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Role of MAP kinases in regulating expression of antioxidants and inflammatory mediators in mouse keratinocytes following exposure to the half mustard, 2-chloroethyl ethyl sulfide

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Abstract

Dermal exposure to sulfur mustard causes inflammation and tissue injury. This is associated with changes in expression of antioxidants and eicosanoids which contribute to oxidative stress and toxicity. In the present studies we analyzed mechanisms regulating expression of these mediators using an in vitro skin construct model in which mouse keratinocytes were grown at an air-liquid interface and exposed directly to 2-chloroethyl ethyl sulfide (CEES), a model sulfur mustard vesicant. CEES (100-1000 μ M) was found to cause marked increases in keratinocyte protein carbonyls, a marker of oxidative stress. This was correlated with increases in expression of Cu,Zn superoxide dismutase, catalase, thioredoxin reductase and the glutathione-S-transferases, GSTA1-2, GSTP1 and mGST2. CEES also upregulated several enzymes important in the synthesis of prostaglandins and leukotrienes including cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-2 (mPGES-2), prostaglandin D synthase (PGDS), 5-lipoxygenase (5-LOX), leukotriene A₄ (LTA₄) hydrolase and leukotriene C₄ (LTC₄) synthase. CEES readily activated keratinocyte JNK and p38 MAP kinases, signaling pathways which are known to regulate expression of antioxidants, as well as prostaglandin and leukotriene synthases. Inhibition of p38 MAP kinase suppressed CEES-induced expression of GSTA1-2, COX-2, mPGES-2, PGDS, 5-LOX, LTA₄ hydrolase and LTC₄ synthase, while JNK inhibition blocked PGDS and GSTP1. These data indicate that CEES modulates expression of antioxidants and enzymes producing inflammatory mediators by distinct mechanisms. Increases in antioxidants may be an adaptive process to limit tissue damage. Inhibiting the capacity of keratinocytes to generate eicosanoids may be important in limiting inflammation and protecting the skin from vesicant-induced oxidative stress and injury.

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Keywords

CEES; sulfur mustard; inflammation; skin

Introduction

Sulfur mustard (bis[2-chloroethyl] sulfide) is a bifunctional alkylating agent and a potent skin vesicant (Dacre and Goldman, 1996). It readily penetrates the skin, causing an early inflammatory response followed by blistering and persistent tissue damage (Dacre and Goldman, 1996; Rice, 2003). Oxidative stress is known to initiate inflammatory responses in the skin (Trouba *et al.*, 2002). Sulfur mustard and related analogs including, 2,2'-dichloro-N-methyldiethylamine (nitrogen mustard) and 2-chloroethyl ethyl sulfide (CEES) or half mustard, have been reported to induce oxidative stress by depleting cells of intracellular antioxidants including glutathione and thioredoxin (Smith *et al.*, 1995; Paromov *et al.*, 2007). CEES has also been shown to cause changes in mitochondrial membrane potential resulting in increased production of reactive oxygen species (ROS) (Gould *et al.*, 2009). Moreover, topical application of CEES to mouse skin results in the generation of 8-oxo-2-deoxyguanosine DNA adducts and increased lipid peroxidation and protein oxidation, markers of ROS and intracellular oxidative stress (Pal *et al.*, 2009). In the guinea pig model, superoxide dismutase (SOD) protects against sulfur mustard-induced skin burns, while desferrioxamine, an iron chelator that prevents the formation of hydroxyl radicals, mitigates nitrogen mustard-induced skin toxicity (Eldad *et al.*, 1998; Karayilanoglu *et al.*, 2003). Sulphoraphane, an antioxidant enzyme inducer, as well as glutathione, also promote keratinocyte survival in culture following treatment with sulfur mustard (Smith *et al.*, 1997; Gross *et al.*, 2006). These findings demonstrate that ROS and oxidative stress play important roles in the toxicity of sulfur mustard and related analogs.

In the skin, a complex network of antioxidant enzymes functions to protect against oxidative stress (see Fig. 1 for summary of ROS detoxification pathways) (Darr and Fridovich, 1994; Sander *et al.*, 2004). These enzymes include ROS scavengers such as SOD, catalase, and thioredoxin reductase (TrxR), as well as glutathione *S*-transferases (GST), a family of glutathione-metabolizing enzymes that detoxify electrophilic compounds (Hayes and McLellan, 1999b; Nordberg and Arner, 2001). Mechanisms mediating expression of vesicant-induced oxidative stress were analyzed in the present studies using a skin construct model consisting of mouse keratinocytes grown at an air-liquid interface. In this system, chemicals are applied directly to the air surface of the cells in order to simulate realistic exposure scenarios. Using the skin construct, CEES was found to cause marked increases in keratinocyte protein oxidation and alterations in expression of several key intracellular antioxidants. Moreover, this was associated with increased expression of enzymes important in the synthesis of prostaglandins and leukotrienes, a response dependent upon JNK and p38 MAP kinase signaling. These data demonstrate that keratinocytes grown in the skin construct system are highly responsive to vesicants, and that this is a useful model for analyzing mechanisms of sulfur mustard-induced skin toxicity. Currently, few effective countermeasures against vesicant-induced dermal toxicity are available and a more complete understanding of the mechanism of action of sulfur mustard using skin models is key to identifying potential therapeutic targets.

Materials and Methods

Chemicals and reagents

Rabbit polyclonal antibodies to p38, phospho-p38, JNK, phospho-JNK, ERK 1/2, and phospho-ERK 1/2 were from Cell Signaling Technology (Beverly, MA). Goat polyclonal

antibodies to COX-2, rabbit polyclonal antibodies to β -catenin and Cu,Zn-SOD, and horseradish peroxidase-labeled donkey anti-goat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to 5-LOX and LTA₄ hydrolase were from Cayman Chemical (Ann Arbor, MI). Horse radish peroxidase-labeled goat anti-rabbit secondary antibodies and detergent-compatible protein assay reagents were from Bio-Rad Laboratories (Hercules, CA). Superscript III Reverse Transcriptase and the MTS CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay were from Promega (Madison, WI), and the Versagene RNA purification kit from Gentra Systems (Minneapolis, MN). The Western Lightning enhanced chemiluminescence kit (ECL) was from Perkin Elmer Life Sciences, Inc. (Boston, MA), the OxyBlot Protein Oxidation Detection kit from Chemicon International (Temecula, CA) and precast gels were from Pierce Biotechnology (Rockford, IL). SYBR Green Master Mix and other PCR reagents were from Applied Biosystems (Foster City, CA). Annexin V-APC and other apoptosis reagents were from BD Pharmingen (San Diego, CA). Cell culture reagents and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) mixed isomers were from Invitrogen Corp (Carlsbad, CA), polyester Transwell permeable supports from Corning Life Sciences Inc. (Acton, MA) and SP600125 from Calbiochem (La Jolla, CA). CEES, SB203580, protease inhibitor cocktail, and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cells and treatments

PAM212 mouse keratinocytes were grown in DMEM supplemented with 10% fetal bovine serum (Yuspa *et al.*, 1980). A three-dimensional skin-like construct was generated by plating the cells 1×10^6 /well on 24 mm Transwell semi-permeable polyester membrane supports (0.4 μ m pore size) in 6-well cell culture plates (Aufderheide *et al.*, 2003). After reaching confluence, the medium was removed from the top portion of the Transwell support such that the keratinocytes were exposed to air at the apical surface and medium at the basolateral surface. After 24 hr, one ml of vehicle control or CEES was added to the apical surface of the construct. A stock CEES solution (100 mM) was prepared fresh in absolute ethanol immediately before use and diluted to the appropriate concentrations in PBS. Cells were incubated for 2 hr at 37°C in a humidified CO₂ incubator. The Transwells were then removed, washed in PBS and immediately replaced in the same 6-well culture plates for 24 hr unless otherwise indicated. In some experiments, one ml PBS/Transwell containing the p38 MAP kinase inhibitor, SB203580 (10 μ M), the JNK kinase inhibitor, SP600125 (20 μ M), or DMSO control were added to the apical surface, and the cells incubated at 37°C for 3 hr prior to CEES treatment.

Western blotting

Cell lysates were prepared using an SDS-lysis buffer (10 mM Tris-base and 1% SDS, pH 7.6 supplemented with 10 μ l protease inhibitor cocktail consisting of 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin). Proteins (20 μ g) from lysates were separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. After incubation in blocking buffer (5% dry milk Tris-buffered saline containing 0.1% Tween 20) for 1 hr at room temperature or overnight at 4°C, the membranes were incubated for 2 hr at room temperature or overnight at 4°C with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at room temperature. Protein expression was visualized using enhanced chemiluminescence reagents. Equal loading of protein was confirmed by expression of β -catenin.

Densitometric analysis of western blots were performed using a Fluor Chem SP imaging system (Alpha Innotech Corp., San Leandro, CA), and absolute values for each protein were normalized with the values for β -catenin. Results are reported in arbitrary units, comparing each value with that obtained from each respective β -catenin measurement.

Determination of cell viability and apoptosis

Cell viability was assessed using an MTS cell viability assay as described by the manufacturer. In this assay, viable cells convert MTS to an aqueous formazan product, which can be quantified by absorbance at 492 nm. Briefly, the cells grown on Transwell supports were treated with CEES (100-1000 μ M) as described above. After 24 hr, cells were treated with the MTS reagent (20 μ l/Transwell support) and incubated for 2 hr at 37°C. The supernatants were then collected, transferred to a 96-well plate and analyzed using a Molecular Devices SpectraMax M2 spectrophotometer (Sunnyvale, CA). Cell viability was determined to be > 80% for all treatments.

For analysis of keratinocyte apoptosis, the cells treated with CEES (100-1000 μ M) as described above. After 24 hr, the cells were removed from the Transwell supports with trypsin, incubated for 15 min at room temperature with Annexin V-APC followed by 10 min with 7-amino-actinomycin (7-AAD). The cells were then analyzed by flow cytometry on a Becton Dickinson FACSArray Bioanalyzer (San Diego, CA). Apoptotic keratinocyte populations were gated electronically and data analyzed using quadrant statistics based on relative Annexin V-APC and 7-AAD fluorescence (Lecoeur *et al.*, 2002). Approximately 13% of the control cells were apoptotic while 13%, 17% and 36% were apoptotic after treatment with 100, 300 and 1000 μ M CEES, respectively.

Measurement of ROS production

Intracellular hydrogen peroxide production was measured using the hydrogen peroxide sensitive dye, CM-H₂DCFDA, in conjunction with flow cytometry as previously described (Black *et al.*, 2008b). Briefly, cells grown on Transwell supports were incubated with CM-H₂DCFDA (5 μ M) in PBS for 15 min and then treated with CEES (100-1000 μ M) or control for 30 min. The cells were then immediately analyzed for DCF fluorescence on a Beckman Coulter Cytomics FC 500 flow cytometer. Data were analyzed using the Beckman Coulter CXP software.

Protein oxidation assay

Protein oxidation was assessed by the formation of carbonyl groups on protein side chains using an OxyBlot Protein Oxidation Detection kit (Chevion *et al.*, 2000). Keratinocyte lysates, prepared as described above with the addition of 20 μ l β -mercaptoethanol per sample, were incubated with 2,4-dinitrophenylhydrazine to derivatize the carbonyl groups on proteins to 2,4-dinitrophenylhydrazone-tagged products. Nonderivatized samples were used as a control. Samples were then separated on pre-cast 4-12% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies to dinitrophenylhydrazone-modified carbonyl groups, followed by HRP-conjugated secondary antibodies. Protein expression was visualized using ECL reagents.

Real-time polymerase chain reaction

For these experiments, three constructs (n = 3) were treated with each CEES concentration or control. RNA was isolated from the cells using the Versagene RNA purification kit following the manufacturer's protocol. RNA was converted to cDNA using Superscript reverse transcriptase. The cDNA was diluted 1:10 in RNase-DNase-free water for PCR

analysis. For each gene to be tested, a standard curve composed of a serial dilution of pooled cDNA from the samples was used as a reference. All values were normalized to GAPDH. The control was assigned a value of one and treated samples calculated relative to control. Real-time PCR was performed on an ABI Prism 7900 Sequence Detection System using 96-well optical reaction plates. SYBR-Green was used for detection of fluorescent signal and the standard curve method was used for relative quantification analysis. The primer sequences for the genes were generated using Primer Express software (Applied Biosystems) and the oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The forward and reverse sequences used are listed in Table 1.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical differences between the means were determined using two-way ANOVA and were considered statistically significant at $p < 0.05$.

Results

Effects of CEES on keratinocyte oxidative stress and expression of antioxidant enzymes

In initial studies, we determined if CEES induces oxidative stress in keratinocytes in the skin construct model by quantifying the generation of intracellular hydrogen peroxide (Fig. 2A). We found that the cells constitutively generated low levels of hydrogen peroxide. Small increases in hydrogen peroxide production were noted following treatment with 100 μ M CEES, while 5-10-fold increases were found after treatment with 300 and 1000 μ M CEES.

CEES treatment was also found to cause increased protein oxidation in the keratinocytes, as measured by protein carbonyl formation. Low constitutive levels of oxidized proteins were detected in control cells, as evidenced by the presence of an oxidized protein with a molecular weight of approximately 75 kDa (Fig. 2B). Treatment of the cells with CEES (100 – 1000 μ M) caused a marked increase in oxidation of this protein, and the appearance of additional oxidized proteins with molecular weights of approximately 36, 43, 50, 66 and 70 kDa. Lower levels of oxidized proteins with molecular weights of 24 and 34 kDa were also noted in CEES-treated cells. CEES-induced protein oxidation was associated with a 4-6 fold increase in mRNA expression of the primary antioxidant enzymes, Cu,Zn-SOD, catalase and thioredoxin reductase (Fig. 3). Cu,Zn-SOD protein expression was also upregulated by CEES (Fig. 4A). In contrast, Mn-SOD mRNA expression decreased (Fig. 3). GST's, including GSTA1-2 (14-fold), GSTP1 (3-fold) and mGST2 (2-fold), were also upregulated in keratinocytes after CEES treatment while no major changes in expression of GSTA3, GSTA4, GSTM1, mGST1 or mGST3 were observed (Fig. 5).

Effects of CEES on keratinocyte expression of enzymes mediating the generation of eicosanoids

In further studies, we analyzed the effects of CEES on enzymes mediating the production of prostaglandins and leukotrienes, which are important in the development of inflammatory responses in the skin (Fogh and Kragballe, 2000). CEES (100-1000 μ M) was found to upregulate mRNA expression of COX-2 (4-fold), mPGES-2 (4-fold) and PGDS (8-fold) (Fig. 6A) in keratinocyte skin constructs. Maximal increases in expression of COX-2 and PGDS were observed with 300 μ M CEES, while mPGES-2 expression increased continuously over all concentrations tested. CEES also upregulated COX-2 protein expression (Fig. 4A). In contrast, a concentration-dependent decrease in mPGES-1 mRNA expression was observed following CEES treatment, while no major changes were noted in mRNA expression of COX-1, PGFS, PGIS or TXAS (Fig 6A).

CEES was also found to upregulate expression of 5-LOX, LTA₄ hydrolase and LTC₄ synthase mRNA, but not FLAP in keratinocytes (Fig. 6B), CEES also increased 5-LOX and LTA₄ hydrolase protein expression in the cells (Fig. 4A). In contrast, CEES had no effect on expression of enzymes mediating production of HETEs, including 8-LOX, epidermal and platelet-type 12-LOX, and 15-LOX (Fig. 6B).

Role of MAP kinases in CEES-induced alterations in expression of antioxidants and eicosanoid generating enzymes

In our next series of studies, we analyzed the role of MAP kinases in CEES-induced alterations in gene expression. Treatment of keratinocytes with CEES resulted in a concentration-dependent activation of JNK and p38 MAP kinase expression in keratinocytes, as measured by increases in the phosphorylated forms of the enzymes (Fig. 4C). In contrast, only small increases in activated ERK1/2 kinase were detected at the highest concentration of CEES tested (1000 μ M) (Fig. 4C). Keratinocytes also constitutively expressed activated phosphorylated forms of p38 and ERK1/2 kinases, but not JNK kinase.

To determine if JNK and p38 MAP kinases mediated CEES-induced changes in mRNA expression in keratinocytes, we used specific inhibitors of these enzymes. Treatment of the cells with the p38 MAP kinase inhibitor, SB203580, was found to markedly suppress CEES-induced expression of COX-2, mPGES-2, PGDS, 5-LOX, LTA₄ hydrolase and LTC₄ synthase mRNA, as well as GSTA1-2 mRNA (Fig. 7). In contrast, inhibition of JNK kinase using SP600125 was only partially effective in suppressing expression of PGDS and GSTA1-2. No major effects of kinase inhibition on CEES-induced expression of Cu,Zn-SOD, catalase, thioredoxin reductase, GSTP1 or mGST2 were noted (Fig. 7 and not shown).

Discussion

Three dimensional *in vitro* skin constructs have been developed as models to assess skin toxicity (Andreadis *et al.*, 2001). By growing cells at an air-liquid interface, an epidermal-like structure is created in which chemicals can be applied directly to the air surface (Nakamura *et al.*, 1990; Greenberg *et al.*, 2006). Some advantages of using skin constructs as models for toxicity testing are that this system provides an alternative to animal experiments, as well as a practical method for mechanistic investigation of large numbers of chemicals and their biological effects (Nakamura *et al.*, 1990). Previous studies with human skin constructs have shown that these models can also be used to evaluate morphological changes in basal keratinocytes and microblister formation in response to CEES and sulfur mustard (Blaha *et al.*, 2000; Blaha *et al.*, 2001; Hayden *et al.*, 2009). The present studies demonstrate that mouse keratinocytes in a construct model are also highly responsive to CEES. Thus, treatment of cells with CEES induced oxidative stress, activated signal transduction pathways, and modulated expression of antioxidants and enzymes that generate eicosanoids, responses known to occur in sulfur mustard-treated rodent and human skin (Papirmeister *et al.*, 1991; Kehe *et al.*, 2009; Shakarjian *et al.*, 2010). Our findings that these responses are dependent on JNK and p38 MAP kinases are novel and suggest a potential therapeutic target for mitigating dermal toxicity induced by vesicants.

Vesicants such as sulfur mustard have been shown to induce oxidative stress in the skin or in skin-derived cells (Papirmeister *et al.*, 1991; Trouba *et al.*, 2002; Bickers and Athar, 2006). This process is the result of excessive production of ROS which can damage lipids, proteins and nucleic acids (Ryter *et al.*, 2007). Using an *in vivo* mouse skin model, Pal *et al.* (2009) have recently demonstrated that topical application of CEES results in oxidative stress, as evidenced by the appearance of several key biomarkers including 4-hydroxynonenal-protein adducts, protein radical adducts, and protein and DNA oxidation products. 4-hydroxynonenal is a highly reactive aldehyde generated following lipid peroxidation (Niki,

2009); in mouse skin, CEES induces the formation of 4-hydroxynonenal protein adducts with molecular weights ranging from 6-92 kDa (Pal *et al.*, 2009). This was correlated with the generation of protein carbonyls, products resulting from the oxidation of amino acids such as lysine, arginine, proline, threonine and cysteine (Stadtman, 1993). In our skin construct model, CEES treatment was found to induce oxidative stress. It readily stimulated hydrogen peroxide production in the keratinocytes and resulted in the formation of several distinct carbonyl protein products with molecular weights ranging from 36-75 kDa. These findings suggest that the skin construct model can be used to recapitulate the process of vesicant-induced oxidative stress. The identity of the oxidized proteins generated by keratinocytes in the skin construct model in response to CEES, and their role in vesicant-induced toxicity remains to be determined. Oxidized proteins and their degradation products can regulate cell growth and differentiation, processes known to be important in the response of the skin to sulfur mustard (Papirmeister *et al.*, 1991). In this regard, oxidative stress has been shown to result in carbonylation of several cytoskeletal proteins important in controlling cell growth including β -actin and tubulin, annexin A1, a protein regulating apoptosis, and mitochondrial ATP synthase, which is critical for cellular energy production (Magi *et al.*, 2004; Je *et al.*, 2008; Wong *et al.*, 2008).

It is well recognized that cells respond to increased oxidative stress with changes in expression of antioxidant enzymes, which is key to limiting cellular damage (Adler *et al.*, 1999; Droge, 2002; Scandalios, 2005). Using monolayer cultures of mouse keratinocytes, we previously showed that paraquat-induced oxidative stress was associated with increased expression of Cu,Zn-SOD, catalase and heme oxygenase-1, as well as the glutathione-S-transferases GSTA1-2, GSTP1, GSTA3 and GSTA4, while UVB light-induced oxidative stress resulted in increased glutathione peroxidase and GSTA1-2 (Black *et al.*, 2008a; Black *et al.*, 2008b). In our skin construct model, CEES-induced protein carbonylation was associated with upregulation of mRNA for catalase, TrxR and Cu,Zn-SOD. These findings suggest that the antioxidant response to oxidative stress varies with the inducer. Catalase is a critical enzyme for detoxifying hydrogen peroxide (Chen *et al.*, 2004; Shim *et al.*, 2005). Increased catalase activity has been reported in the livers of rats following injection of sulfur mustard (Jafari, 2007) and liposomes containing catalase provide significant protection from CEES-induced lung damage in rats (McClintock *et al.*, 2006). These results suggest a key role for hydrogen peroxide in vesicant-induced toxicity. TrxR is a selenocysteine containing enzyme important in retaining thioredoxin in its reduced state, and in maintaining cellular redox balance (Schallreuter and Wood, 2001). CEES has been reported to inhibit TrxR enzyme activity; reducing intracellular production of thioredoxin by inhibiting TrxR may be a trigger for increasing synthesis of the enzyme (Jan *et al.*, 2009). Increased total SOD activity has been described previously in rodents following exposure to sulfur mustard or CEES (Mukhopadhyay *et al.*, 2006; Jafari, 2007); in guinea pig lung, CEES induced mRNA for Cu,Zn-SOD (Mukhopadhyay *et al.*, 2006). Our findings in the present studies that Cu,Zn-SOD increased in keratinocytes are consistent with these reports. In contrast, Mn-SOD mRNA expression decreased in keratinocytes after CEES treatment. Cu,Zn-SOD is a cytosolic enzyme, whereas Mn-SOD is a mitochondrial matrix and inner membrane enzyme; thus, these antioxidants protect different subcellular compartments from oxidative stress and are known to be regulated by distinct mechanisms (Fridovich, 1978). Increases in Cu,Zn-SOD are most likely the result of CEES-induced generation of ROS in keratinocyte cytosol. Sulfur mustard and CEES damage mitochondria, resulting in mitochondrial DNA adducts and altered mitochondrial membrane potential (Shahin *et al.*, 2001; Gould *et al.*, 2009) and this is likely to contribute to reduced expression of Mn-SOD.

CEES was also found to upregulate mRNA expression of GSTA1-2, GSTP1 and mGST2. As members of a large superfamily of cytosolic and microsomal enzymes, the GSTs primarily function in the detoxification of xenobiotics via conjugation to glutathione (Hayes

et al., 2004). Previous studies have shown that sulfur mustard increases total GST activity in human keratinocytes (Gross *et al.*, 2006). GSTA and GSTP enzymes disrupt lipid radical chain reactions, thereby enhancing cellular elimination of lipid and protein peroxidation products (Hayes *et al.*, 2004). Microsomal GSTs including mGST2 are key for detoxifying membrane bound peroxidation products; sulfur mustard has been shown to induce lipid peroxidation in mouse lung (Elsayed and Omaye, 2004) and liver (Vijayaraghavan *et al.*, 1991), and increases in expression of GSTs may be important in repairing damage to cellular membranes (Papirmeister *et al.*, 1991).

Sulfur mustard-induced oxidative stress is correlated with the production of inflammatory mediators in skin including the lipid-derived mediators, prostaglandins and leukotrienes (Dacre and Goldman, 1996; Paromov *et al.*, 2007). Sulfur mustard has been reported to induce the release of membrane-derived arachidonic acid, an important precursor for prostaglandin and leukotriene biosynthesis (Ray *et al.*, 1995; Funk, 2001; Lefkowitz and Smith, 2002). Consistent with previous studies with sulfur mustard-treated mouse skin (Nyska *et al.*, 2001), in our skin construct model, CEES increased expression of mRNA and protein for COX-2, which mediates the metabolism of arachidonic acid to prostaglandin precursors. Findings that sulfur mustard-induced skin inflammation and tissue damage are significantly reduced in COX-2 deficient mice demonstrate the importance of this enzyme in vesicant-induced dermatotoxicity (Wormser *et al.*, 2004a). This is supported by reports that topical anti-inflammatory agents targeting cyclooxygenase are effective in reducing sulfur mustard-induced skin damage (Babin *et al.*, 2000; Casillas *et al.*, 2000; Dachir *et al.*, 2002; Watanabe *et al.*, 2003; Dachir *et al.*, 2004; Wormser *et al.*, 2004b). We also found that CEES treatment of keratinocytes resulted in increased mRNA expression of mPGES-2 and PGDS, two prostanoid synthases downstream of COX-2 that are responsible for the generation PGE₂ and PGD₂, respectively. PGE₂ is known to mediate skin inflammation (Murakami *et al.*, 2002) and sulfur mustard has been reported to stimulate PGE₂ production in full-thickness human skin explants (Rikimaru *et al.*, 1991) and in intact mouse skin (Dachir *et al.*, 2004). Increases in PGDS in response to CEES suggest that PGD₂ may also be formed in the skin. PGD₂ is an important mediator of dermal allergic responses (Satoh *et al.*, 2006), and it may play a similar role in the development of inflammation following exposure to sulfur mustard. Our observation that mPGES-1 is downregulated, while mPGES-2 is upregulated in keratinocytes following CEES treatment, indicates that the mechanisms mediating expression of these two enzymes are distinct, and that mPGES-2 may be more important for generating PGE₂ in keratinocytes in response to vesicants.

Although the effects of sulfur mustard on the biosynthesis of leukotrienes is less well characterized, increased concentrations of these mediators have been measured in inflammatory lesions in the skin of sulfur mustard-treated rabbits (Tanaka *et al.*, 1997). Our findings that CEES caused increases in 5-LOX, LTA₄ hydrolase and LTC₄ synthase, enzymes responsible for the synthesis of leukotriene B₄ (LTB₄) and leukotriene C₄ (LTC₄), are in agreement with these data. mGST2, like LTC₄ synthase, conjugates glutathione to leukotriene A₄ forming LTC₄ (Hayes and McLellan, 1999a). Thus, the increases in mGST2 expression that we observed following CEES treatment of keratinocytes may be representative of a coordinated inflammatory response important not only in protecting against lipid peroxidation, but also in generating leukotrienes. Further studies correlating protein expression of the eicosanoid biosynthetic enzymes in the skin construct model will be important in elucidating their role in sulfur mustard-induced skin injury.

ROS are potent activators of MAP kinase signaling pathways which are key regulators of cell proliferation and cytotoxicity (Kyriakis and Avruch, 2001; Matsuzawa and Ichijo, 2005; McCubrey *et al.*, 2006). MAP kinases have been reported to be activated following treatment of mouse skin or human and mouse keratinocytes in vitro with vesicants (Dillman

et al., 2004; Rebholz *et al.*, 2008; Pal *et al.*, 2009). In SKH-1 hairless mouse skin, CEES activates ERK1/2, JNK and p38 MAP kinases (Pal *et al.*, 2009). Similarly, in our skin construct model, CEES was found to activate JNK and p38 MAP kinases, and to a lesser extent ERK1/2. Apparent differences in MAP kinase activation in response to CEES between the mouse skin construct model and mouse skin may be due to differences in the post exposure times evaluated. Thus, in our studies, MAP kinases were induced within 5 min of CEES exposures, while 3-72 hr was required to activate MAP kinases in mouse skin. Using MAP kinase inhibitors, we demonstrated that p38 MAP kinase regulates keratinocyte expression of COX-2, mPGES-2, PGDS, 5-LOX, LTA₄ hydrolase, and GSTA1-2 in the skin construct system, while JNK kinase regulates PGDS. Our data are largely in agreement with earlier studies showing that p38 MAP kinase regulates proinflammatory cytokine expression in keratinocytes post-sulfur mustard exposure (Dillman *et al.*, 2004). These data indicate that CEES-induced expression of antioxidant enzymes and eicosanoid biosynthetic enzymes can occur via distinct mechanisms. Earlier studies showed that inhibition of p38 MAP kinase can effectively suppress UVB-induced inflammation in mouse skin including expression of proinflammatory cytokines and COX-2 (Hildesheim *et al.*, 2004; Kim *et al.*, 2005). It remains to be determined if inhibitors of this enzyme and/or JNK also suppress sulfur mustard-induced inflammation and skin injury.

In summary, our data demonstrate that CEES induces oxidative stress in a skin construct model as shown by increased keratinocyte protein oxidation. This is associated with increased mRNA expression of antioxidant enzymes including Cu,Zn-SOD, catalase, TrxR, GSTA1-2, GSTP1, and mGST2. At the same time, CEES stimulates expression of key enzymes important in the production of prostaglandins and leukotrienes, including, COX-2, mPGES-2, PGDS, 5-LOX, LTA₄ hydrolase and LTC₄ synthase. The MAP kinase signal transduction pathways appear to be important in mediating expression of several of these enzymes. Whether or not changes in expression of these enzymes mediate inflammation or protect the skin from sulfur mustard toxicity will depend on the levels of expression of functional proteins for these enzymes, as well as their roles in repairing or enhancing cellular damage. Our data also demonstrate that the skin construct model may be useful in understanding the responses of keratinocytes to sulfur mustard-induced toxicity.

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List of Abbreviations

(CEES)	2-chloroethyl ethyl sulfide
(COX-1)	cyclooxygenase-1
(COX-2)	cyclooxygenase-2
(GST)	glutathione S-transferase
(mGST)	microsomal GST
(LTA ₄)	leukotriene A ₄
(LTC ₄)	leukotriene C ₄
(5-LOX)	5-lipoxygenase

(FLAP),)	5-LOX activating protein
(8-LOX),)	8-lipoxygenase
(12-LOX),)	12-lipoxygenase
(15-LOX)	15-lipoxygenase
(PGE₂)	prostaglandin E ₂
(mPGES-1)	microsomal PGE ₂ synthase-1
(mPGES-2)	microsomal PGE ₂ synthase-2
(PGD₂)	prostaglandin D ₂
(PGDS)	PGD ₂ synthase
(PGF₂)	prostaglandin F ₂
(PGFS)	PGF ₂ synthase
(PGH₂)	prostaglandin H ₂
(PGI₂)	prostaglandin I ₂
(PGIS)	PGI ₂ synthase
(ROS)	reactive oxygen species
(SOD)	superoxide dismutase
(TrxR)	thioredoxin reductase
(TXA₂)	thromboxane A ₂
(TXAS)	TXA ₂ synthase

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Figure 1. Summary of ROS detoxification pathways and eicosanoid biosynthetic pathways
Panel A: ROS detoxification pathways. Superoxide anion is metabolized to hydrogen peroxide by SOD. Hydrogen peroxide is then detoxified by catalase and/or various peroxidases and reductases including TrxR. In the presence of transition metals, hydrogen peroxide is converted to highly reactive hydroxyl radicals. Oxidized proteins and lipids are conjugated to glutathione by the GST enzymes to facilitate cellular elimination. **Panel B:** Eicosanoid biosynthetic pathways: Arachidonic acid, mobilized directly from membrane phospholipids, is oxidized to form various eicosanoids by cyclooxygenases (COX), lipoxygenases (LOX) or cytochrome P450 monooxygenases. For prostaglandin metabolism, arachidonic acid is metabolized via cyclooxygenases (COX-1 and COX-2) to produce an intermediate prostaglandin, PGH_2 . Further metabolism by the prostanoid synthases, microsomal prostaglandin E_2 synthase (mPGES), prostaglandin F_2 synthase (PGFS), prostaglandin D_2 synthase (PGDS), prostaglandin I_2 synthase (PGIS) or thromboxane A_2 synthase (TXAS), forms prostaglandins (PGE_2 , $PGF_{2\alpha}$ and PGD_2), prostacyclin (PGI_2) or thromboxane (TXA_2), respectively. For leukotriene biosynthesis, arachidonic acid is metabolized via 5-lipoxygenase (5-LOX), in association with the 5-LOX activating protein

(FLAP), generating a series of intermediate metabolites, 5-hydroperoxyeicosatetraenoic acid (5-HPETE), 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene A₄ (LTA₄). LTA₄ is further metabolized by either LTA₄ hydrolase to form leukotriene B₄ (LTB₄) or conjugated with glutathione by LTC₄ synthase to form leukotriene C₄ (LTC₄).

Figure 2. CEES stimulates intracellular hydrogen peroxide production and protein oxidation
Panel A: Keratinocytes were incubated with DCFH-DA (5 μM) for 15 min.. The cells were then treated with CEES (100, 300 or 1000 μM) or control and analyzed by flow cytometry. Data are presented on a three decade log scale. *Panel B:* Keratinocytes were treated with CEES (100, 300 or 1000 μM) or control. After 24 hr, lysates were prepared and proteins derivatized with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone-modified carbonyl groups. After separation on SDS-polyacrylamide gels, modified proteins were quantified by Western blotting using antibodies to dinitrophenylhydrazone-tagged carbonyl groups.

Figure 3. Effects of CEES on keratinocyte expression of antioxidants

Cells were treated with CEES (100, 300 or 1000 μ M) or control. After 24 hr, expression of Cu,Zn-SOD, Mn-SOD, catalase, and TrxR mRNA was analyzed by real-time PCR. Data are presented as fold change in gene expression relative to control. *Significantly ($p < 0.05$) different from control ($p < 0.05$).

Figure 4. Effects of CEES on protein expression of antioxidants, eicosanoid biosynthetic enzymes and MAP kinases

Cells were treated with CEES (100-1000 μ M) or control and then analyzed for protein expression by Western blotting. β -catenin was analyzed as a control for equal protein loading. *Panel A*: Protein expression was analyzed 24 hr after CEES treatment. *Panel B*: Densitometric analysis of Western blot expression normalized to β -catenin expression. *Panel C*: MAP kinases were analyzed 5 min after CEES treatment.

Figure 5. Effects of CEES on keratinocyte expression of glutathione S-transferases

Cells were treated with CEES (100, 300 or 1000 μ M) or control. After 24 hr, expression of cytosolic and microsomal glutathione S-transferases was analyzed by real-time PCR. Data are presented as fold change in gene expression relative to control. *Significantly ($p < 0.05$) different from control ($p < 0.05$).

Figure 6. Effects of CEES on expression of prostaglandin and leukotriene biosynthetic enzymes
Cells were treated with CEES (100, 300 or 1000 μ M) or control. After 24 hr, expression of eicosanoid biosynthetic enzymes was analyzed by real-time PCR. *Panel A*: Keratinocyte expression of prostaglandin biosynthetic enzymes. *Panel B*: Keratinocyte expression of leukotriene biosynthetic enzymes. Data are presented as fold change in gene expression relative to control. *Significantly ($p < 0.05$) different from control ($p < 0.05$).

Figure 7. Effects of MAP kinase inhibitors on CEES-induced expression of antioxidants and eicosanoid biosynthetic enzymes

Cells were incubated with the p38 MAP kinase inhibitor or JNK MAP kinase inhibitor or control (CTL) for 3 hr and then with CEES (100-1000 μM) or control. After 24 hr, gene expression was analyzed by real-time PCR. Data are presented as fold change in gene expression relative to control. ^aSignificantly ($p < 0.05$) different from control (p38 inhibitor); ^bSignificantly different ($p < 0.05$) from control (JNK inhibitor).

Table 1

Primers used to analyze expression of antioxidants and inflammatory mediators¹.

Gene	Forward (5'→3')	Reverse (5'→3')
catalase	ACCAGGGCATCAAAACTTG	GCCCTGAAGCTTTTGTGTCAG
COX-1	GTCCCAGAACCAGGGTGTCT	CACTGGTAGTTGTGCGAGGCCA
COX-2	CATTCTTTGCCAGCACTTCAC	GACCAGGCACCAGACCAAAGAC
FLAP	GGTGGAGCATGAAAGCAAGG	CCGGTCCTCTGGAAGCTTC
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
GSTA1-2	CAGAGTCCGGAAGATTTGGA	CAAGGCAGTCTTGGCTTCTC
GSTA3	GCAAGCCTTGCCAAGATCAA	GGCAGGGAAGTAACGGTTCC
GSTA4	CCCTTGGTTGAAATCGATGG	GAGGATGGCCCTGGTCTGT
GSTM1	CCTACATGAAGAGTAGCCGCTAC	TAGTGAGTGCCCGTGTAGCAA
GSTP1	CCTTGGCCGCTCTTTGG	GGCCTTCACGTAGTCATTCTTAC
mGST1	GCTTTGGCAAGGAGAGAATG	CCTTCTCGTCAGTGCGAACA
mGST2	TGCAGCCTGTCTGGGTCTC	CAGAAATACTTGTGACGGGCG
mGST3	GGAGGTGTACCTCCCTTCC	TGGTAAACACCTCCCACCGT
LTA ₄	CTCACGGTCCAGTCACAGGA	TGTGTCCAAAGTCAGGCTGC
LTC ₄ synthase	GGACGAAGTGGCTCTTCTGG	TGCAACAGAACTCCCACGAG
5-LOX	AGGGAGAAGCTGTCCGAGT	GCAGAGGCCGTGAAGATCAC
8-LOX	TGTTTGCACACTGGCAGGAA	CATTTAGGAACTGGGAGGCG
12-LOX epi	AAGTTCCTTGGCAGACGCC	TCTTTATGCTGCCCCAGGG
12-LOX plt	ACCAGCAAGGACGACGTGAC	ATCAGGTAGCGACCCCATCA
15-LOX	TCGAGAGCAGAATTCAAGGT	CAGCAGTGGCCCAAGGTATT
PGDS	CTGCTCTGAGCAAATGGCTG	AGGACCAAACCCATCCACAG
mPGES-1	GGCCTTTCTGCTCTGCAGC	GCCACCGCTACATCTTGAT
mPGES-2	AGCCCTTGAAGAGGTCATC	CATTCATGGCCTTCATGGGT
PGFS	GAGGAAGTAGGGCTGGCCAT	CCTCACAGTGCCATCAGCAA
PGIS	ATCTGCTGCTCCCCAACTG	CTTTATCCCCGCTGACAAG
Cu,Zn-SOD	ACCAGTGCAGGACCTCATTTAA	TCTCCAACATGCCTCTCTTCATC
Mn-SOD	CACATTAACGCGCAGATCATG	CCAGAGCCTCGTGGTACTTCTC
TrxR	TTAGAGACCGTGGGCGTGA	GACGGGTATCTTTCCGGTTTTT
TXAS	CCCCAAGCCTTCTCCTTTTG	AAACCCTGGCGGAAAAACAT

¹Primer sequences were designed using Primer Express software (Applied Biosystems).