Efficacy of Glutathione in Ameliorating Sulfur Mustard Analog-Induced Toxicity in Cultured Skin Epidermal Cells and in SKH-1 Mouse Skin In Vivo

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Received August 5, 2010; accepted October 20, 2010

ABSTRACT

Exposure to chemical warfare agent sulfur mustard (HD) is reported to cause GSH depletion, which plays an important role in HD-linked oxidative stress and skin injury. Using the HD analog 2-chloroethyl ethyl sulfide (CEES), we evaluated the role of GSH and its efficacy in ameliorating CEES-caused skin injury. Using mouse JB6 and human HaCaT epidermal keratinocytes, we observed both protective and therapeutic effects of exogenous GSH (1 or 10 mM) in attenuating a CEES-caused decrease in cell viability and DNA synthesis, as well as S and G₂M phase arrest in cell cycle progression. However, the protective effect of GSH was stronger than its ability to reverse CEES-induced cytotoxic effect. The observed effect of GSH could be associated with an increase in intracellular GSH levels after its treatment before or after CEES exposure, which

strongly depleted cellular GSH levels. *N*-Acetyl cysteine, a GSH precursor, also showed both protective and therapeutic effects against CEES-caused cytotoxicity. Buthionine sulfoximine, which reduces cellular GSH levels, caused an increased CEES cytotoxicity in both JB6 and HaCaT cells. In further studies translating GSH effects in cell culture, pretreatment of mice with 300 mg/kg GSH via oral gavage 1 h before topical application of CEES resulted in significant protection against CEES-caused increase in skin bifold and epidermal thickness, apoptotic cell death, and myeloperoxidase activity, which could be associated with increased skin GSH levels. Together, these results highlight GSH efficacy in ameliorating CEES-caused skin injury and further support the need for effective antioxidant countermeasures against skin injury by HD exposure.

Introduction

Sulfur mustard [HD, bis(2-chloroethyl)sulfide], a major chemical warfare agent, has been successfully deployed as a weapon in military conflicts (Graham et al., 2005; Paromov et al., 2007). The primary effect of HD exposure is cutaneous toxicity, in addition to ocular, respiratory, and other tissue injuries (Dacre and Goldman, 1996; Kehe et al., 2008; Rowell et al., 2009). HD penetrates into skin tissue, attacks mainly the dividing basal epidermal cells, and causes massive inflammatory response, apoptotic and necrotic cell death, as well as delayed excruciating vesication (Ray et al., 2000;

Henemyre-Harris et al., 2008; Hayden et al., 2009). HD is a powerful bifunctional alkylating agent, and its exposure causes the depletion of cellular antioxidant thiols, mainly GSH, which may lead to an increased production of ROS and membrane lipid peroxidation (Kehe and Szinicz, 2005; Mukhopadhyay et al., 2006; Paromov et al., 2007; Ruff and Dillman, 2007). These events lead to oxidative stress and macromolecular damage, including DNA damage, which trigger various signaling pathways and modulate gene expression, causing a series of HD-associated toxic responses (Paromov et al., 2007; Ruff and Dillman, 2007; Kehe et al., 2009; Shakarjian et al., 2010). HD-induced increase in inflammatory cells, including neutrophils in skin, also generates ROS, which promotes oxidative stress (Shakarjian et al., 2010).

Apart from HD, its monofunctional analog 2-chloroethyl ethyl sulfide (CEES) also causes several skin toxic effects comparable with HD, and therefore CEES is used extensively to uncover the mechanism of action of HD and to screen

This work was supported by the Countermeasures Against Chemical Threats (CounterACT Program), National Institutes of Health Office of the Director, and the National Institutes of Health National Institute of Environmental Health Sciences [Grant U54-ES015678].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.110.173708.

ABBREVIATIONS: HD, sulfur mustard; ROS, reactive oxygen species; CEES, 2-chloroethyl ethyl sulfide; NAC, *N*-acetyl-cysteine; AEOL 10150, manganese (III) meso-tetrakis (di-*N*-ethylimidazole)porphyrin; BSO, buthionine sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; FBS, fetal bovine serum; BrdU, 5-bromo-2'-deoxy-uridine; MPO, myeloperoxidase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin.

effective therapeutic agents (Han et al., 2004; Paromov et al., 2007; Gould et al., 2009; Tewari-Singh et al., 2009, 2010). HD/CEES-caused oxidative stress results in the 8-oxo-2-deoxyguanosine DNA adduct as well as lipid and protein oxidation, which can cause inflammation and other toxic responses in skin (Pal et al., 2009; Black et al., 2010). The exogenous addition of antioxidants such as GSH, *N*-acetyl cysteine (NAC), vitamin E, superoxide dismutase, catalase, sulforaphane, quercetin, and catalytic antioxidants such as manganese (III) *meso*-tetrakis (di-*N*-ethylimidazole)porphyrin (AEOL 10150) has been reported to attenuate lung and skin injury by HD/CEES (Gross et al., 1993; Smith et al., 1997; Amir et al., 1998; Han et al., 2004; Arfsten et al., 2007; Paromov et al., 2007, 2008; Gould et al., 2009; Black et al., 2010; O'Neill et al., 2010). Whereas most of these efficacy studies with antioxidants indicate that they have beneficial effects in attenuating HD/CEES-caused lung injury, there are few, if any, reports of efficacy studies with GSH and its associated mechanism of action in relevant skin epidermal cells and skin toxicity models of HD/CEES (Paromov et al., 2007).

The results from our recent study in mouse epidermal JB6 and human epidermal HaCaT cells suggest that the DNA-damaging effect of CEES activates ataxia telangiectasia-mutated/ataxia telangiectasia-Rad3-related cell cycle checkpoint signaling as well as caspase-poly(ADP-ribose) polymerase pathways, which lead to apoptosis and necrosis (Tewari-Singh et al., 2010). In addition, the results from our study using the SKH-1 hairless mouse model indicate that CEES induces oxidative stress and the activation of interlinked complex mechanisms of CEES-mediated skin inflammation and injury (Pal et al., 2009; Tewari-Singh et al., 2009). To analyze further the role of GSH and oxidative stress in CEES-induced biological and molecular responses in these skin toxicity models, we evaluated the efficacy of exogenous GSH in attenuating CEES-caused skin injury in both cell culture and mouse skin models. Our results further support the involvement of GSH and oxidative stress in molecular mechanisms that lead to CEES-caused skin injury, which form the basis for the development of effective antioxidant therapies alone or in combination with other identified countermeasures in attenuating HD-caused skin injury.

Materials and Methods

Chemicals, Culture, and Assay Materials. HD ($\text{ClCH}_2\text{CH}_2\text{SCH}_2\text{CH}_2\text{Cl}$) analog, CEES ($\text{CH}_3\text{CH}_2\text{SCH}_2\text{CH}_2\text{Cl}$), buthionine sulfoximine (BSO; $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_3\text{S}$), GSH ($\text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_6\text{S}$), NAC ($\text{C}_5\text{H}_9\text{NO}_3\text{S}$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and saponin and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO). Minimal essential medium and Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin G, streptomycin sulfate, and gentamycin for cell culture were obtained from Invitrogen (Carlsbad, CA). The 5-bromo-2'-deoxy-uridine (BrdU) colorimetric kit was obtained from Roche Applied Science (Indianapolis, IN). The fluorescent myeloperoxidase (MPO) detection kit was purchased from Cell Technology (Mountain View, CA); eosinophil peroxidase and catalase (3-amino-1,2,4-triazole) inhibitors were obtained from Sigma-Aldrich. The GSH-Glo glutathione assay kit was obtained from Promega (Madison, WI), and the Bio-Rad DC protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

Culture and Treatment of Cells. JB6 and HaCaT cell culture and treatments were carried out as described previously (Tewari-Singh et al., 2010). In brief, JB6 cells were cultured in minimal

essential medium containing 5% heat-inactivated FBS and 25 $\mu\text{g}/\text{ml}$ gentamycin, and HaCaT cells were grown overnight in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 100 U/ml penicillin G with 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. Cells were grown overnight under standard culture conditions and treated with DMSO, 1 or 10 mM GSH, 25 or 50 mM NAC as controls, or 1 or 10 mM GSH with 25 or 50 mM NAC before or after exposure to 0.35 or 0.5 mM CEES in DMSO, or they were exposed to CEES in DMSO alone. These treatments and CEES exposures were carried out in the same growth medium without the removal of the GSH/NAC or CEES in the pre- or post-treatments. For BSO studies, 0.2 mM BSO was added to the media 6 h before CEES exposure. A required amount of CEES from the stock prepared in DMSO was mixed into the cell growth media and added to the 70 to 80% confluent cells immediately, as reported previously (Tewari-Singh et al., 2010). Desired GSH and NAC stocks were prepared in the cell growth media and adjusted to pH 7.0 before diluting to the concentration required for the treatment of cells. The final concentration of DMSO in the culture medium during treatments did not increase [0.1% (v/v)].

Measurement of Cell Viability and Cell Proliferation (DNA Synthesis). These measurements were carried out as reported previously (Tewari-Singh et al., 2010). In short, cell viability (MTT) and cell proliferation (BrdU) assays were conducted 24 or 48 h after the desired treatment of cells (2000 cells in every well) cultured overnight in 96-well culture plates. For the MTT assay, cells were incubated with 1 mg/ml MTT in serum-free medium at 37°C for 4 h; the MTT solution was removed thereafter, and absorbance was read after the addition of 100 μl of DMSO at 540 nm. The BrdU assay (which is based on a measurement of incorporation of BrdU, a thymidine analog, during DNA synthesis) was conducted according to the manufacturer's instructions. In brief, treated cells were incubated with BrdU, fixed, DNA denatured, and labeled with anti-BrdU mouse monoclonal antibody Fab. The BrdU antibody bound to newly synthesized cellular DNA, and complexes were detected via product quantification by measuring the absorbance at 370 nm (reference wavelength, 492 nm). For both MTT and BrdU assays, absorbance was read using a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA), and blank control readings were subtracted from all the sample readings taken.

Cell Cycle Analysis. Cell cycle analysis was carried out as described previously (Tewari-Singh et al., 2010), using 70 to 80% confluent cells cultured overnight in 60-mm Petri dishes seeded at a density of 5000 cells/cm². Cells were treated with DMSO, GSH alone, or GSH before or after CEES exposure, or they were exposed to CEES alone for 24 h. Thereafter, cells were harvested, washed twice with 1 $\times$ PBS, and incubated in 0.5 ml of saponin/PI solution (0.3% saponin, 25 $\mu\text{g}/\text{ml}$ PI, 0.1 mM EDTA, and 10 $\mu\text{g}/\text{ml}$ RNase in PBS) at 4°C for 24 h in the dark. Cell cycle distribution was analyzed by flow cytometry using fluorescence-activated cell sorting analysis core services of the University of Colorado Denver Cancer Center (Denver, CO).

Quantification of GSH in Cells. An equal number of cells were seeded in 96-well plates overnight, and after desired treatments with DMSO, GSH alone, GSH before or after CEES exposure, or 0.5 mM CEES alone for 24 h, quantification of GSH was carried out using the GSH-Glo glutathione assay kit from Promega, according to the manufacturer's protocol. In brief, after the desired treatments, wells were washed with PBS. To the total number of cells per well, 100 μl of prepared GSH-Glo reagent (containing 1 μl of Luciferin NT substrate and 1 μl of glutathione transferase) was added, mixed briefly, and incubated at room temperature for 30 min. Thereafter, 100 μl of Luciferin detection reagent was added to each well, mixed briefly, and incubated for 15 min. Next, luminescence was read using a luminescence plate reader (BioTek Instruments, Winooski, VT), and control readings were subtracted from all the sample readings. The GSH concentration was determined as picomoles per 1000 cells using the GSH standard curve.

Animal Treatment. Female SKH-1 hairless mice (4–5 weeks of age; Charles River Laboratories, Inc., Wilmington, MA) were housed

under standard conditions at the Center of Laboratory Animal Care, University of Colorado Denver. The animals were acclimatized for 1 week before their use in experimental studies, carried out according to the specified protocol approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver. CEES was dissolved in acetone for dorsal skin topical application, and GSH was administered in saline via oral gavage to mice. Mice were exposed to either 200 μ l of acetone alone, exposed to 2 mg of CEES in 200 μ l of acetone, or treated with 300 mg/kg GSH in 200 μ l of saline, or they were treated with 300 mg/kg GSH 1 h before topical exposure to 2 mg of CEES. Untreated mice were also included as a control group, and five animals per group were taken for this study. CEES at a dose of 2 mg was chosen because this dose showed maximum changes in the studied biomarkers we previously reported in this mouse strain (Tewari-Singh et al., 2009). In addition, CEES doses of 0.5 to 6 mg/kg per animal have been used in studies demonstrating its toxic responses in the skin tissue (Chatterjee et al., 2004), and skin injury could be observed after exposure to 10 to 40 μ g/cm² HD (Kehe et al., 2008). At the end of above-defined treatments, after euthanizing mice, dorsal skin was collected and either snap-frozen in liquid nitrogen or fixed in formalin as detailed previously (Tewari-Singh et al., 2009).

Measurement of Skin Bifold Thickness, Epidermal Thickness, and Quantification of Apoptotic Cells. The dorsal skin bifold thickness was measured (in millimeters) 3, 6, 9, 12, and 24 h after desired treatments using an electronic digital caliper (Marathon Watch Inc., Belleville, ON, Canada), and hematoxylin and eosin (H&E) staining of the mouse skin sections was carried as detailed previously (Tewari-Singh et al., 2009). The epidermal thickness was measured (in micrometers) in H&E-stained skin tissue sections randomly in at least five fields per tissue sample under a microscope using AxioVision software ver. 4.5 (400 $\times$ magnification; Carl Zeiss GmbH, Jena, Germany). Apoptotic cells were identified in H&E-stained skin sections by virtue of their eosinophilic cytoplasm and dark purple pyknotic nuclei, and they were counted in randomly selected fields (400 $\times$ magnifications) of control and treated skin tissue sections. The apoptotic index was determined as the number of apoptosis-positive cells $\times$ 100/total number of cells.

Measurement of Myeloperoxidase Activity. MPO activity was measured using a kit from Cell Technology as published previously (Tewari-Singh et al., 2009). In brief, $\sim$ 100 mg of clean (with red blood cells removed) skin tissue samples from each group were used to prepare lysates, and protein concentration was determined by the Lowry method (Lowry et al., 1951) using the Bio-Rad DC protein assay kit. For the assay, a reaction mixture was prepared with detection reagent, 20 mM hydrogen peroxide, 1 μ M eosinophil peroxidase inhibitor, 20 mM catalase inhibitor (3-amino-1,2,4-triazole), and 1 $\times$ assay buffer. Equal amounts of 50- μ l reaction mixture and the prepared sample (at a concentration of 50 μ g of protein) or MPO standards were added in 96-well plates. After 1-h incubation at room temperature in the dark, the fluorescence was measured by a fluorescent plate reader using excitation and emission wavelengths of 530 and 590 nm, respectively. Blank control readings were subtracted from all the sample readings. The MPO activity was determined as milliunits per micrograms of protein using the MPO standard curve.

Determination of Reduced GSH Levels in Skin Samples. Skin GSH levels were determined according to the protocol described previously (Kariya et al., 2008). In brief, skin tissues were weighed, sonicated on ice in KPBS buffer (50 mM potassium phosphate buffer, 17.5 mM EDTA, 50 mM serine, and 50 mM boric acid, pH 7.4), and incubated in the dark at room temperature for 30 min in the presence of monobromobimane (3 mM in acetonitrile). The reaction was stopped by the addition of 70% perchloric acid. The samples were then centrifuged at 16,000g at 4°C for 10 min, and the supernatant was removed, transferred to a high-performance liquid chromatography vial, and analyzed for GSH on a Hitachi high-pressure liquid chromatograph (model L-2420; San Jose, CA) equipped with a flu-

orometric detector (model L-2480) as previously described with minor modifications, using excitation and emission wavelengths at 390 and 480 nm, respectively (Kariya et al., 2008).

Microscopic and Statistical Analyses. The microscopic histopathologic analyses of skin samples were performed using a Zeiss Axioscop 2 microscope (Carl Zeiss GmbH); photomicrographs were captured by a Carl Zeiss AxioCam MrC5 camera with the AxioVision Rel 4.5 software. The data were analyzed using SigmaStat software (version 2.03; SPSS, Inc., Chicago, IL) for statistical significance of difference between the CEES-treated group versus the control and other treatment groups. Significance was determined by one-way analysis of variance with a Bonferroni *t* test or Tukey's *t* test for multiple comparisons. *p* < 0.05 was considered statistically significant.

Results

GSH Protects and Reverses CEES-Caused Decrease in JB6 and HaCaT Cell Viability. In our recent studies using these skin epidermal cells, we have reported that decreased cell viability is as an important biomarker of CEES-induced skin toxicity (Tewari-Singh et al., 2010). To determine whether exogenous GSH treatment was effective in reversing and/or preventing CEES-caused cell death, cells were treated with DMSO, 0.35 or 0.5 mM CEES, 1 or 10 mM GSH alone, or 1 or 10 mM GSH 1 to 120 min before or after exposure to 0.35 or 0.5 mM CEES; the MTT assay was carried out 24 h after these treatments.

In JB6 cells, 0.35 mM CEES caused cell viability to decrease to 48% of control, which was significantly (*p* < 0.05) attenuated when cells were treated with GSH 15, 30, and 120 min before or after 0.35 mM CEES exposure (Fig. 1A). Although 10 mM GSH was more effective, causing almost complete protection or prevention in CEES-caused reduction in cell viability, pre- or post-treatment with 1 mM GSH also showed significant (*p* values ranging from <0.001 to <0.01) protective and rescue effects (Fig. 1A). Increasing the CEES concentration to 0.5 mM caused a further decrease in the viable JB6 cell number to 31% of control; however, GSH treatment 15, 30, and 120 min before CEES exposure increased the viable cells to 80, 90, and 91% of control, respectively (Fig. 1B). GSH treatment (10 mM) 15 to 120 min after 0.5 mM CEES exposure also significantly (*p* < 0.001) reversed (from 90 to 67%) CEES-caused decreased cell viability (Fig. 1B). Furthermore, 30-min post-treatment of 1 mM GSH also resulted in a significant (*p* < 0.001) reversal (51%) of CEES-caused cytotoxicity in JB6 cells (Fig. 1B). In HaCaT cells, pretreatment with 10 mM GSH for 15 or 30 min resulted in significant (*p* values ranging from <0.001 to <0.05) protection against 0.5 mM CEES-induced reduction (37% of control) in cell viability, with maximum protection at 30 min before treatment, where 72% of control viable cells were evidenced (Fig. 1C). Treatment of HaCaT cells with 10 mM GSH 1 and 30 min after 0.5 mM CEES exposure showed 68 and 55% of control viable cells, respectively (Fig. 1C). A 1 mM GSH treatment up to 30 min before 0.5 mM CEES exposure was effective in significantly (*p* values ranging from <0.01 to <0.05) protecting the cells from reduction in cell viability (Fig. 1C); however, 1 mM GSH post-treatment was effective (*p* < 0.05) only 1 min after CEES exposure in reversing CEES-caused decrease in HaCaT cell viability (Fig. 1C). A significant difference in the viability of cells between the DMSO control and GSH-treated groups was not observed in either JB6 or HaCaT cells (Fig. 1).

Fig. 1. Effect of GSH on CEES-caused decrease in JB6 and HaCaT cell viability. JB6 (A and B) and HaCaT (C) cells were seeded and grown overnight in 96-well plates as detailed under *Materials and Methods*; they were then treated with either DMSO alone (control), 0.35 mM (A) or 0.5 mM (B and C) CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after exposure to 0.35 mM (A) or 0.5 mM (B and C) CEES for the indicated time periods. After the desired treatments, the MTT assay was carried out 24 h after CEES exposure as described under *Materials and Methods*. MTT assay data shown are mean $\pm$ S.E.M. of four to six independent samples for each treatment. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the CEES-treated group. Similar results were obtained in two independent experiments. VC, vehicle control.

GSH Protects and Reverses CEES-Caused Decrease in DNA Synthesis in JB6 and HaCaT Cells. Our recent studies have shown that exposure of JB6 and HaCaT cells to CEES causes a decrease in DNA synthesis, which is a measure of cell proliferation (Tewari-Singh et al., 2010). Therefore, using similar treatments as above for cell viability, we next assessed whether GSH is also effective against CEES-caused decrease in DNA synthesis in both cell lines. In JB6 cells, 10 mM GSH treatment 15 to 120 min before 0.5 mM CEES exposure caused a significant (p values ranging from <0.001 to <0.01) increase in BrdU-incorporating cells (76–57% of control) compared with CEES alone, where BrdU-incorporating cells were reduced to 7% of control (Fig. 2A). Pretreatment with a lower concentration of 1 mM GSH for 15 to 120 min also resulted in significant (p values ranging from <0.01 to <0.05) protection of JB6 cells from CEES-caused decrease in DNA synthesis (Fig. 2A). Treatment of JB6 cells with 1 or 10 mM GSH 1 min after CEES exposure was also significantly ($p < 0.001$) effective in reversing CEES-caused decrease in DNA synthesis (Fig. 2A); however, GSH treatment at later time points after CEES exposure was not effective. In HaCaT cells, 0.5 mM CEES alone reduced BrdU-incorporating cells to 54% of control, but pretreatment with 10 mM GSH for 15, 30, and 120 min significantly (p values

ranging from <0.01 to <0.05) increased proliferating cells to 114, 104, and 90%, respectively (Fig. 2B). Pretreatment (15 min) with 1 mM GSH resulted in maximum reversal, with 91% ($p < 0.05$) of control proliferating cells (Fig. 2B). Treatment of HaCaT cells with 10 mM GSH 1 min after CEES exposure was also effective in significantly (p values ranging from <0.001 to <0.05) reversing CEES-caused decrease in DNA synthesis (Fig. 2B). A significant difference in the DNA synthesis of cells between the DMSO control and GSH-treated groups was not observed in either cell line. Together, the results above indicate both the protective and therapeutic effects of GSH in attenuating CEES-caused decrease in cell viability and DNA synthesis in both JB6 and HaCaT skin epidermal cells; however, the protective effect of GSH was more profound than its rescue ability.

GSH Protects and Reverses CEES-Caused S and G₂M Phase Arrest in Cell Cycle Progression of JB6 and HaCaT Cells. CEES-induced decrease in DNA synthesis could be associated with the modulation in cell cycle progression, which could allow cells for DNA damage repair, or, in cases of extensive damage, cells consequently undergo apoptotic death. Our recent studies have demonstrated that the DNA-damaging effect of CEES in JB6 and HaCaT cells results in S and G₂M phase arrest (Tewari-Singh et al.,

Fig. 2. Effect of GSH on CEES-caused decrease in DNA synthesis in JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown overnight in 96-well plates as detailed under *Materials and Methods*; they were then treated with either DMSO alone (control), 0.5 mM CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after exposure to 0.5 mM CEES for the indicated time periods. After the desired treatments, the BrdU assay was carried out 24 h after CEES exposure as described under *Materials and Methods*. BrdU assay data shown are mean $\pm$ S.E.M. of five independent samples for each treatment. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the CEES-treated group. Similar results were obtained in two independent experiments. VC, vehicle control.

2010), prompting us to assess the effect of GSH on CEES-caused cell cycle arrest under the experimental conditions used for cell viability and DNA synthesis studies. Exposure of JB6 cells to 0.5 mM CEES for 24 h resulted in arrest of 52% cells in S phase compared with 32% cells in control; however, treatment with GSH at 10 mM concentration 30 min before CEES exposure reduced the S phase cell population to 19% ($p < 0.001$; Fig. 3A). Similar GSH treatment of JB6 cells 30 min after CEES exposure resulted in only a 17% ($p < 0.001$) S phase cell population (Fig. 3A). A significant (p values ranging from <0.01 to <0.05) prevention in CEES-caused G₂M arrest in JB6 cells was also observed with 10 mM GSH pretreatment for 30 min, but similar GSH post-treatment did not show any significant reversal in CEES-caused increase in the G₂M phase cell population (Fig. 3A). In other studies, only 1 mM GSH pretreatment was significantly (p values ranging from <0.001 to <0.05) effective in reversing CEES-caused S and G₂M phase cell cycle arrest in JB6 cells (Fig. 3A). In HaCaT cells, 0.5 mM CEES exposure for 24 h resulted in a 31% G₂M phase cell population compared with 16% in control (Fig. 3B) and was reduced to 18% ($p < 0.001$) after 30-min pretreatment with 10 mM GSH (Fig. 3B). Treatment with GSH 1 min after CEES exposure was also effective in significantly (p values ranging from <0.01 to <0.05) reversing CEES-caused increase in the G₂M phase HaCaT cell population (Fig. 3B). A significant difference in the percentage of cells in G₁, S, or G₂M phases between JB6 and HaCaT cells treated with DMSO and with GSH alone was not observed. Together, these results also show that GSH before or after treatment reverses CEES-caused cell cycle arrest in both JB6 and HaCaT cells; however, the protective effect of GSH was found to be more potent than its rescue ability.

NAC Protects and Reverses CEES-Caused Decrease in JB6 and HaCaT Cell Viability. Because the GSH precursor NAC is more readily taken up by cells than GSH

Fig. 3. Effect of GSH on CEES-caused S and G₂M phase arrest in cell cycle progression of JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown overnight as detailed under *Materials and Methods*; they were then treated with either DMSO alone (control), 0.5 mM CEES, 1 or 10 mM GSH, or 1 or 10 mM GSH before or after exposure to 0.5 mM CEES for the indicated time periods. After the desired treatments, cells were collected and incubated with saponin/PI at 4°C for 24 h in the dark and subjected to fluorescence-activated cell sorting analysis as detailed under *Materials and Methods*. Data shown are mean $\pm$ S.E.M. of three independent samples for each treatment. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the CEES-treated group. VC, vehicle control.

(Paromov et al., 2007), we next evaluated the protective and therapeutic effects of NAC in attenuating CEES-caused decrease in JB6 and HaCaT cell viability. Cells were subjected to the MTT assay after exposure to either DMSO alone, 0.5 mM CEES, 25 or 50 mM NAC, or 25 or 50 mM NAC at 5 or 1 h before or 30 min or 1 h after CEES exposure. Compared with 25 mM treatment, treatment with 50 mM NAC before or after CEES exposure was more effective in ameliorating CEES-caused cytotoxicity in both JB6 and HaCaT cells (Fig. 4, A and B). In JB6 cells, CEES exposure reduced cell viability to 22% of control, and 50 mM NAC treatment demonstrated complete protection and reversal (Fig. 4A). Treatment of HaCaT cells with 50 mM NAC for 1 h before CEES exposure resulted in 82% of control viable cells, which were reduced to 28% after exposure to CEES alone (Fig. 4B). Similar NAC treatment after CEES exposure resulted in a complete reversal in CEES-caused cytotoxicity (Fig. 4B). Similar results were obtained with 50 mM NAC treatment given 5 h before or 30 min after CEES exposure (data not shown). A significant difference in the viability of cells between the DMSO control and NAC-treated groups was not observed in either JB6 or HaCaT cells (Fig. 4).

GSH Prevents CEES-Caused Inflammatory Responses in SKH-1 Hairless Mouse Skin. Our recent findings have established dose- and time-dependent quantitative inflammatory biomarkers of CEES-caused skin injury in our SKH-1 hairless mouse model (Tewari-Singh et al., 2009). Here, we used some of the prominent CEES-induced injury

Fig. 4. The effect of NAC on CEES-caused decrease in JB6 and HaCaT cell viability. JB6 (A) and HaCaT (B) cells were seeded and grown overnight in 96-well plates as detailed under *Materials and Methods*; they were then treated with either DMSO alone (control), 0.5 mM CEES, 25 or 50 mM NAC, or 25 or 50 mM NAC before or after exposure to 0.5 mM CEES for the indicated time periods. After the desired treatments, the MTT assay was carried out 24 h after CEES exposure as described under *Materials and Methods*. Data shown are mean $\pm$ S.E.M. of five independent samples for each treatment. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the CEES-treated group. Similar results were obtained in two independent experiments. VC, vehicle control.

biomarkers—i.e., increased skin bifold thickness, skin epidermal thickness, apoptotic cell death, and tissue MPO activity—to evaluate the protective effect of GSH. Although GSH exhibited both protective and therapeutic effects in reversing CEES-caused toxic consequences in cell culture studies, its protective effect was more prominent than its rescue potential. Therefore, we focused only on the preventive effect of GSH in our SKH-1 mouse skin studies as summarized below.

Fig. 5. Effect of GSH on CEES-caused inflammatory responses in SKH-1 hairless mouse skin. Mice were treated topically with either acetone or CEES (2 mg), given GSH (300 mg/kg) alone via oral gavage or given GSH (300 mg/kg) via oral gavage 1 h before CEES topical treatment. After 3, 6, 9, 12, and 24 h of the indicated treatments, skin bifold thickness (A) was measured using a digital caliper as detailed under *Materials and Methods*. Mice were sacrificed, and dorsal skin tissue samples were collected 24 h after the desired treatments. Skin sections were processed for H&E staining and analyzed for histological changes, epidermal thickness (B; 400 $\times$ magnification and C), and apoptotic cell death (D) as detailed under *Materials and Methods*. To determine the neutrophil infiltration, treated skin tissue samples were used for lysate preparation, and MPO activity (E) was determined by a fluorescence assay as detailed under *Materials and Methods*. Data presented are mean $\pm$ S.E.M. of five animals in each treatment group. Statistical significance of difference between the groups exposed to CEES and control groups was determined by one-way analysis of variance with a Bonferroni t test for pairwise multiple comparisons. UC, untreated control; VC, vehicle control; e, epidermis; d, dermis. Black arrows, epidermal necrosis; white arrows, dead cells. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the CEES-treated group.

Skin Bifold Thickness. Treatment with GSH 1 h before CEES exposure resulted in significant (p values ranging from <0.001 to <0.01) protection against CEES-caused increase in skin bifold thickness (Fig. 5A). At 9 and 24 h, untreated controls showed 0.61 ± 0.04 - and 0.598 ± 0.01 -mm skin bifold thickness that increased to 2.11 ± 0.19 and 2.14 ± 0.07 mm ($p < 0.001$), respectively, after CEES exposure (Fig. 5A). GSH pretreatment reduced CEES-caused increase in skin bifold thickness to 1.29 ± 0.10 and 1.30 ± 0.27 mm (p values ranging from <0.001 to <0.01) at these time points, respectively (Fig. 5A). No significant difference was observed in skin bifold thickness between untreated or vehicle control groups and groups treated with GSH alone at any of the study time points (Fig. 5A).

Epidermal Thickness and Apoptotic Cell Death. Because we observed a significant effect of GSH in preventing the CEES-caused increase in skin bifold thickness, we next assessed its effect on other important histological changes related to CEES-caused inflammatory and toxicity responses (Tewari-Singh et al., 2009). Evaluation of H&E-stained skin sections showed that GSH pretreatment also resulted in a significant protection against CEES-caused increase in epidermal thickness, upper epidermal cell necrosis and desquamation (Fig. 5B, black arrows), and cytoplasmic swelling with shrinkage or condensation of nuclei indicating apoptotic cell death (Fig. 5B, white arrows). In terms of quantitative analysis, compared with untreated controls with epidermal thickness of 18.46 ± 0.28 μ m, CEES treatment caused an increase to 33.52 ± 1.55 μ m; however, GSH pretreatment caused a significant ($p < 0.01$) reduction in epidermal thickness, to 23.16 ± 1.38 μ m (Fig. 5C). We also quantified apoptotic cells in the epidermal layer, where H&E-stained skin sections from the CEES-treated group showed 54% apoptotic cells in the epidermis compared with 14% in untreated controls; GSH pretreatment decreased the apoptotic cell number to 39% ($p < 0.01$; Fig. 5D). No significant difference was observed in epidermal thickness or the percentage of apoptotic cells between untreated or vehicle control groups and

groups treated with GSH alone at any of the study time points.

MPO Activity. We also analyzed GSH efficacy on CEES-caused increase in skin tissue MPO activity, which is a measure of neutrophil infiltration at the sites of acute inflammation (Bradley et al., 1982). After 24 h of CEES exposure, 3.15 ± 0.42 mU/ μ g MPO activity was observed in skin tissue lysates compared with 1.22 ± 0.11 mU/ μ g protein in untreated controls (Fig. 5E). A significant decrease in MPO activity (1.80 ± 0.25 mU/ μ g protein activity, $p < 0.01$) was observed in skin tissues from mice treated with GSH before CEES exposure (Fig. 5E). No significant difference was observed in MPO activity between untreated or vehicle control groups and groups treated with GSH alone at any of the study time points.

GSH Treatment Attenuates CEES-Caused Decrease in Intracellular GSH Levels in JB6 Cells, HaCaT Cells, and SKH-1 Hairless Mouse Skin. Because we observed that GSH treatment causes both protection and reversal of CEES-induced skin injury in cell culture and mouse skin (only protection in this case), we next assessed whether changes in intracellular GSH levels were associated with these effects. After treatment of JB6 and HaCaT cells for the above-summarized cytotoxicity studies, intracellular GSH levels were measured. In JB6 cells, 0.5 mM CEES exposure decreased GSH levels to 3.2 pmol in every 1000 cells from 10.97 pmol in every 1000 cells in controls, and treatment with 10 mM exogenous GSH alone increased the GSH level to 101.6 pmol in every 1000 cells (Fig. 6A). It is noteworthy that GSH treatment 30 min before or after CEES exposure resulted in significantly ($p < 0.001$) elevated GSH levels of 110.1 and 149.4 pmol in every 1000 cells, respectively, compared with only 3.2 pmol GSH in every 1000 cells in JB6 cells exposed to CEES alone (Fig. 6A). Likewise, exposure of HaCaT cells to 0.5 mM CEES resulted in GSH levels of 7.0 pmol in every 1000 cells compared with 26.7 pmol in every 1000 cells in respective controls. However, treatment with GSH alone and with GSH 30 min before and 30 min after CEES exposure resulted in elevated GSH levels of 53.3, 75.6, and 68.1 pmol in every 1000 cells, respectively (Fig. 6B). Together, these results clearly show that exogenous GSH treatment before or after CEES exposure increases intracellular GSH levels that were depleted by exposure to CEES alone in both cell types.

On the basis of our results showing the protective and reversal effects of NAC against CEES-caused cytotoxicity, we next measured GSH levels in cells treated with NAC and either with or without CEES to decipher whether NAC treatment increases cellular GSH levels or acts through antioxidant properties to attenuate CEES-caused cytotoxicity. Compared with DMSO control and CEES-alone-treated JB6 cells showing GSH levels of 13.5 and 6.3 pmol, respectively, in every 1000 cells, 25 and 50 mM NAC treatments 60 min before CEES increased GSH levels to 16.93 and 16.91 pmol in every 1000 cells, respectively (Fig. 6C). In HaCaT cells, both 25 and 50 mM NAC treatments 60 min before CEES exposure showed an increase in cellular GSH levels, to 20.5 pmol in every 1000 cells, which was 18.1 pmol in every 1000 cells in vehicle controls and was reduced to 9.5 pmol in every 1000 cells after exposure to CEES alone (Fig. 6D). In neither cell line did treatment with NAC after CEES exposure show an increase in CEES-depleted cellular GSH levels.

Fig. 6. Effect of CEES exposure and GSH treatment on intracellular GSH levels in JB6 cells, HaCaT cells, and SKH-1 hairless mouse skin. JB6 (A and C) and HaCaT (B and D) cells were seeded, grown in 96-well plates overnight, and treated with either DMSO alone (control), 0.5 mM CEES, 10 mM GSH (A and B), 25 or 50 mM NAC (C and D) before or after 0.5 mM CEES for the indicated time periods. After the desired treatments, the intracellular GSH level was determined as detailed under *Materials and Methods*. Cellular GSH levels were calculated as picomoles in every 1000 cells and estimated in three separate treatments per group. E, mice were treated topically with either acetone or CEES (2 mg), given GSH (300 mg/kg) alone via oral gavage or given GSH (300 mg/kg) via oral gavage 1 h before CEES topical application. Mice were sacrificed, and dorsal skin tissue samples were collected 24 h after the desired treatments and processed for the determination of total GSH levels in the skin as described under *Materials and Methods*. The amount of GSH was calculated as picomoles per micrograms of protein. Data shown are mean $\pm$ S.E.M. of three to five independent samples for each treatment. *, $p < 0.05$; and ***, $p < 0.001$ compared with the CEES-treated group. UC, untreated control; VC, vehicle control.

Similar to our cell culture findings, our animal study also showed GSH efficacy in protecting against CEES-caused skin injury, and therefore, we also questioned whether topical application of CEES depletes GSH levels in skin and whether an oral gavage feeding of GSH to mice indeed increases the GSH level in skin, which could be associated with the observed protective effect of GSH on CEES-caused inflammatory responses in SKH-1 hairless mouse skin. Our results show that 2 mg of CEES exposure decreased GSH levels from 3.28 to 1.29 pmol/ μ g protein in vehicle control-treated skin; however, GSH feeding 1 h before CEES exposure either retained or reversed CEES-caused depletion in GSH levels in skin. We measured a 3.79 pmol/ μ g protein GSH level, which was comparable with that of the mouse skin samples treated with vehicle alone (Fig. 6E). These results suggest that the changes in skin GSH levels could be related to the observed protective effect of GSH against CEES-caused skin injury in mice.

BSO Treatment Further Increases CEES-Caused Cytotoxicity in JB6 and HaCaT Cells. As alluded to previously, depletion of GSH and oxidative stress play an important role in CEES/HD-caused cytotoxicity (Kehe and Szincz,

2005; Paromov et al., 2007; Black et al., 2010). In our present study, treatment of skin epidermal cells with GSH or its precursor NAC attenuated CEES-caused cytotoxic responses in cell culture and skin injury in mouse skin; moreover, CEES exposure decreased GSH levels in cell culture as well as in mouse skin systems. Therefore, we also assessed the involvement of GSH in CEES-caused skin injury by questioning whether a decrease in cellular GSH level would cause an increase in CEES cytotoxic effects in skin epidermal cells. In JB6 cells, 0.35 mM CEES exposure reduced viable cells to 57% of control; however, pretreatment of cells with BSO followed by CEES resulted in a viable cell count to be 3% of the control (Fig. 7A). Likewise, after BSO treatment, 0.5 mM CEES exposure resulted in complete cell death compared with 75% cell death when exposed to 0.5 mM CEES alone (Fig. 7A). In HaCaT cells, 47 and 31% of control cell viability was observed after 0.35 and 0.5 mM CEES exposure, which was reduced to 18 and 3%, respectively, when the cells were exposed to BSO before CEES (Fig. 7B). A significant difference in cell viability between the DMSO control group and the group treated with BSO alone was not observed. Together, these results clearly show that pretreatment with BSO, which is known to reduce GSH levels in cells, further increases CEES-caused cytotoxicity in both JB6 and HaCaT cells.

Discussion

The results of our present report demonstrate the protective and therapeutic efficacy of GSH in attenuating CEES-

caused epidermal cell toxicity and skin injury, asserting that GSH plays at least a protective role under in vivo conditions against CEES/HD-caused skin injury and possibly vesication. This study also provides additional insights and further emphasizes the involvement of oxidative stress in biological and molecular mechanisms associated with CEES-caused skin injury we recently reported (Pal et al., 2009; Tewari-Singh et al., 2010).

HD and CEES are alkylating agents that deplete GSH, which enhances lipid peroxidation and ROS generation, causing damage to lipids, proteins, and nucleic acids and eventually leading to toxic responses (Kehe and Szinicz, 2005; Paromov et al., 2007; Ruff and Dillman, 2007; Black et al., 2010). CEES-caused mitochondrial oxidative damage and altered mitochondrial membrane potential are also reported in lung, liver, and other tissues, further indicating a role of oxidative stress in CEES-caused toxicity (Jafari, 2007; Gould et al., 2009; Black et al., 2010). Therefore, supplementing GSH or its precursors, including NAC, could help minimize this oxidative stress and reduce HD/CEES-caused toxicity, although its associated mechanism and defined biological systems are not well known (Amir et al., 1998; Atkins et al., 2000; Han et al., 2004; Arfsten et al., 2007; Paromov et al., 2007, 2008). The protective role of extracellular GSH is highlighted in a study using the macrophage monocyte cell line J774 as well as in the mitotically active SVK 14 keratinocyte cell line (Smith et al., 1997; Amir et al., 1998). Using the biomarker we recently established in JB6 and HaCaT cells (Tewari-Singh et al., 2010), our present study demonstrates both the protective and therapeutic efficacy of GSH in attenuating CEES-caused cytotoxic responses; however, the protective efficacy was greater than its rescue potential in terms of DNA synthesis and cell cycle progression parameters. Our results also show that exogenous GSH given before CEES exposure could avert CEES-induced GSH depletion and hence could diminish oxidative stress, which is, in part, responsible for CEES-caused DNA damage that leads to the observed decrease in DNA synthesis and cell cycle arrest. However, the addition of GSH after CEES exposure, which possibly has already induced oxidative stress and DNA damage, could not effectively reverse these toxic effects of CEES.

The protection and reversal in CEES-caused cell viability by exogenous GSH could also be due in part to the involvement lipid peroxidation and/or protein damage reported previously in CEES-caused skin toxicity (Pal et al., 2009). Another mechanism by which extracellular GSH could protect the cells from CEES-induced damage is its possible extracellular conjugation with CEES, preventing CEES from entering the cell and causing its toxic effects (Salmen et al., 2005). Although the protective effects of 10 and 50 mM NAC have been shown in lipopolysaccharide-stimulated macrophages and endothelial cells (Atkins et al., 2000; Paromov et al., 2008), respectively, we observed both the protective and therapeutic effects of NAC at a 50 mM concentration against CEES-caused decrease in cell viability. The efficacy of NAC in ameliorating CEES-caused toxicity was stronger than that of GSH in HaCaT cells, which could be due to better uptake of GSH in JB6 cells compared with HaCaT cells as observed in this study, even though the GSH levels in control JB6 cells were lower than those in HaCaT cells. Important also is our observation that NAC treatments before or after CEES exposure caused a strong reversal in CEES-caused cytotoxicity;

Fig. 7. Effect of BSO on CEES-caused cytotoxicity in JB6 and HaCaT cells. JB6 (A) and HaCaT (B) cells were seeded and grown overnight as detailed under *Materials and Methods*; they were then treated with media without or with 0.2 mM BSO. After 6 h, media were removed, cells were exposed to either DMSO alone (control) or 0.35 or 0.5 mM CEES for 24 h in fresh media, and the MTT assay was carried out as described under *Materials and Methods*. Data shown are mean $\pm$ S.E.M. of five independent samples for each treatment. **, $p < 0.01$; and ***, $p < 0.001$ compared with the control and CEES-treated groups. Similar results were obtained in two independent experiments. VC, vehicle control.

however, the CEES-caused decrease in intracellular GSH levels was reversed only with pretreatment of NAC, and post-treatment was not effective. This finding suggests that the protective effect of NAC against CEES-caused toxicity occurs in part through an increase in GSH levels, and the other mechanisms could be related to its activity as a strong ROS scavenger; NAC is also known to act via the nuclear factor- κ B pathway and inhibit prostaglandin synthesis apart from being a GSH precursor (Atkins et al., 2000; Arfsten et al., 2007). CEES-induced depletion of GSH and generation of ROS have been shown in human lymphocytes that induce cell death and in human skin cells where treatment with GSH-depleting agent BSO showed an increased toxicity to HD (Han et al., 2004; Simpson and Lindsay, 2005). In the present study, we also observed that BSO pretreatment to both the skin epidermal cells caused an increase in CEES-caused toxicity. We also observed that CEES exposure depletes cellular GSH levels and that GSH supplementation results in its elevated intracellular levels both in cell culture and mouse skin, further strengthening the role of GSH in CEES-caused skin injury.

The role of oxidative and/or nitritative stress in HD/CEES-caused skin inflammation and injury is reported in various animal models (Paromov et al., 2007; Ruff and Dillman, 2007; Kehe et al., 2009; Pal et al., 2009; Black et al., 2010; Shakarjian et al., 2010). Our recent study in SKH-1 hairless mice demonstrated that induction of oxidative stress by CEES possibly leads to lipid, protein, and DNA oxidation together with the activation of mitogen-activated protein kinases and Akt pathways and activator protein-1 and nuclear factor- κ B transcription factors (Pal et al., 2009), followed by inflammatory responses in mouse skin (Tewari-Singh et al., 2009). Using the inflammatory biomarkers from our previous study, in this article we demonstrate the protective effect of GSH in attenuating CEES-caused increase in inflammation, which could be associated with the observed changes in the skin GSH levels. The observed reversal of CEES-induced increase in MPO activity by GSH feeding could also lead to a decrease in lipid peroxidation, as the oxidative products of MPO could cause lipid peroxidation (Brennan et al., 2001). Treatment with NAC or lipoic acid was previously shown to significantly reduce skin, eye, and lung toxicity associated with HD exposure in rats (Paromov et al., 2007), and superoxide dismutase treatment to guinea pigs is shown to reduce the skin lesion area caused by HD (Eldad et al., 1998; Arfsten et al., 2007; Paromov et al., 2007). However, most studies with antioxidants have shown protection against CEES/HD-induced lung injury (Kumar et al., 2001; McClintock et al., 2006; Paromov et al., 2007; O'Neill et al., 2010). With inadequate reports on the use of GSH and its precursors in attenuating HD/CEES-caused skin injury, the present study demonstrates the protective role of GSH in decreasing CEES-caused inflammatory responses in mice, which further supports our previous findings on the role of oxidative stress in CEES-induced signaling pathways that lead to inflammatory responses in the skin of an SKH-1 mouse (Pal et al., 2009; Tewari-Singh et al., 2009).

Authorship Contributions

Participated in research design: Tewari-Singh, C. Agarwal, Day, White, and R. Agarwal.

Conducted experiments: Tewari-Singh, C. Agarwal, and Huang.

Performed data analysis: Tewari-Singh, C. Agarwal, Day, White, and R. Agarwal.

Wrote or contributed to the writing of the manuscript: Tewari-Singh, Day, White, and R. Agarwal.

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