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Research Article

Nrf2 Regulates the Sensitivity of Mouse Keratinocytes to Nitrogen Mustard via Multidrug Resistance-Associated Protein 1 (Mrp1)

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ABSTRACT

Sulfur mustard and nitrogen mustard (mechlorethamine, HN2) are potent vesicants developed as chemical warfare agents. These electrophilic, bifunctional alkylating agents cause skin injury, including inflammation, edema, and blistering. HN2 covalently modifies macromolecules such as DNA, RNA, and proteins or is scavenged by glutathione, forming adducts that can contribute to toxicity. Multidrug resistance-associated protein 1 (Mrp1/MRP1) is a transmembrane ATPase known to efflux glutathione-conjugated electrophiles. In the present studies, we examined the effects of modulating Mrp1-mediated transport activity on the sensitivity of primary and PAM212 mouse keratinocytes to HN2. Primary keratinocytes, and to a lesser extent, PAM212 cells, express Mrp1 mRNA and protein and possess Mrp1 functional activity, as measured by calcein efflux. Sulforaphane, an activator of Nrf2, increased Mrp1 mRNA, protein, and functional activity in primary keratinocytes and PAM212 cells and decreased their sensitivity to HN2-induced growth inhibition ($IC_{50} = 1.4$ and 4.8 μM in primary keratinocytes and 1 and 13 μM in PAM212 cells, in the absence and presence of sulforaphane, respectively). The Mrp1 inhibitor, MK-571, reversed the effects of sulforaphane on HN2-induced growth inhibition in both primary keratinocytes and PAM212 cells. In primary keratinocytes from $Nrf2^{-/-}$ mice, sulforaphane had no impact on Mrp1 expression or activity, or on sensitivity to HN2, demonstrating that its effects depend on Nrf2. These data suggest that Mrp1-mediated efflux is important in regulating HN2-induced keratinocyte growth inhibition. Enhancing HN2 efflux from keratinocytes may represent a novel strategy for mitigating vesicant-induced cytotoxicity.

Key words: xenobiotic transporters < biotransformation and toxicokinetics; chemical & biological weapons < agents; alkylating agents < agents; glutathione < biotransformation and toxicokinetics; cutaneous or skin toxicity < systems toxicology; membrane biology < systems toxicology.

Sulfur mustard (2,2′-dichlorodiethyl sulfide) and nitrogen mustard (mechlorethamine, methylbis(2-chloroethyl)-amine, HN2) are nonspecific bifunctional alkylating agents that were produced as chemical warfare agents. The skin is a major target for mustards and depending on the concentration and duration of exposure, these electrophiles can cause inflammation, edema, and blistering (Jan *et al.*, 2014; Rajski and Williams, 1998; Shakarjian *et al.*, 2010). Accumulating evidence suggests that a

major detoxification mechanism of 2 related mustards, chlorambucil and melphalan, is their rapid transport out of cells (Barnouin *et al.*, 1998; Cui *et al.*, 1999; Dean *et al.*, 2001; Higgins and Hayes, 2011; Jedlitschky *et al.*, 1996; Paumi *et al.*, 2001). Multidrug resistance protein 1 (ABCB1, P-glycoprotein, or Mdr1/MDR1) was discovered as a protein overexpressed in tumors that catalyzes the extracellular transport of many mainly lipophilic cytotoxic agents (Van der Bliek *et al.*, 1988). Of interest are findings that cells lacking functional Mdr1/MDR1 still export a wide variety of toxic agents including doxorubicin, daunorubicin, and vincristine (Barrand *et al.*, 1994; Grant *et al.*, 1994; Klaassen and Aleksunes, 2010; Stride *et al.*, 1997). This discovery led to the identification of a distinct class of transporters known as multidrug resistance-associated proteins (ABCCs, Mrps/MRPs) (Chen and Tiwari, 2011). Mdr1/MDR1 and Mrps/MRPs are members of the ATP-binding cassette superfamily of proteins that catalyze the efflux of ligands from cells at the expense of ATP (Choi, 2005; Dean *et al.*, 2001). Mrp1/MRP1 was the first Mrp/MRP cloned and is thought to function by transporting organic anions including various lipid mediators, the electrophile scavenger and antioxidant glutathione, cysteinyl leukotriene C4, a pro-inflammatory eicosanoid mediator, and other electrophiles modified by glutathione, including nitrogen mustards (Barnouin *et al.*, 1998; Cui *et al.*, 1999; Higgins and Hayes, 2011; Jedlitschky *et al.*, 1996; Paumi *et al.*, 2001). A number of additional Mrp/MRP efflux pumps have since been identified including Mrp2/MRP2, Mrp3/MRP3, Mrp4/MRP4, and Mrp6/MRP6 which are also thought to mediate export of glutathione-conjugated electrophiles (Klaassen and Aleksunes, 2010). MRP2 has been reported to transport glutathione-conjugated chlorambucil, although much less efficiently than MRP1 (Smitherman *et al.*, 2004). MRP2 is also known to efflux glutathione-conjugated cisplatin, a crosslinking agent with a generally similar molecular mechanism of action as HN2 (Ishikawa and Ali-Osman, 1993; Wen *et al.*, 2014a). Mrp3/MRP3 stimulates the efflux of glutathione-conjugated prostaglandin J2, and also effluxes various glucuronides, as well as endogenous metabolites including bile acids and glycine-conjugated cholic acid (Paumi *et al.*, 2003; Zelcer *et al.*, 2003). Mrp4/MRP4 catalyzes the efflux of glutathione-conjugated bimane (Bai *et al.*, 2004), and Mrp6/MRP6 has been associated with the transport the endogenous glutathione conjugate leukotriene C_4, as well as the glutathione conjugate of 2, 4-dinitrophenyl (Belinsky *et al.*, 2002).

Because glutathione readily modifies HN2, and because Mrp1/MRP1, Mrp2/MRP2, Mrp3/MRP3, Mrp4/MRP4, and Mrp6/MRP6 are the predominant mediators of glutathione adduct efflux, our studies were aimed at examining the role of these transporters in HN2-induced cytotoxicity in mouse keratinocytes. We also assessed the role of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in mediating the response of keratinocytes to HN2-induced growth inhibition. To do so, we used sulforaphane, an activator of this transcription factor pathway (McMahon *et al.*, 2001), known to induce Mrp1/MRP1 through the Nrf2 pathway (Sibhatu *et al.*, 2008; Telang *et al.*, 2009). Our data indicate that efflux transporters are important in regulating sensitivity to HN2 in keratinocytes. Strategies that enhance efflux of sulfur mustard or HN2 from target cells may be effective in ameliorating vesicant-induced skin injury.

MATERIALS AND METHODS

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), trypsin, and penicillin/streptomycin were purchased from Life Technologies (Rockville, Maryland). Rabbit primary antibody to Nrf2 was from Santa Cruz Biotechnologies (Santa Cruz, California). Rat primary Mrp1 antibody, rabbit heme oxygenase-1 (Ho-1) primary antibody, and mouse Mrp2 primary antibody were from Enzo Life Sciences (Farmingdale, New York). Goat NAD(P)H dehydrogenase [quinone] 1 (Nqo1) primary antibody was from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit- and rabbit anti-mouse secondary antibodies, precast polyacrylamide gels, Coomassie blue, and the bicinchoninic acid (BCA) protein assay kit were from Bio-Rad Laboratories (Hercules, California). Chemiluminescence reagents were purchased from Millipore (Billerica, Massachusetts). Sulforaphane, HN2, MK-571 (3-[[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid), mouse primary antibody to Gapdh, protease inhibitor cocktail (P2714) consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64 [trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane], bestatin, leupeptin, and aprotinin, goat anti-rat secondary antibody, rabbit anti-goat secondary antibody, and all other chemicals were purchased from Sigma-Aldrich (St Louis, Missouri), unless otherwise specified.

Cells. PAM212 cells were obtained from Dr Stuart Yuspa (National Institutes of Health) and were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml) and cultured at 37°C in 5% CO_2 in a humidified incubator. Primary mouse epidermal keratinocytes were isolated from the skin of newborn C57BL/6J wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, Maine) or C57BL/6J Nrf2$^{-/-}$ mice (Chan *et al.*, 1996; Shen *et al.*, 2005), bred at the Rutgers University animal care facility, as previously described (Hager *et al.*, 1999; Zheng *et al.*, 2014). Cells were grown in CnT-Prime medium (ZenBio, Inc, Research Triangle Park, North Carolina) on culture plates coated with collagen IV (Black *et al.*, 2008; Zheng *et al.*, 2014). The genotype of Nrf2$^{-/-}$ keratinocytes was confirmed using PCR (Chan *et al.*, 1996; Shen *et al.*, 2005).

Western blotting. Western blotting was performed as previously described (Hu *et al.*, 2006; Zheng *et al.*, 2014). Briefly, to prepare total cell lysates, cells were suspended in buffer containing 20 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, pH 7.4, and protease inhibitor cocktail, transferred into 1.5 ml Eppendorf microcentrifuge tubes, and centrifuged (750 × g, 10 min, at 4°C). Total protein in supernatants was determined by the BCA protein assay kit using bovine serum albumin as the standard. Nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, Illinois). Lysates (18–27-μg protein/well) were electrophoresed on 10.5–14% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in Tris buffer supplemented with 5% milk at room temperature for 1 h. Blots were then incubated overnight at 4°C with primary antibodies, washed with tTBS (Tris-buffered saline supplemented with 0.1% Tween 20), and incubated with horseradish peroxidase-conjugated secondary antibodies. After 1 h at room temperature, proteins were visualized by chemiluminescence.

RNA isolation and real-time quantitative PCR (qPCR). The medium of PAM212 cells and primary mouse keratinocytes, grown to confluence in 6-well tissue culture dishes, was replaced with 3-μM sulforaphane or vehicle control. After 3 h, RNA was isolated and analyzed by qPCR as previously described (Aleksunes *et al.*, 2013; Wen *et al.*, 2014a,b). Total RNA was isolated using an RNeasy Mini kit (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. cDNA was

synthesized using Moloney murine leukemia virus, (M-MLV) reverse transcriptase, diluted in RNase-DNase-free water and Mrp1, Mrp2, Mrp3, Mrp4, Mrp5, Mrp6, Ho-1, and Nqo1 mRNA quantified by qPCR using Sybr Green for detection of amplified products using a Viia7 RT-PCR system (Applied Biosystems, Carlsbad, California). The forward (5′–3′) and reverse (3′–5′) primer sequences used are shown in Table 1. The housekeeping gene ribosomal protein l13a (Rpl13a) was used to normalize all values. Average raw C_t values in control PAM212 cells were 22.4 for Mrp1, 28.5 for Mrp2, 24.7 for Mrp3, 22.4 for Mrp4, 22.9 for Mrp5, 33.4 for Mrp6, 30.3 for Nqo1, 20.4 for Ho-1, and 15.0 for Rpl13a. Average raw C_t values in untreated WT primary mouse keratinocytes were 19.7 for Mrp1, 28.2 for Mrp2, 23.3 for Mrp3, 21.9 for Mrp4, 22.6 for Mrp5, 30.2 for Mrp6, 24.2 for Nqo1, 20.4 for Ho-1, and 15.6 for Rpl13a, and average raw C_t values for untreated primary keratinocytes from Nrf2$^{-/-}$ mice were 19.9 for Mrp1, 28.6 for Mrp2, 27.1 for Mrp3, 21.9 for Mrp4, 21.3 for Mrp5, 31.2 for Mrp6, 27.7 for Nqo1, 21.4 for Ho-1, and 15.6 for Rpl13a. Although differences in primer efficiency can account for some variability, differences in raw C_t values indicate that Mrp1 is much more highly expressed than Mrp2, Mrp3, or Mrp6. Mrp4 and Mrp5 are expressed at similar levels to Mrp1. C_t values for Mrp1-6, Nqo1, and Ho-1 mRNA were first converted to delta C_t values by comparing to the reference gene, Rpl13a, and then to delta delta C_t values by designating the average delta C_t values for vehicle control samples in each group.

Cell growth inhibition studies. Cell growth inhibition by HN2 was determined as previously described (Mariano *et al*., 2002; Martey *et al*., 2002). Briefly, the medium of PAM212 cells or primary mouse keratinocytes, plated at a density of 1.8–3.0 × 10^4 cells/well in 24-well tissue culture dishes, was replaced with 0.25 ml of serum-free growth medium supplemented with 3μM sulforaphane or vehicle control. After 3 h, cells were then treated with increasing concentrations of HN2. Thirty minutes later, the cells were washed twice with 'Hanks' buffered salt solution and refed with fresh drug-free growth medium. After an additional 72 h, the cells were removed from the dishes with trypsin and counted using a Z1 Coulter Particle Counter (Beckman Coulter, Brea, California). Concentrations of HN2 that caused 50% growth inhibition (IC_{50}) were then determined. In some experiments, cells were pretreated with 25 μM MK-571 or control for 1 h, prior to treatment with sulforaphane.

Assay for MRP functional activity. The fluorescent ligand calcein was used to monitor Mrp functional activity in suspensions of intact cells as previous described (Bircsak *et al*., 2013). Briefly, cells (10^5 cells/0.1 ml) were seeded into 96-well round bottom plates, centrifuged (500 × g, 5 min, 4°C), resuspended in serum-free medium supplemented without and with 3-μM sulforaphane and incubated for 3 h at 37°C. The cells were then washed with PBS and resuspended in 100 μl of serum-free medium containing 1 μM calcein acetoxymethyl ester (calcein-AM) in the presence or absence of MK-571. After 30 min at 37°C (*uptake period*), cells were washed, resuspended in 50 μl PBS and analyzed for fluorescence using a Cellometer Vision cell counter fitted with a VB-595-402 (excitation/emission 495/515 nm) filter cube (Nexcelom Bioscience, Lawrence, Massachusetts). Cells were then incubated in serum-free medium with or without MK-571 for 60 min to allow calcein efflux from the cells (*efflux period*). After washing, cell fluorescence was assessed. Differences in fluorescence after the uptake phase and the efflux phase provided an indication of MRP-mediated transport (Bircsak *et al*., 2013; Olson *et al*., 2001).

Data analysis. GraphPad Prism (GraphPad software, San Diego, California) was used for calculation of all IC_{50} values and for all statistical analysis. ImageStudioLite (LI-COR Biosciences, Lincoln Nebraska) was used for all densitometric analysis of Western blots.

RESULTS

Expression of Mrp1 in Mouse Keratinocytes

PAM212 cells and primary mouse keratinocytes were found to express Mrp1 mRNA and protein (Figs. 1 and 2). Mrp1 was functionally active in both cell types as indicated by their ability to export calcein, an Mrp transporter substrate (Bircsak *et al*., 2013; Olson *et al*., 2001). Treatment with the Mrp1 specific inhibitor, MK-571, inhibited calcein efflux in both cell types; primary keratinocytes were significantly more sensitive to MK-571 than PAM212 cells (Figs. 1 and 2). Mrp2, Mrp3, and Mrp6 mRNA were also expressed in the cells, but at lower levels than Mrp1, while Mrp4 and Mrp5 mRNA were expressed at similar levels to Mrp1 (see Materials and Methods for raw C_t values). Both PAM212 cells and primary keratinocytes were highly sensitive to growth inhibition by HN2 (IC_{50} = 1.0 μM for PAM212 cells and 1.4 μM for primary keratinocytes). Treatment of primary keratinocytes with MK-571 markedly enhanced growth inhibition induced by HN2 (IC_{50} = 0.48 μM, Table 2) but caused only a small increase in sensitivity in PAM212 cells (IC_{50} = 0.73 μM, Figure 3).

Effects of Sulforaphane on Mrp1 in Mouse Keratinocytes

Sulforaphane is an activator of the Nrf2 transcription factor pathway, which is known to induce Mrp1/MRP1 (Sibhatu *et al*., 2008; Telang *et al*., 2009). Sulforaphane was found to upregulate expression of Mrp1 mRNA in both PAM212 cells and primary keratinocytes (mRNA expression increased 1.8- and 2.1-fold in PAM212 cells and primary mouse keratinocytes, Figs. 1 and 2) by statistically significant amounts. Mrp2-6 mRNA were expressed in both cell types; their expression was not altered by sulforaphane (Figs.

TABLE 1. PCR Primer Sequences

Gene	Forward 5′–>3′	Reverse 5′–>3′
Mrp1/Abcc1	GCTGTGGTGGGCGCTGTCTA	CCCAGGCTCAGCCACAGGAA
Mrp2/Abcc2	AGCAGGTGTTCGTTGTGTGT	AGCCAAGTGCATAGGTAGAGAAT
Mrp3/Abcc3	CTGGGTCCCCTGCATCTAC	GCCGTCTTGAGCCTGGATAAC
Mrp4/Abcc4	CCAGACCCTCGTTGAAAGAC	TGAAGCCGATTCTCCCTTC
Mrp5/Abcc5	AGGGCAGCTTGTGCAGGTGG	TGCTGTTCCCGCTTCCTTGCT
Mrp6/Abcc6	TGTCTGCAAGCCATCGGACTG TTTG	TGGAAAAGCGGTTCAGCAGGTTCC
Nqo1	CTCAACATCTGGAGCCATGG	CAGCTCACCTGTGATGTCATT
Ho-1	GGTGACAGAAGAGGCTAAGACCGC	GCAGTATCTTGCACCAGGCTAGCA
Rpl13a	GGGCAGGTTCTGGTATTGGAT	GGCTCGGAAATGGTAGGGG

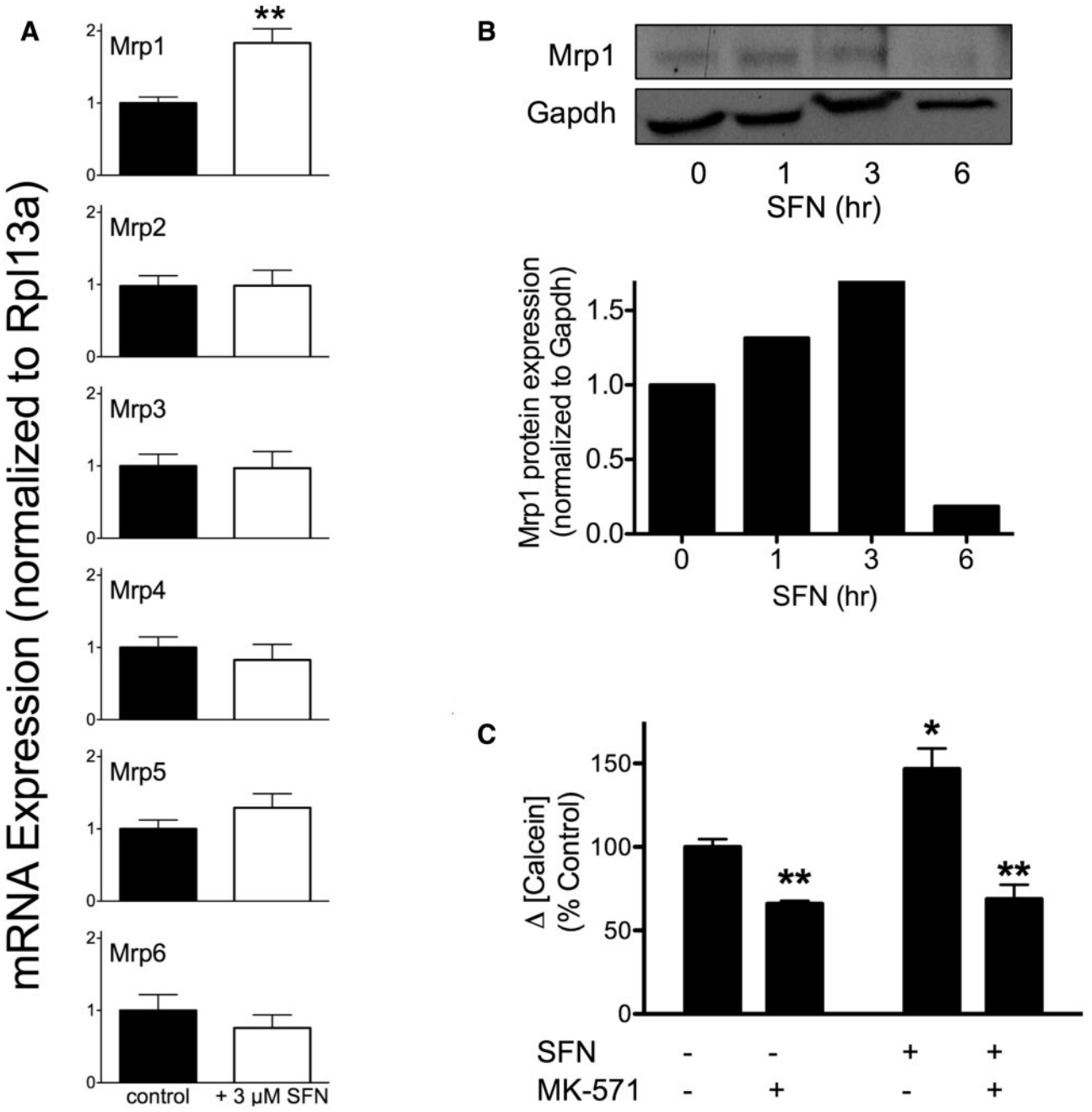

FIG. 1. Effects of sulforaphane on Mrp expression in PAM212 cells. Panel A, PAM212 cells were treated with 3 μM sulforaphane (SFN) or vehicle control for 3 h. mRNA was then isolated and analyzed for Mrp1-6 expression. Data are mean ± SEM ($n = 4$–6). $^{**}P < .01$ compared with control. Panel B, Cells were treated with 3 μM sulforaphane or control; cell homogenates were prepared at the indicated times and analyzed for Mrp1 protein expression by Western blotting. Densitometric quantification of the Western blot appears below the blot. Panel C, Cells were analyzed for Mrp1 functional activity using calcein-AM in the absence or presence of 25 μM MK-571, an Mrp1 inhibitor, and/or 3 μM sulforaphane as described in the Materials and Methods. Fluorescence differences not inhibited by MK-571 are nonspecific efflux from the cells. Data are mean ± SEM ($n = 3$). $^{**}P < .01$, $^{*}P < .05$ compared with untreated control.

1 and 2). Treatment with sulforaphane for 1 or 3 h increased Mrp1 protein expression in both PAM212 cells and primary mouse keratinocytes (protein expression increased 1.3- and 1.8-fold in PAM212 cells following sulforaphane treatment for 1 and 3 h, respectively; protein expression increased 2.1- and 1.9-fold in primary mouse keratinocytes following sulforaphane treatment for 1 and 3 h, respectively). Upregulation of Mrp1 by sulforaphane resulted in an increase in calcein efflux in both cell types, which was inhibited by MK-571 (calcein efflux increased to 150% and 210% of control for PAM212 cells and primary mouse keratinocytes, respectively, Figs. 1 and 2). In PAM212 cells and primary keratinocytes, increases in Mrp1 by sulforaphane were associated with decreased sensitivity of the cells to HN2-induced growth inhibition, an effect that was blocked by MK-571 (Figure 3 and Table 2).

We next determined if the effects of sulforaphane on Mrp1 were Nrf2-dependent using keratinocytes isolated from $Nrf2^{-/-}$ mice. The constitutive levels of Mrp1 mRNA and protein expressed in these cells were not altered by sulforaphane (Figure 2). As observed in keratinocytes from WT mice, these cells

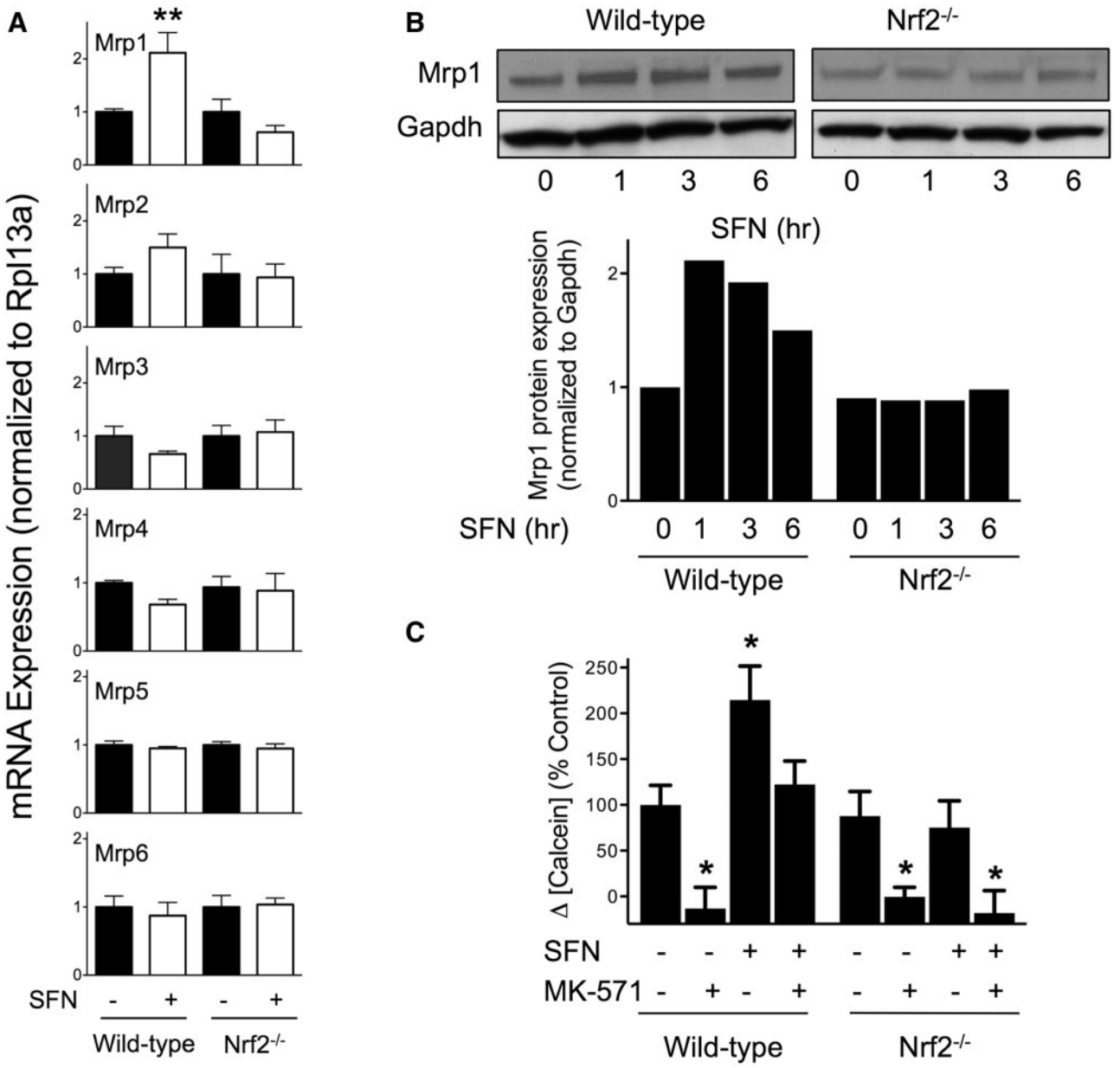

FIG. 2. Effects of sulforaphane on Mrp transporters in primary mouse keratinocytes. Panel A, Primary WT and Nrf2$^{-/-}$ keratinocytes were treated with 3 μM sulforaphane (SFN) or vehicle control for 3 h. mRNA was then isolated and analyzed for Mrp1-6 expression. Data are mean ± SEM ($n = 4$–6). **$P < .01$ compared with control. Panel B, Cells were treated with 3 μM sulforaphane or control; cell homogenates were prepared at the indicated times and analyzed for Mrp1 protein expression by Western blotting. Densitometric quantification of the Western blot appears in the panel below the blot. Panel C, Cells were analyzed for Mrp1 functional activity using calcein-AM in the absence or presence of 25 μM MK-571, an Mrp1 inhibitor, and/or 3 μM sulforaphane as described in the Materials and Methods. Data are means ± SEM ($n = 5$). *$P < .05$ compared with untreated control for each cell type.

also expressed Mrp2-Mrp6 mRNA, and sulforaphane did not alter expression of these genes. In Nrf2$^{-/-}$ keratinocytes, Mrp1 was functionally active as MK-571 readily inhibited calcein efflux; however, sulforaphane did not alter Mrp1 functional activity (Figure 2). Nrf2$^{-/-}$ keratinocytes were found to be more sensitive than WT cells to HN2 ($IC_{50} = 0.31$ vs 1.4 μM, respectively, Table 2). Treatment of Nrf2$^{-/-}$ cells with MK-571, like WT cells, increased their sensitivity to HN2 ($IC_{50} = 0.08$, Table 2). In contrast to WT cells, sulforaphane did not protect Nrf2$^{-/-}$ cells from HN2-induced growth inhibition ($IC_{50} = 0.14$ μM), and it did not protect these cells from the actions of MK-571 ($IC_{50} = 0.07$ μM).

Effects of Sulforaphane on Nrf2 and Target Genes Nqo1 and Ho-1 in Mouse Keratinocytes

In PAM212 cells, sulforaphane increased expression of nuclear (1.9- and 2.1-fold following treatment with sulforaphane for 3 and 6 h, respectively) and total cellular Nrf2 (3.4- and 4.7-fold following treatment with sulforaphane for 3 and 6 h, respectively). We observed upregulation of mRNA expression of 2 downstream gene products regulated by Nrf2, Nqo-1, and Ho-1, in both PAM212 cells and primary mouse keratinocytes following treatment with sulforaphane. For Nqo1, mRNA expression increased 2.5- and 4.8-fold in PAM212 cells and primary mouse keratincoytes, respectively, following

TABLE 2. Effects of Sulforaphane on the IC_{50} of HN2-Induced Growth Inhibition in Mouse Keratinocytes

	Keratinocytes		
	PAM212[a]	WT	$Nrf2^{-/-}$
Growth Inhibition by HN2 (IC_{50}, μM)[b]			
Control	1.0	1.4	0.31
+ 3 μM SFN	13.0	4.8	0.14
+ 25 μM MK-571	0.73	0.48	0.08
+ 3 μM MK-571 + 25 μM SFN	0.63	0.27	0.07

[a]Mouse keratinocytes were treated with 3 μM sulforaphane or vehicle control in serum-free medium. Three hours later, cells were treated with increasing concentrations (0.01–30 μM) of HN2. After an additional 30 min, cells were washed and refed with drug-free growth medium. Seventy-two hours later, cell number was determined using a Coulter Counter. In some experiments, cells were treated with 25 μM MK-571 in serum-free medium 1 h prior to sulforaphane. MK-571 remained in the medium when the cells were treated with sulforaphane and HN2.

[b]IC_{50} is the concentration of HN2 inhibiting cell growth by 50%.

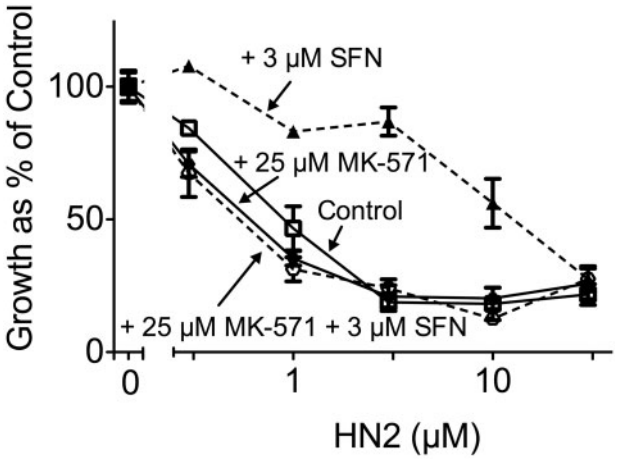

FIG. 3. Effects of sulforaphane on HN2-induced growth inhibition. PAM212 cells were treated with 3 μM sulforaphane or vehicle control in serum-free medium. After 3 h, increasing concentrations of HN2 were added to the medium. After an additional 30 min, cells were washed and refed with drug-free growth medium. Seventy-two hour later, cell number was determined using a Coulter Counter. In some experiments, cells were pretreated with 25 μM MK-571, an Mrp1 inhibitor, for 1 h prior to treatments with sulforaphane. MK-571 remained in the medium when the cells were treated with sulforaphane and HN2. Data are the mean ± SEM ($n = 3$).

sulforaphane treatment, while Ho-1 mRNA expression increased 8.1- and 12-fold in PAM212 cells and primary mouse keratincoytes, respectively.

In PAM212 cells and primary keratinocytes, sulforaphane also increased protein expression of Nqo1 and Ho-1 (Figs. 4 and 5). In primary keratinocytes from $Nrf2^{-/-}$ mice, Nqo1 and Ho-1 mRNA and protein expression were unaffected by treatment with sulforaphane.

DISCUSSION

Mrp1/MRP1 is a transmembrane protein important in limiting the toxicity of a number of cytotoxic compounds largely via its ability to mediate ATP-dependent cellular efflux of glutathione-, glucuronide-, and other amphipathic toxin conjugates (Klaassen and Aleksunes, 2010). Earlier studies reported that glutathione S-conjugates of HN2 derivatives, including chlorambucil and melphalan, are exported from cells by Mrp1/MRP1, an activity that contributes to reducing their toxicity by eliminating inhibitors of cytosolic glutathione S-transferase enzymes (Barnouin *et al.*, 1998; Jedlitschky *et al.*, 1996; Paumi *et al.*, 2001). Our studies show that mouse keratinocytes express Mrp efflux transporters. We also demonstrate that activity of Mrp1 can regulate sensitivity to HN2-induced cytotoxicity.

Both PAM212 cells and primary WT mouse keratinocytes were found to express Mrp1 mRNA and protein; greater levels of Mrp1 protein were noted in primary WT keratinocytes. We also found significantly more Mrp1 efflux activity in primary WT keratinocytes when compared with PAM212 cells. Differences in Mrp1 activity between WT keratinocytes and PAM212 cells may be due to differences in expression of the transporter in primary cells and a transformed cell line. In this regard, varying amounts of Mrp1 have been reported in different tumor cell lines isolated from human stomach tumors and this may be due to differences in expression of regulatory elements controlling MRP1 gene expression (Obuchi *et al.*, 2013). The reduced activity of Mrp1 in PAM212 cells likely accounts for our findings that MK-571 caused a relatively small increase in sensitivity to HN2-induced growth inhibition in these cells ($IC_{50} = 1.0$ vs 0.73 μM, in the absence and presence of MK-571, respectively). In contrast, in WT keratinocytes, MK-571 causes a marked increase in sensitivity to HN2-induced growth inhibition ($IC_{50} = 1.4$ vs 0.48 μM, in the absence and presence of MK-571, respectively). These results are consistent with earlier reports showing that the sensitivity of stomach cancer cell lines to etoposide, an Mrp1/MRP1 substrate, was directly correlated with levels of expression of MRP1, and that MK-571 was most effective in increasing the sensitivity of cells to etoposide that highly expressed MRP1 (Obuchi *et al.*, 2013).

Mrp1/MRP1 genes have 2 antioxidant/electrophile response elements—the sequences that signal the transcription factor Nrf2 to initiate RNA synthesis—in their promoter regions (Ji *et al.*, 2013). These sequences are TGAGCGGGC starting from base pairs -66 to -57 and GTGACTCAGC from positions -499 to -490 (Ji *et al.*, 2013). It has previously been shown that sulforaphane, an isothiocyanate found in cruciferous vegetables such as broccoli, bok choy, cauliflower, and Brussels sprouts, that activates the Nrf2 pathway, upregulates MRP1 mRNA and protein expression in HepG2 human liver cells and A549 human lung cells (Harris and Jeffery, 2008). Similarly, we found that sulforaphane upregulates Mrp1 mRNA and protein expression, and efflux activity in PAM212 cells and primary WT mouse keratinocytes. In contrast, Mrp2, Mrp3, and Mrp6, efflux pumps that also transport glutathione-conjugated electrophiles (Klaassen and Aleksunes, 2010), were expressed only at low levels in keratinocytes and their expression, as well as expression of Mrp4 which was expressed at similar levels to Mrp1, was not altered by sulforaphane. These findings support the notion that expression of effux transporters is cell type specific (Klaassen and Aleksunes, 2010). In both PAM212 cells and primary keratinocytes, sulforaphane was found to be functionally active as reflected by its ability to readily activate Nrf2 causing its translocation from the cytosol to the nucleus. Sulforaphane is thought to activate Nrf2 by binding to cysteine-151 on Keap1, a chaperone protein that sequesters Nrf2 in the cytosol (Niture *et al.*, 2014). Formation of a sulforaphane-Keap1 adduct results in a conformational change in the protein, causing it to dissociate from Nrf2, which then translocates into the nucleus, binds to antioxidant/electrophile response elements and regulates transcription of antioxidant/electrophilic response genes including Nqo1, Ho-1, and Mrp1

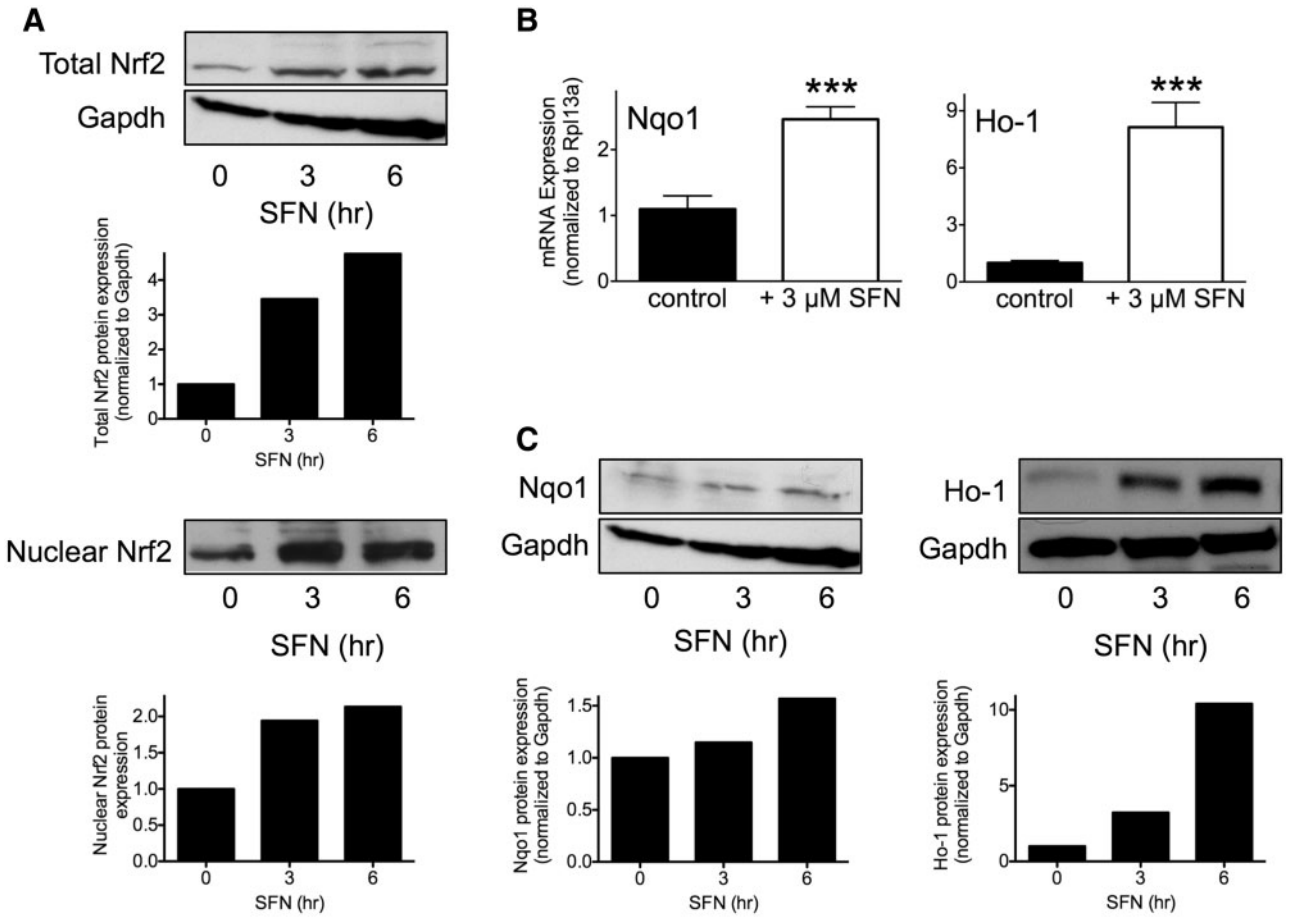

FIG. 4. Activation of Nrf2 and downstream targets by sulforaphane in PAM212 cells. Panel A, Cells were treated for 0, 3, or 6 h with 3 μM sulforaphane in serum-free medium, washed, and refed with growth medium. After 24 h, total cell lysates and nuclear fractions were prepared and analyzed for Nrf2 expression by Western blotting. Gadph was used as a protein loading control for total cell homogenates. Coomassie blue stain was used to confirm even loading of nuclear samples (data not shown). Densitometric quantification of each Western blot appears below the blots. Panel B, Cells were treated with 3 μM SFN or control for 3 h. RNA was then extracted and analyzed for Nqo1 and Ho-1 mRNA expression. Data are mean ± SEM ($n = 4$–6). ***$P < .001$ compared with control. Panel C, Cells were treated with 3 μM sulforaphane for 0, 3, or 6 h in serum-free medium, washed, and refed with growth medium. After an additional 24 h, cell homogenates were prepared and analyzed for Nqo1 and Ho-1 protein expression by Western blotting. Gapdh was used as a protein loading control. Densitometric quantification of each Western blot appears below the blots.

(Niture *et al.*, 2014). This is consistent with our data showing that sulforaphane upregulated mRNA and protein expression of the Nrf2 target genes, Nqo1 and Ho-1, in the keratinocytes. The fact that increases in Nqo1 and Ho-1, as well as Mrp1, were not observed in primary keratinocytes from $Nrf2^{-/-}$ mice indicates that upregulation of these genes by sulforaphane is Nrf2-dependent (Ade *et al.*, 2009; Alfieri *et al.*, 2013; Sibhatu *et al.*, 2008). In the absence of an activator, Nrf2 is tagged by ubiquitin for proteosomal degradation (Niture *et al.*, 2014). Because Nrf2 translocates to the nucleus and escapes proteosomal degradation, total Nrf2 protein, as well as nuclear Nrf2 protein increases. Consistent with this model, we found increases in both total and nuclear Nrf2 in PAM212 cells treated with sulforaphane. Similar activation of Nrf2 following sulforaphane treatment has been described in several different cell types, including mouse (Manandhar *et al.*, 2007) and human keratinocytes (Gao *et al.*, 2001).

Sulforaphane was found to partially protect PAM212 cells and primary WT keratinocytes from HN2-induced growth inhibition, an activity blocked by MK-571. These data are consistent with the idea that sulforaphane-induced increases in efflux of glutathione-conjugated HN2 can reduce HN2-induced growth inhibition. The monoglutathionyl conjugates of melphalan and chlorambucil are transported by MRP1 (Paumi *et al.*, 2001), and it is likely that efflux of HN2 via Mrp1 in mouse keratinocytes is similarly mediated via a monoglutathionyl conjugate. However, because HN2 is a bifunctional alkylating agent, the monoglutathionyl HN2 conjugate still contains a highly reactive chloroethyl side chain that has the potential to react with macromolecules in cells resulting in toxicity. In this regard, crosslinks between glutathione, sulfur mustard, and guanine have been reported in mouse skin, presumably formed in the tissue via glutathionyl sulfur mustard conjugates reacting with DNA (Batal *et al.*, 2015). Analogous crosslinks have also been observed between glutathione and DNA with other bifunctional alkylating agents, including 1,2-dibromoethane (diethylene bromide) (Cmarik *et al.*, 1990; Guengerich *et al.*, 1990; Inskeep and Guengerich, 1984; Kim and Guengerich, 1997; Koga *et al.*, 1986; Ozawa and Guengerich, 1983) and 1,2,3,4-diepoxybutane (Cho and Guengerich, 2012a,b; Cho *et al.*, 2010). Endogenously generated glutathione conjugates of bifunctional lipid peroxidation products such as 4-oxo-2(E)-nonenal have also been reported to react with cellular macromolecules and initiate toxicity (Blair, 2006). We speculate that sulforaphane-induced suppression of HN2-induced growth inhibition in keratinocytes is due to its ability to increase monoglutathionyl HN2 efflux, and thus

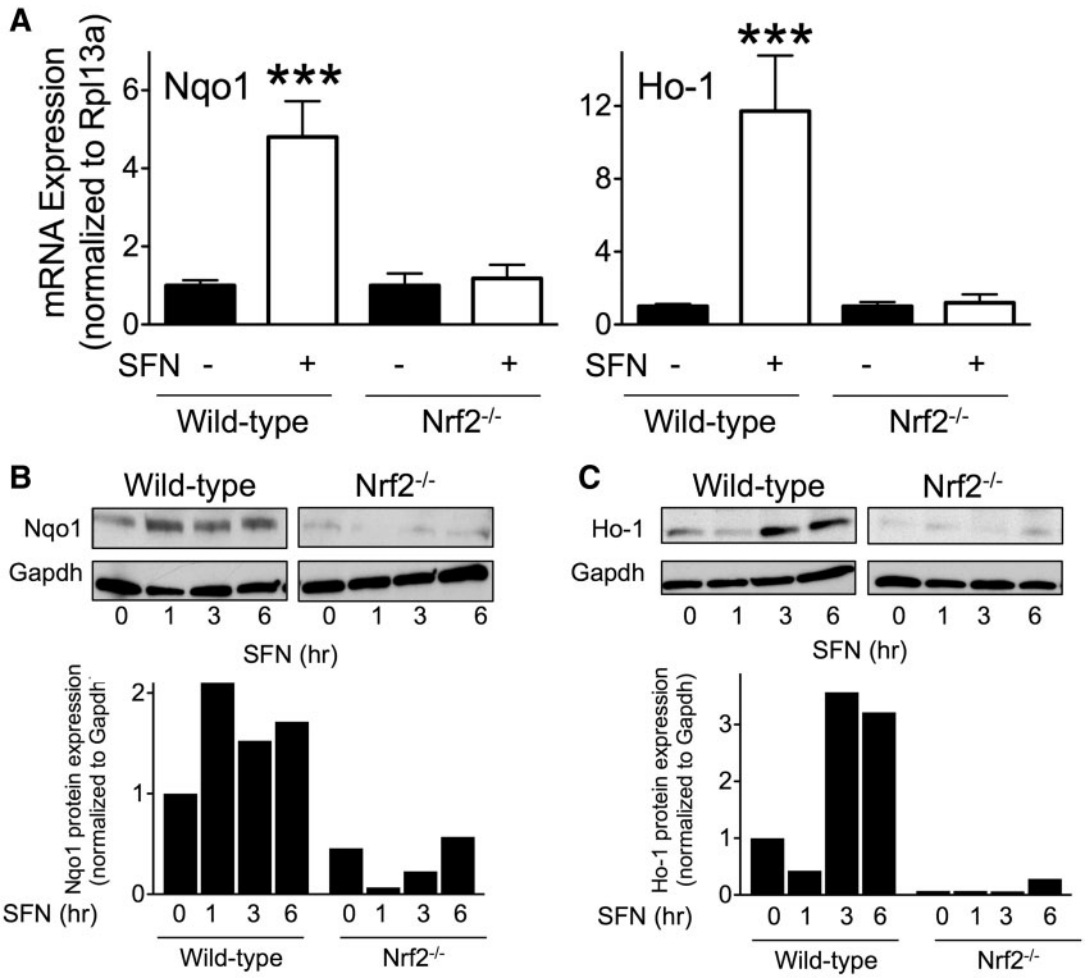

FIG. 5. Effects of sulforaphane on Nqo1 and Ho-1 expression in primary mouse keratinocytes. Panel A, Primary keratinocytes from WT and Nrf2$^{-/-}$ mice were treated with 3 μM SFN or vehicle control in serum-free medium. After 3 h, RNA was extracted and analyzed for Nqo1 and Ho-1 mRNA expression. Data are mean ± SEM ($n = 4$–6). ***$P < .001$ compared with control. Panels B and C, Cells were treated with 3 μM SFN for 0, 1, 3, or 6 h, washed, and refed with growth medium. After 24 h, total cell lysates were prepared and analyzed for Nqo1 (Panel B) and Ho-1 (Panel C) protein expression. Gapdh was used as a protein loading control. Densitometric quantification of each Western blot appears below the blots.

reduce intracellular levels of a reactive and cytotoxic monofunctional alkylating agent. Conversely, decreasing monoglutathionyl HN2 efflux with MK-571 increases intracellular levels of this alkylating agent and increases HN2-induced toxicity. It is worth noting that sulforaphane has been previously shown to protect human keratinocytes against sulfur mustard (Gross *et al.*, 2006), as well as against the monofunctional half-mustard chloroethyl ethyl sulfide (Abel *et al.*, 2011, 2013); however, none of these previous works investigated the role that efflux transporters play in mediating this protection.

Of interest is our finding of increased sensitivity of keratinocytes from Nrf2$^{-/-}$ mice to HN2-induced growth inhibition ($IC_{50} = 1.4$ vs 0.31 μM, in WT and Nrf2$^{-/-}$ keratinocytes, respectively). In Nrf2$^{-/-}$ cells, a number of key gene products that protect against HN2-induced injury are altered, including those important in antioxidant defense, drug metabolism and DNA repair (Jain *et al.*, 2015; Owuor and Kong, 2002; Piberger *et al.*, 2014). These alterations likely result in increased cellular damage and greater cytotoxicity. In this regard, cells that lack functional Nrf2 have been reported to be more sensitive to cytotoxic agents, such as doxorubicin (Li *et al.*, 2014), arsenic (Yang *et al.*, 2012), and ionizing radiation (Mathew *et al.*, 2014). Similarities in expression and functional activity of Mrp1 in WT and Nrf2$^{-/-}$ keratinocytes indicate that differences in the sensitivity of these cells to HN2 are not due to differences in HN2 efflux. The fact that MK-571 sensitized Nrf2$^{-/-}$ keratinocytes to HN2 is consistent with the idea that Mrp1 is functionally active in the cells. Because Mrp1 was not altered following treatment of Nrf2$^{-/-}$ keratinocytes with sulforaphane, increases in sensitivity of the cells to HN2 following treatment with this isothiocyanate appear to be Mrp1-independent. This is supported by our findings that MK-571 further increased the sensitivity of sulforaphane-treated Nrf2$^{-/-}$ keratinocytes to HN2-induced growth inhibition.

We also observed that sulforaphane caused an increase in sensitivity to HN2-induced growth inhibition ($IC_{50} = 0.31$ vs 0.14 μM, without and with sulforaphane treatment, respectively) in Nrf2$^{-/-}$ keratinocytes. Sulforaphane is known to bind cysteine residues in many proteins (Hong *et al.*, 2005; Mi *et al.*, 2008), and it may be that, in the absence of Nrf2, sulforaphane targets additional proteins that contribute to toxicity. For example, sulforaphane has been reported to inhibit critical antioxidant enzymes, including thioredoxin reductase and glutathione reductase (Hu *et al.*, 2007) and this could result in increased

sensitivity to HN2. In this regard, previous studies from our laboratory have shown that HN2 can target thioredoxin reductase and inhibit its disulfide reductase activity (Jan *et al.*, 2014).

It is well recognized that the sensitivity of cells to many drugs is dependent on their expression of efflux transporter proteins. Our studies provide evidence that Mrp1 activity contributes to resistance to HN2; thus, enhancing Mrp1 activity by sulforaphane protects keratinocytes from HN2-induced cytotoxicity. Many clinical trials are ongoing with sulforaphane or sulforaphane-containing dietary supplements based on the idea that these agents will induce a protective response against exposures to electrophiles and oxidants (Riedl *et al.*, 2009; Shiina *et al.*, 2015; Singh *et al.*, 2014; Yanaka *et al.*, 2009). Our studies suggest that one pathway involved in cytoprotection is upregulation of efflux transporter activity. Further studies are required to determine whether sulforaphane enhances mustard efflux *in vivo* and whether it will be effective in mitigating mustard-induced skin injury.

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