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An inhibitor of p38 MAP kinase downregulates cytokine release induced by sulfur mustard exposure in human epidermal keratinocytes [☆]

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Abstract

Sulfur mustard (2,2'-dichlorodiethyl sulfide, SM) is a potent alkylating agent that induces skin vesication after cutaneous exposure. Previous work has revealed that SM induces the production of inflammatory cytokines, including IL-8, IL-6, TNF- α , and IL-1 β , in keratinocytes. The p38 MAP kinase (MAPK14) signaling pathway is activated via phosphorylation in response to cellular stress and has been implicated in the upregulation of cytokines in response to stress. We investigated the role of p38 MAP kinase in inflammatory cytokine upregulation following SM exposure. A dose response study in cultured human epidermal keratinocytes (HEK) revealed increasing phosphorylation of p38 MAP kinase in response to increasing concentrations of SM. A time course at the 200 μ M exposure revealed that p38 MAP kinase phosphorylation is induced by 15 min post-exposure, peaks at 30 min and is sustained at peak levels until 8 h post-exposure. Phosphorylation of the upstream kinase MKK3/6 was also detected. Assay of the SM-exposed HEK culture media for cytokines revealed that exposure to 200 μ M SM increased IL-8, IL-6, TNF- α , and IL-1 β . When cells exposed to 200 μ M SM were treated with the p38 MAP kinase inhibitor SB203580, the levels of IL-8, IL-6, and TNF- α and IL-1 β were significantly decreased when compared with cells that were untreated. These results show that p38 MAP kinase plays a role in SM-induced cytokine production in HEK and suggest that inhibiting this pathway may alleviate the profound inflammatory response elicited by cutaneous SM exposure.

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Keywords: Sulfur mustard; p38; p38 MAP kinase; MAPK14; Cytokine

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1. Introduction

The chemical warfare agent sulfur mustard (2,2'-dichlorodiethyl sulfide, SM) is a potent bifunctional alkylating agent. In aqueous environments SM forms a reactive ethylene sulfonium ion intermediate that undergoes nucleophilic attack with biological molecules.

Exposure to SM results in ocular, respiratory and cutaneous toxicity (Papirmeister et al., 1991). The primary manifestation of cutaneous SM exposure is delayed erythema followed by severe skin vesication. A component of the cutaneous toxicity is likely due to the profound inflammatory response induced by cutaneous SM exposure. Work from several investigators utilizing a variety of model systems has shown that cutaneous SM exposure induces a significant increase in the production of inflammatory cytokines including interleukin-8 (IL-8), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), and interleukin 1-beta (IL- β) (Schlager et al., 2002; Ricketts et al., 2000; Sabourin et al., 2000; Arroyo et al., 1999; Lardot et al., 1999; Tsuruta et al., 1996; Rikimaru et al., 1991).

Understanding the molecular and cellular pathways activated in response to SM exposure would provide a rational basis for development of pharmacological

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intervention to alleviate toxicity. However, little is known about the signal transduction pathways activated by SM immediately after exposure that result in cytokine production and a delayed inflammatory response. It is likely that SM exposure induces a cell stress response due to alkylation and/or crosslinking of cellular macromolecules and alteration of the cellular redox state (Papirmeister et al., 1991). Analysis of stress gene induction in several lines of HepG2 cells stably transformed with either a stress gene promoter or upstream stress gene regulatory element associated with a reporter gene revealed activation of several of these stress gene constructs, including MT2A metallothionein IIA response element, GADD45 response element, NF- κ B response element, and p53 response element (Schlager and Hart, 2000). In endothelial cells, cell stress has been shown to activate the transcription factor NF- κ B, and SM exposure induces binding of NF- κ B to its DNA consensus binding site in electrophoretic mobility shift assays (Atkins et al., 2000). An irritant response via cytokine production and release often occurs in response to cellular stress events (e.g. SM exposure). Several stress signaling pathways have been identified that utilize protein kinase cascades to activate transcription of genes responsive to cellular stress (Kyriakis and Avruch, 2001). The p38 MAP kinase (MAPK14) signaling pathway is activated in response to cellular stress and has been implicated in the upregulation of cytokines in response to inflammatory stimuli (Adams et al., 2001; Lee et al., 1999). The p38 MAP kinase family is related to the extracellular signal-regulated kinases (ERKs) and the c-Jun N-terminus kinases (JNKs) or stress-activated protein kinases (SAPKs) (Chang and Karin, 2001). p38 MAP kinases differ from the ERKs in that ERKs are activated by mitogens and several 7-transmembrane receptor agonists. However, p38 kinases are similar to the JNKs in that both families are activated by various stress stimuli including UV radiation, heat, chemical or osmotic shock, IL-1, TNF, and endotoxin. Activation of p38 kinase is accomplished by phosphorylation of threonine and tyrosine in the motif TGY by upstream kinases. Once phosphorylated, p38 kinase is activated and phosphorylates a variety of targets, including transcription factors involved in upregulation of cytokine gene expression (Adams et al., 2001; Lee et al., 1999). The p38 MAP kinase pathway has been implicated in the upregulation of IL-8, IL-6, and TNF- α in a variety of paradigms (Holtmann et al., 1999; Winzen et al., 1999; Hoffmeyer et al., 1999).

Given the role of p38 MAP kinase in cell stress responses and inflammation, we were interested in determining whether p38 MAP kinase plays a role in inflammatory cytokine upregulation following SM exposure. Using primary human epidermal keratinocytes (HEK) as a model system, we found that p38 MAP kinase is rapidly activated following SM exposure. The

production of the cytokines IL-8, IL-6, IL-1 β , and TNF- α is increased in the culture media of HEK exposed to SM. Upregulation of these cytokines by SM is inhibited by pharmacological inhibition of p38 MAP kinase, suggesting that the p38 MAP kinase pathway represents a potential therapeutic target for attenuating the pro-found inflammatory response resulting from cutaneous SM exposure.

2. Materials and methods

2.1. Cell culture and exposure

HEK from breast skin were obtained as cyropreserved first passage stocks from Cascade Biologics (Eugene, OR). Cells were seeded at 2500/cm² into 75 cm² flasks. Cells were grown in serum-free supplemented keratinocyte growth medium (EpiLife, Cascade Biologics) to 70–80% density prior to trypsin detachment and reseeding at 2500 cells/cm² into 150 cm² flasks (for samples for gel electrophoresis and Western blotting) or reseeding at 4700 cells/well in 24-well plates (for samples for pharmacological treatments and cytokine determination). Third through sixth passage flasks of HEK at 75–90% confluence were used for SM exposure. A frozen aliquot of neat SM in keratinocyte growth medium was thawed and vortexed to generate a 4 mM SM stock solution. This stock solution was placed on ice and immediately diluted into media to expose cells to SM. The HEK were maintained at 37 °C throughout the duration of the experiment.

2.2. Gel electrophoresis

The culture media was removed from the SM-exposed cells and the cells washed with Hank's balanced salt solution. The cells were lysed in 2X SDS sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate), a protein assay was performed (Coomassie Plus, Pierce, Rockland, IL), reducing agent (286 mM β -mercaptoethanol) was added and the samples were boiled. Equivalent amounts of total cellular protein were fractionated on a 10% SDS polyacrylamide gel.

2.3. Western blotting

Proteins were transferred from the gel to polyvinylidene fluoride membrane (PVDF, Hybond, Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked in 3% bovine serum albumin and probed with a rabbit polyclonal antibody against the phosphorylated form of p38 (Phospho-p38 MAP Kinase (Thr 180/Tyr 182) antibody, Cat#9211L, Cell Signaling Technology, Beverly, MA) or the phosphorylated form of MKK3/6 (Phospho MKK3/MKK6 (Ser189/207)

antibody, Cat#9231, Cell Signaling Technology). The primary antibody was detected via an alkaline phosphatase conjugated mouse anti-rabbit secondary antibody and enhanced chemifluorescence (ECF, Amersham Pharmacia Biotech, Piscataway, NJ). The fluorescent signal was detected and visualized using a Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant software (Molecular Dynamics). The membrane was stripped of antibody using low pH glycine buffer and reprobed using an antibody that recognized the total cellular pool of p38 (p38 MAP Kinase antibody, Cat#9212, Cell Signaling Technology) or MKK3 (MKK3 antibody, Cat#9232, Cell Signaling Technology) as described and quantitated as a loading and normalization control. The fold-induction of p38 phosphorylation was determined by dividing the pixel volume of the phospho-p38 signal by the pixel volume of the p38 signal. The same calculations were performed for phospho-MKK3/6 and MKK3/6.

2.4. Pharmacological inhibition of p38 MAP kinase

SB203580 was obtained from Tocris-Cookson (St. Louis, MO), dissolved in DMSO to make a 10 mM (1000 $\times$) stock, frozen in aliquots at -80°C and thawed immediately before use. Each of the four rows of a 24-well plate received one of the following treatments: row A 200 μM SM; row B 200 μM SM and dimethyl sulfoxide (DMSO, drug vehicle, final dilution 1:1000, final concentration 14.1 mM); row C 200 μM SM and 10 μM SB203580 (final concentration; Davies et al., 2000) in DMSO (14.1 mM final concentration); and row D 10 μM SB203580 (final concentration) in DMSO (14.1 mM final concentration). The cell culture media from each well in a row was collected and pooled at the indicated times post-exposure and frozen at -20°C .

2.5. Assay of cytokine production

Cell culture media was assayed for the production of cytokines using the Beadlyte Human Multi-Cytokine Detection System 2 (Upstate Biotechnology, Charlottesville, VA) according to the manufacturer's instructions. The Luminex 100 instrument was used for analysis (Luminex Corp, Austin, TX). This system allows the simultaneous assay of multiple cytokines from a single 50 μl culture media sample. The system used in these studies was designed to quantitate IL-8, IL-6, IL-1 β , IL-10, IL-12, and TNF- α . Duplicate assays were performed on each of three individual cell platings ($n = 3$). A standard curve was generated by resuspending 5000 pg Multi-Cytokine 2 (IL-8, IL-6, IL-1 β , IL-10, IL-12, and TNF- α) standard in 2 ml of assay buffer according to the manufacturer's protocol. Serial dilutions were performed to produce a standard curve of 2500, 1000, 500, 250, 125, 62.5, 31.25, 15.6, and 0 pg/ml (assay buffer alone).

2.6. Statistical analysis

Cytokine production (pg cytokine/ml) was expressed as the average $\pm$ SEM ($n = 3$) and was analyzed for statistical significance using Bonferroni's adjusted t -test to control the experimentwise error rate at 5%. The efficacy of SB203580 as an inhibitor of cytokine production was determined by comparing cytokine production induced by SM in the presence or absence of 10 μM SB203580 and expressed as percent inhibition (average percent inhibition $\pm$ SEM [$n = 3$] for the time points at which cytokines are produced).

3. Results

To determine if the p38 pathway is activated in response to SM exposure, we examined the phosphorylation state of p38 MAP kinase in cultured primary HEK exposed to a range of SM concentrations. HEK were exposed to SM for 1 h at the doses indicated and cellular proteins were extracted, fractionated by gel electrophoresis, and transferred to PVDF membrane. To determine the phosphorylation state of p38, we probed the

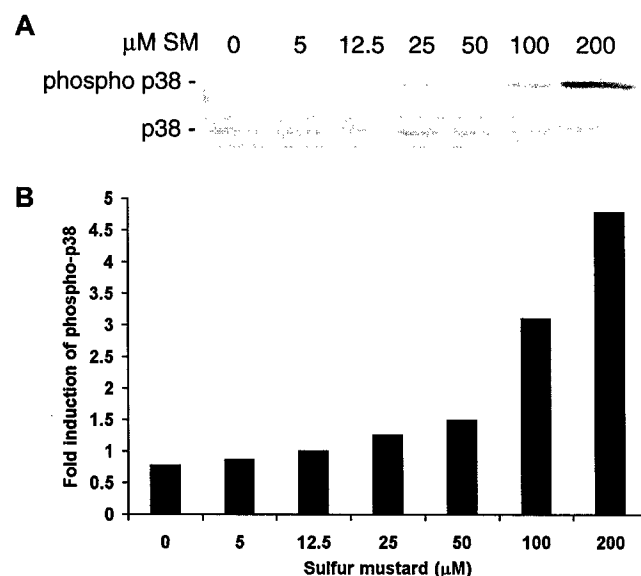


Fig. 1. Phosphorylation of p38 MAP kinase is increased by increasing concentrations of SM. Cultured primary HEK were exposed to various doses (0–200 μM) of SM for one hour. Cell extracts were prepared, total cellular protein was fractionated by gel electrophoresis, transferred to PVDF membrane and probed with an antibody that specifically recognizes the phosphorylated (activated) form of p38 MAP kinase. Phosphorylation of p38 MAP kinase is strongly induced at concentrations ≥ 100 μM SM. The membranes were stripped and reprobed with an antibody that recognizes the total cellular pool of p38 MAP kinase. (A) Western blot of phospho-p38 and p38 in human epidermal keratinocytes exposed to various concentrations of SM for 1 h. (B) Fold-induction of p38 phosphorylation in human epidermal keratinocytes exposed to SM. This experiment was repeated twice with similar results.

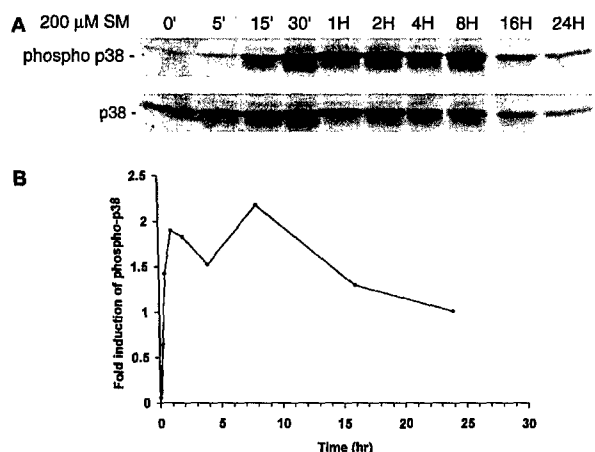


Fig. 2. Phosphorylation of p38 MAP kinase is rapidly induced and sustained by SM exposure. Cultured primary HEK were exposed to 200 μ M SM and harvested at 0, 5, 15, 30 min, 1, 2, 4, 8, 16, and 24 h post-exposure. Cell extracts were prepared, total cellular protein was fractionated by gel electrophoresis, transferred to PVDF membrane and probed with an antibody that specifically recognizes the phosphorylated (activated) form of p38 MAP kinase. Phosphorylation of p38 MAP kinase is rapid and sustained out to 8 h post-exposure. The membranes were stripped and reprobed with an antibody that recognizes the total cellular pool of p38 MAP kinase. (A) Western blot of phospho-p38 and p38 in human epidermal keratinocytes exposed to 200 μ M SM for the times indicated. (B) Fold-induction of p38 phosphorylation in human epidermal keratinocytes exposed to SM. This experiment was repeated twice with similar results.

membrane with an antibody specific to the phosphorylated form of p38. We found that p38 is phosphorylated in a dose-dependent manner in response to SM exposure in HEK (Fig. 1). Maximal phosphorylation of p38 occurs at SM concentrations which are 100 μ M or greater. To determine the kinetics of p38 phosphorylation in response to SM exposure, a time course at 200 μ M was performed. We found that p38 phosphorylation occurs within 15 min following SM exposure and is sustained for over 8 h after the initial exposure (Fig. 2). In addition, we examined the phosphorylation state of MKK3/MKK6, a kinase upstream of p38, using an antibody that specifically recognizes the phosphorylated form of MKK3/MKK6 (Fig. 3). We found that MKK3/MKK6 is also phosphorylated in response to SM exposure, and this phosphorylation occurs in a time frame consistent with upstream activation of the p38 pathway.

Since SM exposure activates the p38 signaling pathway, we wanted to determine whether pharmacologic inhibition of this pathway would block inflammatory cytokine release, specifically release of IL-8, IL-6, TNF- α , and IL-1 β , which have been shown previously to be upregulated in HEK in response to SM exposure. We exposed HEK to 200 μ M SM and harvested the culture media at various timepoints post-exposure for multiplex analysis of cytokine release. Exposure of HEK to 200 μ M SM results in increased production of IL-8, IL-6, TNF- α , and IL-1 β over a 24-h period after exposure

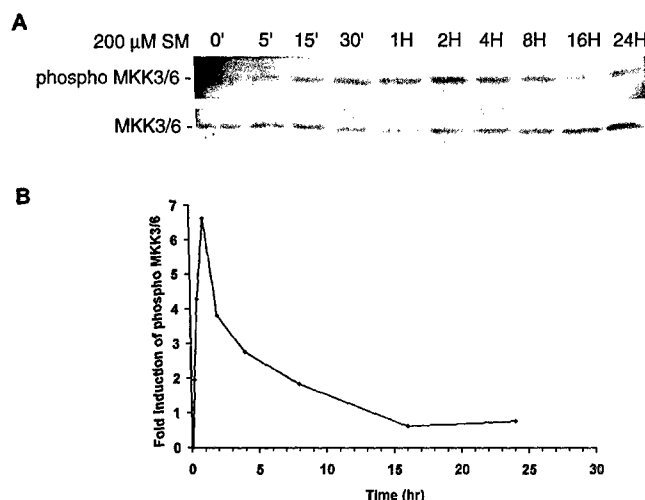


Fig. 3. Phosphorylation of MKK3/6 is rapidly induced and sustained by SM exposure. Western blot of phospho-MKK3/6 and total MKK3/6 in cultured primary HEK exposed to 200 μ M SM for the indicated times. Cell extracts were collected and analyzed as described above. (A) Western blot of phospho-MKK3/6 and MKK3/6 in human epidermal keratinocytes exposed to 200 μ M SM for the times indicated. (B) Fold-induction of MKK3/6 phosphorylation in human epidermal keratinocytes exposed to SM. This experiment was repeated twice with similar results.

(Fig. 4). Our multiplex analysis also determined the levels of IL-10 and IL-12(p70). The levels of these cytokines were not increased by SM exposure (data not shown). Interestingly, the production of IL-8 and IL-6 in response to SM exposure appears to be biphasic in nature with peaks of cytokine production at 8 and 24 h and a decrease in cytokine production at 16 h. This is in contrast to the response of TNF- α and IL-1 β production after SM exposure (compare Fig. 4A–D at 8, 16, and 24 h). Pharmacological inhibition of p38 with the specific p38 inhibitor SB203580 (10 μ M, Davies et al., 2000) results in attenuation of the production of IL-8, IL-6, IL-1 β , and TNF- α in response to SM exposure in cultured HEK (Fig. 4). SB203580 treatment significantly decreased SM-induced IL-8, IL-6, TNF- α , and IL-1 β production at time points between 2 and 24 h. Interestingly, DMSO alone appeared to decrease TNF- α production, however this was not statistically significant. The efficacy of SB203580 was also expressed as percent inhibition of cytokine production (Table 1). No effect of SB203580 was seen on the constitutive levels of IL-10 and IL-12(p70) (data not shown).

4. Discussion

Cutaneous SM exposure results in erythema and then severe vesication following an asymptomatic period. The cutaneous toxicity is likely due, at least in part, to the profound inflammatory response elicited during this asymptomatic period. We have found that p38 MAP

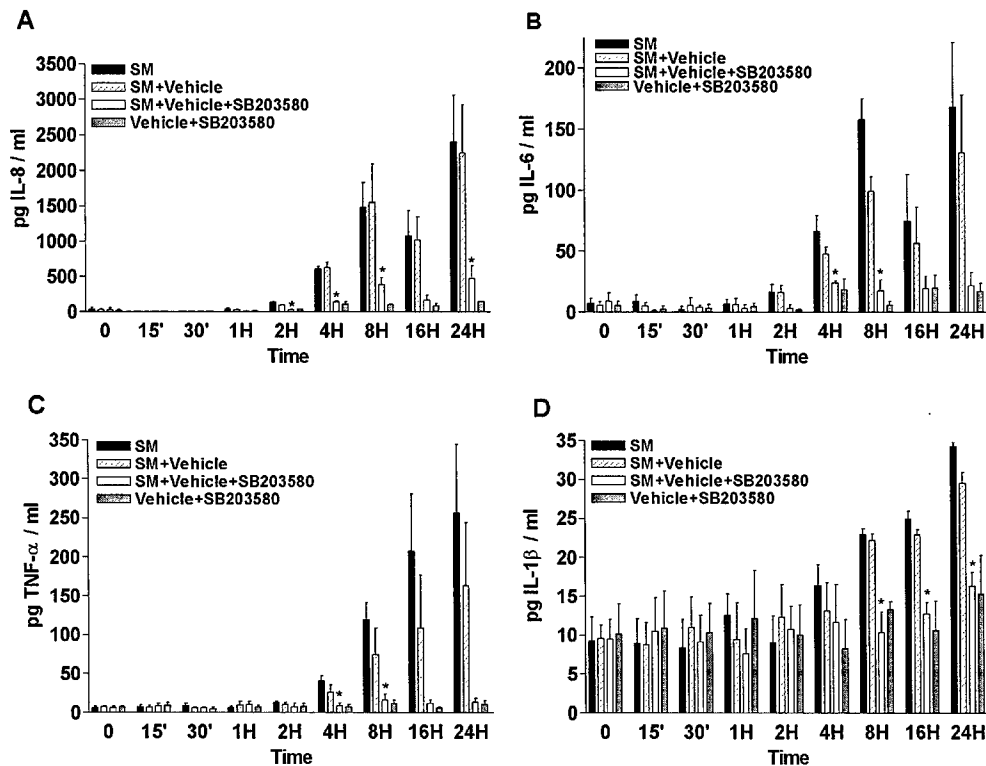


Fig. 4. The p38 MAP kinase inhibitor SB203580 attenuates SM-induced cytokine production. Cultured human epidermal keratinocytes were exposed to 200 μ M SM, 200 μ M SM in the presence of drug vehicle (DMSO) at a vehicle dilution of 1:1000, 200 μ M SM in the presence of 10 μ M SB203580, or 10 μ M SB203580 alone. At the times indicated, the cell culture media was collected and assayed for cytokine production. The average of three separate cell platings ($n = 3$) was determined ($\pm$ SEM). Asterisks indicate statistically significant decreases in cytokine production by treatment with 10 μ M SB203580 ($p < 0.05$). (A) SM-induced IL-8 production. (B) SM-induced IL-6 production. (C) SM-induced TNF- α production. (D) SM-induced IL-1 β production.

Table 1

Percent inhibition of cytokine production by 10 μ M SB203580 in human epidermal keratinocytes exposed to 200 μ M sulfur mustard

Time (h)	IL-8	IL-6	TNF- α	IL-1 β
2	77 $\pm$ 3%	85 $\pm$ 15%	48 $\pm$ 25%	n.c.
4	76 $\pm$ 2%	60 $\pm$ 11%	80 $\pm$ 6%	35 $\pm$ 19%
8	74 $\pm$ 1%	90 $\pm$ 5%	87 $\pm$ 6%	55 $\pm$ 10%
16	85 $\pm$ 3%	49 $\pm$ 25%	91 $\pm$ 7%	48 $\pm$ 7%
24	82 $\pm$ 3%	90 $\pm$ 6%	92 $\pm$ 5%	52 $\pm$ 5%

Note: calculations are the average percent inhibition ($n = 3$) $\pm$ SEM. n.c.—not calculated.

kinase, a signaling molecule associated with inflammation, is activated in cultured primary HEK in a time- and dose-dependent manner in response to SM exposure. p38 MAP kinase activation is rapid and sustained, and is maximal at in vitro concentrations that result in induction of apoptosis (Rosenthal et al., 1998) and cytokine production in HEK. Pharmacological inhibition of p38 MAP kinase by the well characterized inhibitor SB203580 (Davies et al., 2000) blocks the SM-induced production of the inflammatory cytokines IL-8, IL-6, IL-1 β , and TNF- α . Thus the p38 MAP kinase pathway is a potential therapeutic target to combat the inflammatory response elicited by cutaneous SM exposure.

Previously, it had been shown that SM exposure results in the production of inflammatory cytokines in

skin and cultured keratinocytes (Schlager et al., 2002; Ricketts et al., 2000; Sabourin et al., 2000; Arroyo et al., 1999; Lardot et al., 1999; Tsuruta et al., 1996; Rikimaru et al., 1991). IL-8 is a chemokine and a potent chemo-attractant that strongly induces neutrophil chemotaxis. Of the cytokines in this study, it was the cytokine most strongly induced by SM exposure. Regulation of IL-8 expression has been shown to be controlled by the p38 pathway (Holtmann et al., 1999). Using an inducible expression system that allows rapid transcriptional shutoff, Holtmann and coworkers found that an active mutant of MKK6, the upstream activator of p38, which we have shown is also activated by SM exposure, stabilized the IL-8 mRNA (Holtmann et al., 1999). Stabilization of the IL-8 mRNA appears to be related to the

presence of a 161-nucleotide AU-rich region in the 3' untranslated region of the mRNA (Winzen et al., 1999). Thus, activation of the p38 pathway by SM may contribute, at least in part, to increased IL-8 protein production via enhanced mRNA stabilization. In a similar study, Winzen et al. (1999) found that p38 MAP kinase activation was also involved in stabilization of IL-6 mRNA. IL-6 is a multifunctional cytokine that is involved in the stimulation of leukocytes, the production of acute phase proteins, and the induction of B-cells to differentiate into antibody-forming cells. IL-1 β expression induced by SM exposure in keratinocytes is also downregulated by pharmacological inhibition of p38 MAP kinase. IL-1 β is involved in inflammatory responses including edema, prostaglandin production, and corticosteroid release.

TNF- α expression is also increased in response to SM exposures in keratinocytes. Previous work has shown that the MAP kinase pathways, including p38, are involved in TNF- α expression, but the data appears to be dependent on cell type. In T cells, Erk, JNK, and p38 all play an important role in TNF- α production, with p38 playing a post-transcriptional role in TNF- α production (Hoffmeyer et al., 1999). Interestingly, the kinetics of TNF- α production in keratinocytes exposed to SM are different from those of IL-8, IL-6 and IL-1 β . Whereas production of these cytokines appears to be biphasic, production of TNF- α does not. In addition, the effects of vehicle treatment alone (DMSO) seem to be more pronounced on TNF- α than on IL-8, IL-6, and IL-1 β production. This suggests that other signaling pathways may be involved in crosstalk with the p38 pathway in ways that differentially regulate TNF- α and IL-8, IL-6 and IL-1 β . Furthermore, the effects of DMSO suggest that membrane dynamics may play a more important role in the stimulation of TNF- α production by SM than in the stimulation of IL-8, IL-6 and IL-1 β . Future experiments will involve investigating the roles of membrane receptors and the other MAP kinase pathways in SM-induced cytokine production.

In conclusion, we have shown that p38 MAP kinase is activated in a time- and dose-dependent manner in response to SM exposure. Pharmacological inhibition of p38 MAP kinase inhibits production of the inflammatory cytokines IL-8, IL-6, IL-1 β , and TNF- α . Thus, the p38 signal transduction pathway represents a potential therapeutic target for the development of medical countermeasures against cutaneous SM exposure in military and civilian populations.

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