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Multidrug Resistant Protein-Three Gene Regulation by the Transcription Factor Nrf2 in Human Bronchial Epithelial and Non-Small Cell Lung Carcinoma

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Abstract

Multidrug Resistant Proteins (MRP) are members of the ATP-binding cassette superfamily that facilitate detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. Chemotherapy, radiation, and other xenobiotic stresses have been shown to increase levels of select MRPs, although, the underlying mechanism remains largely unknown. Additionally, MRP3 is suspected of playing a role in the drug resistance of non-small cell lung carcinoma (NSCLC). Analysis of the MRP3 promoter revealed the presence of multiple putative electrophile responsive elements (EpRE), sequences that suggested possible regulation of this gene by Nrf2, the key transcription factor that binds to EpRE. The goal of this investigation was to determine whether MRP3 induction was dependent upon the transcription factor Nrf2. Keap1, a key regulator of Nrf2, sequesters Nrf2 in the cytoplasm, preventing entry into the nucleus. The electrophilic lipid peroxidation product, 4-hydroxy-2-nonenal (HNE) has been shown to modify Keap1 allowing Nrf2 to enter the nucleus. We found that HNE up-regulated MRP3 mRNA and protein levels in cell lines with wild type Keap1 (human bronchial epithelial cell line HBE1 and the NSCLC cell line H358), but not in the Keap1 mutant NSCLC cell lines (A549 and H460). Cell lines with mutant Keap1 had constitutively higher MRP3 that was not increased by HNE treatment. In HBE1 cells, silencing of Nrf2 with siRNA inhibited induction of MRP3 and by HNE. Finally, we found that silencing Nrf2 also increased the toxicity of cisplatin in H358 cells. The combined results therefore support the hypothesis that MRP3 induction by HNE involves Nrf2 activation.

Keywords

electrophile response element; lung cancer; 4-hydroxynonenal; Keap1; H460 cell line; H358 cell line; A549 cell line; HBE1 cell line; Nrf2; multidrug resistant proteins

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INTRODUCTION

Lung cancer is the most common cause of cancer death, accounting for more deaths than colorectal, prostate, and breast cancer combined [1]. One class of lung cancer is non-small cell lung carcinoma (NSCLC) [2]. NSCLC constitutes 75% of primary lung cancers and are comprised of large-cell undifferentiated carcinomas, squamous carcinomas, and adenocarcinomas. Unfortunately, most patients present with locally advanced or metastatic disease and are considered incurable. Stage-based therapies may include surgery, radiation, and chemotherapy. In advanced disease current front-line therapies consist of chemotherapeutic doublets (e.g. etoposide with cisplatin). However, as the five-year survival rate for metastatic NSCLC is a dismal 2%, current treatment options are largely ineffective. One factor that contributes to the poor clinical outcome is that a significant proportion of NSCLC are intrinsically chemoresistant [3]. Resistance to chemotherapeutic agents in drug resistant cancers is facilitated, in part, by an increased capacity for detoxification. Like other xenobiotics, chemotherapeutic agents are processed in the body through the detoxification system, which includes Phase II and III enzymes. Phase II genes are family of enzymes that under increased oxidative stress are up-regulated, eliciting a response which detoxifies the stressors, often through conjugation to glutathione. Exposure to carcinogens and various xenobiotics can increase both Phase II and III enzymes [4–6]. Several transcription factors such as AP-1, NF-KB, and Nrf2 are known to be activated by oxidants [7]. After activation, these transcription factors can regulate Phase II genes through cis-acting elements [8,9]. Among them, the electrophile response element (EpRE) has been shown to be an important regulator of Phase II enzyme expression [10,11]. A well-established EpRE binding protein, Nrf2, is a member of the basic-leucine zipper NF-E2 family [12]. Upon formation of heterodimers with c-Jun, small Maf, or other proteins, Nrf2 binds to the EpRE domain located in the upstream regulatory region of multiple Phase II genes [13–16]. Nrf2 is negatively regulated by a cysteine-rich cytoplasmic protein known as Kelch-like ECH-associated protein1 (Keap1) [17]. Keap1 is attached to cytoskeletal actin and binds to Nrf2 directly [18]. Once bound, Keap1 has been shown to facilitate ubiquitination of Nrf2 and its proteosomal degradation.

Phase III enzymes, which include Multidrug Resistance Proteins (MRP), do not interact with xenobiotics directly but facilitate the excretion of water-soluble compounds (including products of Phase II enzymes) out of the cell. MRPs are a branch of the ATP-binding cassette superfamily (ABC) of transmembrane proteins [19]. The MRP (ABCC) family consists of nine different members [20]. Several MRPs are known to transport a wide range of chemotherapeutic compounds and thus reduce the cellular accumulation of anti-cancer agents [21]. MRP family members have been shown to be significant contributors to multidrug resistance in cell lines and have been found in numerous classes of cancer types [22–24]. Increased expression of MRPs has been associated with negative clinical outcomes in a variety of cancer types including breast cancer, gastric cancer, neuroblastoma, retinoblastoma, and lung cancer [25–31].

The MRP3 (ABCC3) gene is located on chromosome 17, encodes a protein of 1527 amino acids, and is known to be expressed in a variety of tissues including lung, adrenal glands, pancreas, gut, gall bladder, liver, kidney, and prostate [32–36]. MRP3 is the closest structural ABCC family member to MRP1, sharing roughly 58% amino acid homology [37]. MRP3 has an N-terminal region that is comprised of three membrane-spanning domains, which include five transmembrane helices, an intracellular loop and an extracellular region at the N-terminus [38]. Currently identified substrates of MRP3 include anticancer drugs asglucuronate, sulfate, or glutathione conjugates [39,40]. Analysis of NSCLC cell lines and clinical specimens revealed that MRP3 is expressed at higher levels in NSCLC than in SCLC [41]. Young *et al.* found that MRP3 protein levels correlated with decreased sensitivity of lung cancer cell lines

to frontline chemotherapeutic compounds such as vincristine, etoposide, and cisplatin [41]. Additionally, MRP3 expression has been associated with increased resistance to methotrexate and doxorubicin in NSCLC cell lines and patient tumor samples [21,37]; however, the mechanism underlying its activity and regulation are largely unknown.

The α,β -unsaturated aldehyde 4-hydroxynonenal (HNE) is a major lipid peroxidation product formed by the reaction of reactive oxygen or nitrogen species with arachidonic acid in cellular membranes [21]. HNE has been previously established to cause the activation of the Nrf2-EpRE signaling and cytoprotective gene induction in the human bronchial epithelial (HBE1) cell line [42,43]. The purpose of this study was to examine the involvement of Nrf2 in the up-regulation of MRP3 in lung epithelial cells in response to oxidative stress. Here we demonstrate that activation of Nrf2 by HNE leads to the induction of MRP3 in human epithelial lung and *Keap1* wild type NSCLC cell lines.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO). Antibodies (MRP3: 6D568 mouse monoclonal IgG sc-71605), and small interfering RNAs were obtained from Santa Cruz (Santa Cruz, CA). HNE was purchased from Cayman Chemical (Ann Arbor, MI). TRIzol Reagent is from Life Technologies (Grand Island, NY). DNA-free reagent was obtained from Ambion (Austin, TX). TaqMan Reverse Transcription Reagent and SYBR Green PCR Master Mix were obtained from Applied Biosystems (Foster City, CA). Luciferase activity assay kit was obtained from Promega (Madison, WI). FuGENE 6 transfection reagent was obtained from Roche (Indianapolis, IN). M-PER mammalian protein extraction reagents were obtained from Pierce (Rockford, IL).

Cell Culture and Treatments

The human bronchial epithelial cell line (HBE1) was cultured in collagen-coated dishes. Cells were grown in serum-free Ham's F-12 medium supplemented with seven additives (5 μ g/ml insulin, 3.7 μ g/ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor, 3×10^{-8} M triiodothyronine, 1×10^{-6} M hydrocortisone, 5 μ g/ml transferrin) in T-75cm² collagen-coated flasks. The NSCLC cell line H460 (*p53* wild type/*Keap1* mutant), H358 (*p53* null/*Keap1* wild type), and A549 (*p53* wild type/*Keap1* mutant) were obtained from the Mack laboratory at the UC Davis Cancer Center, Sacramento CA. NSCLC cell lines were maintained in DMEM (Biowhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA) to which was added MEM Essential Vitamin Mix, Penicillin-Streptomycin, L-Glutamine. All cultures were grown at 37°C in 5% CO₂ atmosphere. Cells were plated on 10cm cell culture dishes at density of 1,000,000 cells/dish. Cells were treated at approximately 85% confluency. The time point studied for both mRNA and protein collection was 24 h. HNE was dissolved in ethanol. HBE1 cells close to confluence were treated with vehicle control (0.05% ethanol) or different concentrations of HNE as indicated in RESULTS.

RT-PCR

The content of MRP3 mRNA was determined by real-time PCR. RNA samples were treated with DNA-free reagent and reverse transcribed using the TaqMan reverse transcription system. Real-time PCR was carried out using the SYBR GreenER qPCR Supermix Universal (Invitrogen) as specified by the manufacturer. Real-time PCR was performed with a Cepheid 1.2 real-time PCR machine (Cepheid, Sunnyvale, CA) and an iQ5 Real-Time PCR detection system (BioRad, Hercules, Ca). GAPDH and beta-actin were used as internal controls. The primers are as follows: MRP3, sense 5'-CAGAGAAGGTGCAGGTGACA-3', antisense 5'-

CTAAAGCAGCATAGACGCCC-3': GAPDH, sense 5'-
TGGGTGTGAACCATGAGAAG-3, antisense 5'-CCATCACGACACAGTTTCC-3; beta-
actin, sense 5'-GAGCGCGGCTACAGCTT-3', antisense 5'-
TCCTTAATGTCACGCACGATTT-3'.

Immunofluorescence

HBE1 cells were grown on glass coverslips pretreated with collagen I (20ug/ul) for 2 h at 20°C in 30-mm plates. NSCLC cells were plated on VRW Scientific glass coverslips in 30-mm plates. Cells were washed twice in PBS and fixed with 95% methanol at 20°C for 5 min. Slides were washed twice in PBS containing 1% BSA, .02% saponin, and .05% sodium azide. Fixed cells were incubated for 30 min at 37°C with primary antibody at a 1:200 dilution in PBS. Slides were washed and incubated with FITC conjugated anti-mouse secondary antibody (Santa Cruz, Ca: sc3699) at a 1:50 dilution for another 30 min. Slides were washed and coverslips were mounted. At least 100 cells were scored for localization and concentration of MRP3 protein under a microscope. Images were obtained using a Nikon eclipse- TE 2000-U/confocal microscope and an Olympus BX61 confocal system microscope. The images were analyzed for luminosity values, and were subsequently exported from the native .ids Nikon file format to Bmp files using the Nikon Ez-C1 viewer software ver. 3.2, and then Adobe Photoshop was used to construct the figures. Experiments were performed at least in triplicate.

siRNA Transfection: Nrf2

Transfection of Nrf2 siRNA was performed using the target sequence 5'-AAGAGTATGAGCTGGAAAAAC-3' for human Nrf2 siRNA. Nonspecific siRNA (NS siRNA) was used as a negative control. HBE1 cells were seeded at 1.5×10^5 cells per well into six-well plates. After 24 h, the cells were transfected with Nrf2 siRNA. Appropriate amounts of Nrf2 siRNA in 250 μ L serum-free DMEM/F12 medium and 5 μ L of transfection reagent in 245 μ L serum-free DMEM/F12 medium was prepared in separate centrifuge tubes. After incubation for 5 minutes, the siRNA and transfection reagent were mixed, incubated for an additional 20 minutes, and added to each well.

Statistics

The comparative $\Delta\Delta C_T$ method was used for relative mRNA quantitation. Comparisons of variants between experimental groups were conducted using one-way analysis of variance (ANOVA). All data was expressed as the mean $\pm$ standard deviation. In-Stat software was used for statistical analysis. Statistical significance was accepted when $p < 0.05$.

RESULTS

Induction of MRP3 in *Keap1* Wild Type Cells

RT-PCR analysis was used to determine which MRP family members were present in normal bronchial epithelial cells. We demonstrated the presence of all known MRPs, with the exception of MRP6 in HBE1 cells. Of the detected MRPs, MRP3 was found to be a prevalent MRP species (Figure 1). We then sought to evaluate whether HNE was capable of up-regulating the expression of MRP3 in the selected cell lines. HNE can be toxic, depleting glutathione, the principal antioxidant made by cells. Normal human plasma contains the equivalent of 0.3–0.7 μ M HNE (much of it reversibly bound to plasma proteins through Schiff base formation); however, during oxidative stress such as occurs in inflammation and tobacco smoking, plasma HNE concentrations can increase more than ten times with affected tissue concentrations in the millimolar range [42,44–47].

Upon exposure to sublethal concentrations of HNE, MRP3 mRNA increased in *Keap1* wild type cells, but not in *Keap1* mutant cells. We observed an approximate four-fold increase in MRP3 mRNA in the *Keap1* wild type cell lines when compared to untreated controls (Figure 2). The expression and localization of MRP3 protein was determined by immunofluorescence using monoclonal antibodies. MRP3 protein levels were markedly induced after exposure to HNE in *Keap1* wild type cell lines. The increase in MRP3 protein by HNE treatment correlated with the observed increase in mRNA levels in these cells. Conversely, we found that MRP3 expression in *Keap1* mutant cell lines were relatively higher than in *Keap1* wild type cells and was unaffected by the addition of HNE (Figure 3). These results were expected as *Keap1* mutants had higher constitutively active Nrf2 than *Keap1* wild type cells, and therefore were expected to also have high constitutive levels of MRP3.

Nrf2 Silencing

Analysis of the promoter sequence of MRP3 located in the 5'-untranslated region revealed the presence of four putative EpRE (TGA(C/T)NNNGC) sites at -434bp, -628bp, -805bp, -1049bp from the 5' UTR of MRP3 (Figure 4) [48]. In order to specifically determine Nrf2 involvement in MRP3 induction, we examined the effect of depleting Nrf2, the principal transcription factor involved in EpRE regulation. We have previously demonstrated the effectiveness of Nrf2 siRNA in HBE1 cells [49]. The non-specific siRNA-treated samples displayed a reduction in cytosolic Nrf2 and an increase in levels of nuclear Nrf2 after exposure to HNE. Transfection with Nrf2 siRNA was shown to reduce cytosolic and nuclear Nrf2 alone, or in the presence of HNE (Figure 5).

We measured the effects of inhibiting the rate of Nrf2 expression with siRNA in cells exposed to HNE. RT-PCR analysis demonstrated that transfection with Nrf2 siRNA 24 h prior to exposure to HNE inhibits the induction of MRP3 mRNA when compared to the non-specific siRNA treated cells (Figure 6). Moreover, the levels of MRP3 mRNA in the Nrf2 siRNA HNE treatment group were lower than the basal level of the control group, which was likely due to a dependence upon Nrf2 for basal expression. Figure 6 shows HBE1 cells that were transiently transfected 24 h prior to exposure to HNE. The difference in the effectiveness of HNE in Figure 6 compared to Figure 2 was likely due to the toxicity of the transfecting agent, FuGENE 6, which alone caused a 25% decrease in viability (data not shown).

Quantitative immunofluorescence analysis of HBE1 cells indicated that MRP3 expression was increased in a dose dependent manner when compared to the control following exposure to HNE (Figure 7). We found that the differences between the luminosity measurements of the control group were statistically significant ($p > 0.001$) when compared to that of either the 10 μM or 15 μM HNE groups. Additionally, the luminosity measurements of the 10 μM or 15 μM HNE groups were statistically significant ($p > 0.001$) when compared to those of the Nrf2 siRNA group (Figure 8). Taken together these observations support the involvement of Nrf2 in MRP3 induction in response to HNE induced oxidative stress.

Additionally, we sought to measure the change in toxicity to cisplatin in a NSCLC when Nrf2 expression was knocked down using siRNA. While not quite statistically significant, we found that in the NSCLC cell line H358 the Nrf2 siRNA→CP group was approximately 25% more sensitive to 48 h of 2.5 μM cisplatin treatment than the non-specific siRNA→CP group (Figure 9). While this was consistent with a role for MRP3, the expected global decrease in activity of other Nrf2 regulated genes had unpredictable consequences.

DISCUSSION

One of the primary treatments for combating NSCLC is the use of chemotherapy. However the majority of NSCLCs are inherently drug resistant. This presents a major obstacle in

successfully treating this disease. One component of this observed multifactorial drug resistance is an increased capacity to efflux chemotherapeutic compounds out of the cell. Studies of clinical specimens and cell lines have shown an increased capacity for drug transport, and subsequent resistance, due to increased levels of MDRs and MRPs. Given that not all NSCLCs possess this ability it would seem that some additional dysregulation has occurred.

Our analysis of the human MRP3 gene (*ABCC3*) revealed multiple EpREs in the tentative promoter region. These findings lead us to hypothesize that activation of Nrf2 could contribute to the induction of MRP3. To test this we used HNE, a known Nrf2 activator, in an effort to evaluate the response of MRP3. Our results demonstrate that Nrf2 activation can up-regulate the expression of the endogenous MRP3 gene, producing increases in both mRNA and protein in both human bronchial epithelial and *Keap1* wild type NSCLC cells. In addition, we demonstrate that selectively inhibiting the expression of Nrf2 has the capacity to abolish induction of MRP3 by HNE. While regulation of MRP3 by Nrf2 has not been described previously, a relationship between Nrf2 and MRP1 and MRP2 expression has been suggested by studies conducted in human cancer specimens including NSCLC [50].

Recent studies have indicated the potential “negative role” for the Nrf2 pathway, as it is been found to be upregulated in a number of drug resistant human malignancies, and that inhibiting expression of Nrf2 during chemotherapy could be clinically beneficial [51]. Additionally, Wang *et al.* showed that overexpression of Nrf2 increased resistance to chemotherapeutic drugs, and that knocking down Nrf2 decreased resistance to these drugs [52]. Our own experiment was in agreement with the aforementioned study, demonstrating that knocking out Nrf2 produced increased toxicity to the front line chemotherapeutic drug cisplatin, which is consistent with a decrease in MRP3 expression. Despite that consistency, we cannot definitively state that the increased toxicity was due to the decrease in MRP3 alone as Nrf2 regulates the expression of numerous Phase II genes that could also have contributed.

It has been recently demonstrated by Singh *et. al.*, that approximately fifty percent of NSCLC cell lines and eighteen percent of NSCLC clinical specimens examined in their study had inactivating mutations in *Keap1* [50]. These *Keap1* mutant NSCLCs had increased accumulation of Nrf2 and Nrf2-target genes, possibly leading to increased drug resistance [50]. We observed elevated basal levels of MRP3 in *Keap1* mutant cells, which were comparable to the levels observed in the *Keap1* wild type cells under oxidative stress. Activation of the Keap1/Nrf2 signaling pathway in normal cells confers protection against oxidative stress and carcinogens, while deregulation of this transcriptional program in cancer cells may provide a selective survival advantage via the up-regulation of MRP3.

In addition to Keap1, other potential mechanisms may lead to aberrant expression of MRP3. One such possibility may involve p53. Dysregulation of the *p53* gene (mutant or null) occurs in approximately 45% of NSCLC cases and is associated with drug resistance [53,54]. Additionally, p53 has been demonstrated to repress Nrf2-regulated expression of several phase II genes (NQO1, X-CT, and GST- α 1), suggesting cross talk between the p53 and Nrf2 signaling pathways [40]. Studies have demonstrated a negative link between p53 activation and MRP1 expression. The correlation between high levels of MRP1 expression and *p53* mutation is supported by in vitro studies of the MRP1 gene regulation in human and murine systems, which have shown that wild type p53 is a strong suppressor of MRP1 transcription [55]. Additionally, the human papilloma E6 protein initiates the rapid degradation of p53, which subsequently increased MRP1 levels in a HPV16 transformed cell line [56]. Whether p53 is involved in the regulation of the MRP3 gene, as it is with MRP1, has not been determined. Of note, p53 is functionally disrupted in both of the cell lines, HBE1 and H358, in which we observed to be inducible for MRP3. If p53 acts as a negative regulator of MRP3 as it does for MRP1, it would

be expected that these cells would be more responsive to the types of cytotoxic stresses that induce MRP3.

While some cell lines express higher levels of MRPs intrinsically; [19,22] chemotherapy, radiation, and other xenobiotic stresses have been shown to increase levels of select MRPs independently [20,21]. Several classes of antineoplastic agents produce oxidative stress leading to the formation of lipid peroxidation products, such as HNE. An additional factor to consider is that chemo- and radiation therapy will induce lipid peroxidation that generates HNE and could potentially activate Nrf2. This effect of inadvertent activation of the genes involved in drug resistance in “real time” may explain one component of the drug-resistance phenotype common to this malignancy. As this is a normal response to chemotherapy, it suggests that even under conditions where there is no aberrant signaling in the drug metabolic pathway; tumors may nevertheless naturally develop resistance over time to therapy, in part through an accumulation of MRP3. Ideally, one solution to resolve this issue may be to determine markers of sensitivity in drug metabolism (e.g. lack of MRP expression) as opposed to resistance.

In conclusion, our results demonstrate that MRP3 belongs to the family of detoxification enzymes whose expression is regulated by Nrf2. This study shows that HNE, at physiologically achievable levels, produces a marked up-regulation of the multidrug resistance protein transporter, MRP3, in human bronchial epithelial and *Keap1* wild type NSCLC cells. In addition, we demonstrated that inhibiting Nrf2 significantly attenuates this response. Elucidating the exact mechanisms involved in the up-regulation and/or suppression of MRP3 could lead to the identification of patient subsets, which may benefit from different chemotherapy regimens thereby improving upon current therapeutic options for NSCLC.

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The abbreviations used are

MRPs	Multidrug resistant proteins
NSCLC	non-small cell lung carcinoma
HNE	4-hydroxynonenal
EpRE	electrophile response element

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Figure 1. RT-PCR analysis of MRPs 1–9 in the HBE1 cell line. Total RNA was isolated, reverse transcribed, and amplified by PCR. Products were analyzed on a 1.5% agarose gel. Numbers above the lanes correspond to the MRP member. MRP3 was loaded at 1/10 the initial concentration during PCR amplification.

Figure 2.

Relative levels of MRP3 mRNA were determined after 24 h exposure to 15 μ M of HNE using real-time PCR. GAPDH and beta-actin were used as internal controls. Results are expressed as standard deviation ($n = 3$)

Figure 3. Immunofluorescence. Selected cell lines were fluorescently labeled for MRP3 protein (green) and counter stained with dapi (blue) for nuclear detection and examined after 24 h exposure to 15 μ M HNE.

5' Untranslated Sequence of MRP3 (*ABCC3*)

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5'TTCTGAGCCCCAACAGCGGTGCTAGATTGGCGTCTGGGATTGTGTTAAGGAAGACAGGGCAACAGCGGGA
TTGAGAGATGGAGGGATCCCCGCATCTGAGGGGATACCGGGAGAAGGAGAGCTGAGGCAAGCAGGAGAAGGA
GAGCACTGACAAGCCCATTTTACAGATCAGGAATGTAGAGTTTCAGCCTTAGGAGGAGGACGAGGACGAGGAC
ACTCACGTGGGGCTCCTCCTGGGGCTTCACCTGTCTTCCCCCCCCAACCCTGATCAGGCTGAAAGCAGAGGGA
ATTACACATGTTGGGTGCCCACTAAGTGTGCCAGGAAATACGCTTTGCCTACCCTTTCCATTCAATCCTCAGAG
AACCTGGGAGGAAGATTCTCTTCTATGATTGTCTCTATTTTACGGATGTGAAAGTAGAGGCCAGAGACGTGC
CGAGGTGATGTGGCAAAGGTCAGGGAGCCACAGAGCCTAAGAGGGCAGCAGTTTAAGAGGACATAAGAGGCC
CCCGCTGGACGATGCTGCGGCCCCACCCCGCTCACGGGTACACACCCGCGGAGCACACCCCGTGTGCTCC
CCGTGTGGCGCCTGCCAGTGGTCTCTGGAACCTGATGGGGAAGAGGTCAGCTTCCTGATTGAGCACAGAAGC
CCTTCCCATTTTACAGATGAGGAACTGACCTCCGGAGAGGAACGTGACCTTCTCAAAGATTCCCTTTTACTA
GGAAAGATTGTAAATCCCTCTCCCTACGTCCCTACTCAATGACTCATCGGCCCCACGGACTTGTTCGGGAGTG
GGAGCGCCTGTGATCCCAGTCCCCCTTGGAATAGACTCGACTCCTGGACTGTTAGCCTCGGTATTAGAGA
GACATCCCCCTGGCTTGGCCAGGGGCGCTCGCAGGGGCTGTGCGCGTGCCAAGGGCCCCCACCTCTGCC
CCAAAGTCCCTCCAGGGAGGCGCTGTGCAACCCGCGGGACGGGGCAGGGAGACCGGACCCAGAGGCACT
GGGGTAGGGCTCGGCAGGGCGGGTCCGGGCGGAGCGCGGGGTGCGGAGGGCGGAGGCGG-3' (start)

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Figure 4.

MRP3 (*ABCC3*) promoter region spans from −11 bp through −1103 bp. Putative EpRE binding sites (blue) are located −434 bp, −628 bp, −805 bp, and −1049 bp upstream from the *ABCC3* gene.

Figure 5.

Effect of Nrf2-siRNA on the activation and constitutive expression of Nrf2 protein. HBE1 cells were transfected with Nrf2 siRNA or nonspecific RNA (NS) 24 h prior to treatment with 10 μ M of HNE for 1 h. Cytosolic and nuclear fractions were extracted for Western blot analysis. *A*, The NS siRNA samples displayed a reduction in cytosolic Nrf2 after HNE treatment. Transfection with Nrf2 siRNA reduced cytosolic Nrf2 alone, or in the presence of HNE. *B*, The NS siRNA samples demonstrated increased nuclear Nrf2 after exposure to HNE. Transfection with Nrf2 siRNA reduced nuclear Nrf2 alone, or in the presence of HNE.

Figure 6. RT-PCR analysis of Nrf2 siRNA silencing. Transfection with Nrf2 siRNA 24 h prior to exposure to 15 μ M HNE inhibits induction of MRP3 mRNA compared to the non-specific siRNA group. Results are expressed as standard deviation ($n = 3$)

Figure 7.

Fluorescent microscopy and digitized images of representative human bronchial epithelial cells. MRP3 protein levels (green) were analyzed 24 h after exposure to 10 µM and 15 µM of HNE, and 24 h after transfection with Nrf2 siRNA.

Figure 8.

Quantitative Immunofluorescence. Twenty intact HBE1 cells in each treatment group were individually measured for luminosity. Control vs. HNE 10 μ M and 15 μ M 24 h = $P < 0.001$, and HNE 10 μ M and 15 μ M 24 h vs. Nrf2 siRNA->15 μ M HNE = $P < 0.001$. Results are expressed as standard deviation ($n = 3$)

Figure 9. MTT Viability Assay. We examined the change in toxicity by Nrf2 silencing by using siRNA in the NSCLC cell line H358 after exposure to 2.5 μ M of cisplatin for 48 h. Treatment groups included NS siRNA + CP, NS siRNA – CP, Nrf2 siRNA + CP, and Nrf2 siRNA – CP. Results are expressed as standard deviation ($n = 3$).