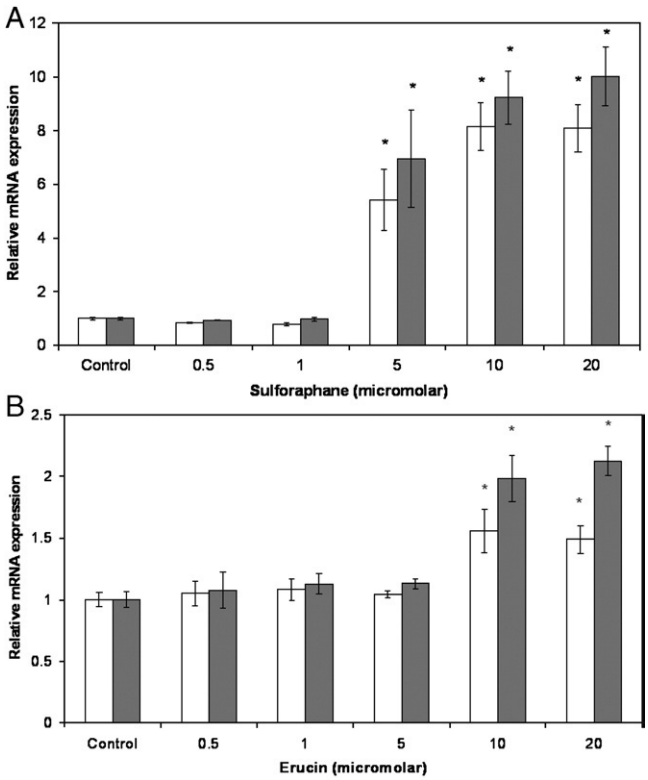

Fig. 3. The effect of SF and ER on MRP1 (open bars) and MRP2 (gray bars) mRNA expression in A549 lung cells. Cells were exposed to treatments for 48 h. Data are presented as mean±S.E. (n=6); values that are significantly different from controls are indicated by an asterisk (P<05).

also increased MRP1-dependent efflux, although to a lesser extent (Fig. 4B).

4. Discussion

We have shown for the first time that SF and ER cause dose-dependent increases in both MRP1 and MRP2 in human hepatoma HepG2 cells, without a concomitant increase in P-gp (Fig. 2). In this study, the MRP1 protein level was similarly increased in human A549 lung carcinoma cells but was not detectable in Caco-2 cells, whereas MRP2 levels were increased in Caco-2 cells but not in A549 cells. These data show that differential effects on MDR expression occur depending on cell type, emphasizing that changes in MDR expression in response to a single compound may vary among tissues or cell types.

Neither SF nor ER had any effect on P-gp expression in any of the three cell lines tested. Whereas another broccoli component (indole-3-carbinol) has been implicated in the down-regulation of *mdr1* (the gene encoding P-gp), presently, evidence that might support a regulatory role for SF or ER on P-gp is lacking. It is possible that a longer treatment time may be required in order to induce the expression of P-gp. However, most effects of SF and ER on cellular mRNA and protein levels are thought to involve the transcription factor Nrf2. In response to SF or ER, Nrf2 translocates to the nucleus, where it interacts with antioxidant response element (ARE) in the promoter region of target genes, increasing the transcription of these genes. When the promoter region of the human *mdr1* gene was isolated and sequenced, several regulatory elements were implicated in the regulation of mdr1 expression, including Y-box (CCAAT box), p53, AP-1, CAAT, C/EBP and heat shock element [24–28]. Unlike MRP1 and MRP2, the P-gp promoter does not appear to contain an ARE sequence. Therefore, any increase in P-gp expression in response to SF or ER treatment is not likely to occur through the traditional Nrf2/ARE pathway.

SF concentrations of >20 μM caused loss of cell viability and have been previously shown to cause cell death by apoptosis and/or necrosis [4,29–32]. To rule out the possibility that a general stress response to toxicity caused the observed increases in MDR transporters, cell viability in response to the 48-h exposure to SF and ER (0–40 μM) was evaluated using MTS assay (Fig. 1). A previous study has shown that treatment of Caco-2 cells with concentrations SF and ER above 20 μM leads to a significant decrease in cell

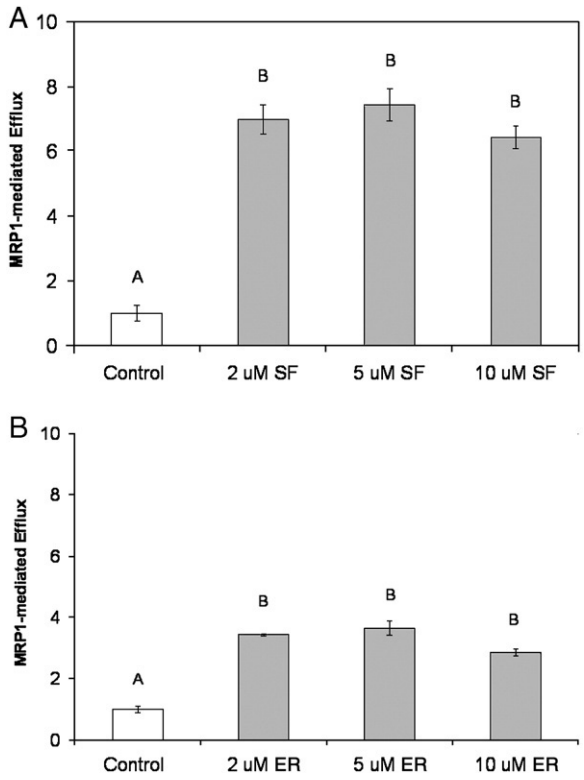

Fig. 4. The effect of SF (A) and ER (B) treatments on MRP1-dependent efflux in A549 cells. Cells were exposed to treatments for 48 h. MRP1-mediated efflux was determined as mean fluorescence in the presence of the MRP1-specific inhibitor MK-571 less mean fluorescence in the absence of MK-571. Data are presented as mean±S.E. (n=3); values with different superscript letters are significantly different (P<05).

viability [21]. Here we report similar results showing no loss of viability at ≤10 μM — the SF concentration that increased protein levels of both MRP1 and MRP2. These data indicate that MDR response was not due to the stress of cytotoxicity. Treatment of cells with low concentrations of SF and ER leads to a significant increase in cell proliferation, as estimated by the MTS assay. To our knowledge, no one has reported an increase in the proliferation of cancer cells in culture following treatment with physiological doses of SF or ER. However, this phenomenon has been observed for a few other phytochemicals such as the soy isoflavone genistein [33]. In the CellTiter 96 Aqueous One assay (MTS assay), factors that affect the cytoplasmic volume or physiology of cells can affect metabolic activity, altering the relationship between cell number and absorbance. As such, the observed apparent increase in cell proliferation with low-dose SF/ER may reflect changes in the cytoplasmic volume of cells, rather than an actual increase in cell growth rate. More detailed studies analyzing cell cycle and cell counts will be necessary to determine this.

Recently, the isothiocyanates ER and SF were shown to induce mRNA levels of MRP2 in primary hepatocytes and Caco-2 cells [19,21,34]. We have confirmed this and extended the study to show a corresponding increase in MRP2 protein expression in both Caco-2 cells and HepG2 cells (Fig. 2B). Although there was no measurable MRP2 protein increase in A549 cells, MRP2 mRNA levels were increased by both SF and ER treatment in a dose-dependent fashion (Fig. 3). The lack of correlation between MRP2 mRNA and protein in the A549 cell line may be due to timing. Protein and mRNA levels may not correlate at any given time point due to variations in mRNA and protein stability and degradation [35].

A recent report showed an increase in MRP1 in primary mouse hepatocytes following treatment with 5 and 10 μM SF for 24 h [34]. The increase in MRP1 protein level was accompanied by a decrease in the cellular accumulation of arsenic, suggesting that the increase in protein translated into an increase in the export of this xenobiotic. Here, we show that SF increases MRP1 mRNA, protein and transporter activities in human lung carcinoma cells. MDR is of particular concern in the treatment of lung cancers; initial chemotherapy and radiation are generally successful, but the majority of lung cancer patients become resistant to treatment and >90% of patients succumb to the disease [36]. Our findings show that treatment of human lung cells with SF increases the expression of MRP1 at the mRNA level and that this amplification is associated with an increase in protein expression and transporter activity, indicating that the newly synthesized protein has been correctly inserted into the plasma membrane and is functional. Such an increase in MRP1-mediated transport may contribute to MDR phenotype, and studies in whole animals are needed to determine this.

The data presented here show that SF increased MRP1 and MRP2 mRNA and protein expressions at lower concentrations than did ER. SF induced MRP1 and MRP2 mRNA and protein expressions at 5 μM, whereas an increase was not observed below 10 μM for ER (Fig. 3). Furthermore, the magnitude of the increase in response to ER was much less than that of SF. The concentration of SF needed to achieve an increase in MRP expression is particularly important in determining whether such an effect may have physiological consequences. In humans, plasma levels of total SF (SF plus SF metabolites) can reach 1–2.25 μM following the consumption of a single serving of broccoli and up to 7 μM after eating "super" broccoli — a broccoli cultivar that contains ~3.5 times the level of glucosinolates [37,38]. Therefore, increases in MRP1 and MRP2 expression in normal tissues or in a tumor following a broccoli meal are certainly plausible. Unfortunately, few studies have evaluated the bioactivity of ER, and typical plasma concentrations of ER following crucifer consumption are not known at this time. ER is primarily derived from arugula, and while the popularity of arugula has been increasing due to its inclusion in gourmet salad greens, consumption is unlikely to reach that of broccoli. However, ER can be formed from SF and glucoraphanin in vivo, making the study of ER particularly interesting [6].

To date, transporter activity studies with isothiocyanates and MDR transporters have focused on acute treatment times and direct inhibition of transporters. One study found that, acutely, 100 μM SF did not alter the accumulation of the MRP1 substrate daunomycin in human pancreatic cells overexpressing MRP1, whereas another study showed that similar concentrations of benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) caused significant inhibition of the MRP1-mediated efflux of daunomycin in human colon adenocarcinoma cells and in pancreatic cells overexpressing mrp1 [39,40]. At low (1–20 μM), but not high (50–100 μM), PEITC concentrations, a significant increase in intracellular daunomycin accumulation was observed, suggesting that lower more physiologically relevant concentrations of isothiocyanate may not have the same acute effect on MRP1-mediated transport as supraphysiological concentrations. In addition, we observed significant cytotoxicity after a 48-h treatment with 40 μM SF and ER. Whereas such short-term studies with high concentrations may not show observable cellular injury, such supraphysiological isothiocyanate concentrations can lead to significant damage that does not become overt until later. In the present study, treatment of A549 cells with physiological concentrations of SF and ER for 48 h resulted in a significant increase in MRP1-dependent transport, rather than the inhibition of efflux reported for acute treatment with supraphysiological PEITC and BITC levels.

SF has been shown to induce the transcription of target genes such as *nqo1* through the activation of the Nrf2 transcription factor. Activated Nrf2 enters the nucleus and interacts with the ARE in the promoter region of target genes, altering expression. In studies utilizing rats, two

compounds known to trigger the Nrf2/ARE system (ethoxyquin and oltipraz) have been shown to increase MRP2 protein levels, although MRP2 mRNA expression was not significantly up-regulated [41]. A more recent study utilizing mice found that butylated hydroxyanisole (BHA), a classic Nrf2 activator, increased the level of hepatic MRP2 mRNA, with an increase in MRP2 in the canalicular membrane [42]. Site-directed mutagenesis of the mouse mrp2 promoter indicated that an ARE sequence at −95 to −85 was responsible for BHA-induced MRP2 expression, supporting the idea that agents known to trigger the Nrf2/ARE pathway (such as SF and ER) cause up-regulation of mrp2. Although not well characterized, an ARE-like sequence (TGCCTCAGC) has been identified in the human mrp2 promoter as well [43].

Analysis of the promoter region of human mrp1 reveals a putative ARE sequence located approximately 468 bp upstream of the transcription start site, which is identical to the ARE in the Nqo1 promoter [44]. In addition, a recent study utilizing mouse embryo fibroblasts derived from Nrf2 knockout mice indicated that Nrf2 is essential for both constitutive and inducible expressions of MRP1 [45]. They found that constitutive levels of MRP1 mRNA and protein were significantly lower in nrf2(−/−)-derived cells than in wild-type-derived cells. In addition, microarray analysis indicated a coordinated up-regulation of γ-GCS, GST and MRP1 in Nrf2-overexpressing glial cells [46]. In a study utilizing the small intestine of nrf2(−/−) mice, microarray analysis indicated that Nrf2 was required for the basal expression of mrp1 [47]. Given these observations and the requisite relationship between Nrf2 and ARE, the potential role of ARE in mrp1 expression is evident. Furthermore, up-regulation of mrp2 expression has been shown to occur through the Nrf2/ARE pathway. Our results show similar responses to SF for both MRP1 and MRP2 (Fig. 3), indicating that SF and ER likely induce the expression of MRP1 and MRP2 though a common Nrf2/ARE-dependent mechanism [42]. Additional studies are necessary to evaluate the role of Nrf2 and putative ARE in the induction of MRP1 by SF.

Acknowledgement

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