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Nitrogen mustard exposure of murine skin induces DNA damage, oxidative stress and activation of MAPK/Akt-AP1 pathway leading to induction of inflammatory and proteolytic mediators

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Abstract

Our recent studies in SKH-1 hairless mice have demonstrated that topical exposure to nitrogen mustard (NM), an analog of sulfur mustard (SM), triggers the inflammatory response, microvesication and apoptotic cell death. Here, we sought to identify the mechanism/s involved in these NM-induced injury responses. Results obtained show that NM exposure of SKH-1 hairless mouse skin caused H2A.X and p53 phosphorylation and increased p53 accumulation, indicating DNA damage. In addition, NM also induced the activation of MAPKs/ERK1/2, JNK1/2 and p38 as well as that of Akt together with the activation of transcription factor AP1. Also, NM exposure induced robust expression of pro-inflammatory mediators namely cyclooxygenase 2 and inducible nitric oxide synthase and cytokine tumor necrosis factor alpha, and increased the levels of proteolytic mediator matrix metalloproteinase 9. NM exposure of skin also increased lipid peroxidation, 5,5-dimethyl-2-(8-octanoic acid)-1-pyrroline N-oxide protein adduct formation, protein and DNA oxidation indicating an elevated oxidative stress. We also found NM-induced increase in the homologous recombinant repair pathway, suggesting its involvement in the repair of NM-induced DNA damage. Collectively, these results indicate that NM induces oxidative stress, mainly a bi-phasic response in DNA damage and activation of MAPK and Akt pathways, which activate transcription factor AP1 and induce the expression of inflammatory and proteolytic mediators, contributing to the skin injury response by NM. In conclusion, this study for the first time links NM-induced mechanistic changes with our earlier reported murine skin injury lesions with NM, which could be valuable to identify potential therapeutic targets and rescue agents.

Graphical abstract

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Conflict of interest

The authors state no conflict of interest.

Keywords

Nitrogen mustard; Skin injury; DNA damage; Oxidative stress; Mitogenic and survival signaling; Inflammatory mediators; Proteolytic mediator

1. Introduction

Sulfur mustard [SM; bis(2-chloroethyl) sulfide] is a highly reactive bi-functional alkylating agent that primarily targets skin, eyes and respiratory system causing adverse toxic effects (Ghabili et al., 2010; Ghanei and Harandi, 2007). Skin is the maximally exposed tissue to this agent; symptoms take several hours to develop and start with irritation and erythema that convert to severe inflammation and blistering (Dacre and Goldman, 1996; Graham et al., 2005). The specific mechanism of SM-induced skin tissue inflammation, blistering, and epidermal cell death is not well understood. This is a major limitation in developing and optimizing effective targeted therapeutic interventions against skin injuries from vesicating agent SM, a potent chemical warfare/ terrorism agent, and its bifunctional alkylating analog nitrogen mustard [NM; bis(2-chloroethyl) methylamine, HN2], which also poses a similar threat (Balali-Mood and Hefazi, 2005, 2006; Ghabili et al., 2011; Rosenthal et al., 1998; Wormser et al., 2002).

SM- and NM-induced skin injury responses are histopathologically similar mainly due to their bi-functional alkylating properties targeting cellular components including lipids, nucleic acids and proteins (Kehe and Szinicz, 2005; Rosenthal et al., 1998; Wormser et al., 2002). Their interaction with nucleic acids in DNA causes the formation of nonfunctional adducts and interstrand crosslinks resulting in DNA damage, which is the major lesion causing direct cytotoxicity or triggering downstream repair and/or cell death signaling pathways (Brimfield et al., 2009; Kehe and Szinicz, 2005). Studies with vesicating agents have shown that DNA damage and epidermal cell death involve the activation of p53-related signaling and DNA damage repair pathways, poly (ADP-ribose) polymerase (PARP), and calmodulin pathway (Inturi et al., 2013; Kehe et al., 2008; Korkmaz et al., 2006; Minsavage and Dillman, 2007; Rebholz et al., 2008; Ruff and Dillman, 2007; Tewari-Singh et al., 2010). Several reports indicate that SM exposure-induced skin toxic effects and DNA damage are associated with enhanced production of inflammatory cytokines including TNF α and chemokines, and increased oxidative stress as well nitric oxide production (Das

et al., 2003; Kehe and Szinicz, 2005; Mukhopadhyay et al., 2006; Paromov et al., 2007), which could stimulate injury and escalate inflammatory responses (Kehe and Szinicz, 2005; Ruff and Dillman, 2007). Apart from the DNA damage, the toxic consequences of SM exposure could encompass the activation of mitogen-activated protein kinases (MAPKs). MAPKs are the serine/threonine kinases activated in response to various stimuli including genotoxic stress and inflammatory cytokines, and play an important role in inflammatory response, cell survival, and cellular proliferation and differentiation (Zhang and Liu, 2002). In addition, PI3K/Akt pathway, which is involved in the regulation of processes like transcription, cell growth, proliferation and apoptosis and could be activated by anti-apoptotic and diverse cell survival stimuli including oxidative stress, could also be involved in vesicant skin toxicity (Franke et al., 2003). Both MAPK and Akt pathways can activate the transcription factor AP1, which plays a key role in epithelial cell growth, differentiation, transformation and apoptosis (Angel et al., 2001; Shaulian and Karin, 2001). SM-induced inflammation and vesication induce a number of pro-inflammatory proteins implicating the role of pro-inflammatory mediators like cyclooxygenase 2 (COX2), as well as up-regulate matrix metalloproteinase (MMP) family proteases that degrade the extracellular matrix proteins, which are the major component of basement membrane and separate epidermis from dermis leading to vesication (Casillas et al., 2000; Nyska et al., 2001; Wormser et al., 2004).

Taken together, several reports employing cell culture and different animal species have implicated various mechanistic aspects of SM- and its monofunctional alkylating analog 2, chloroethyl ethyl sulfide (CEES)-induced skin inflammation, microvesication and cell death (Black et al., 2010a; Inturi et al., 2011; Jain et al., 2011a; Jain et al., 2011a,b; Tewari-Singh et al., 2012, 2009). However, precise molecular mechanisms and their interconnection with the progression of vesicant skin injury and healing remain to be fully elucidated. Accordingly, here we carried out detailed mechanistic studies with NM in SKH-1 hairless mouse skin, where our recent studies have shown that the clinical and histopathological sequelae of skin injuries by NM were comparable to those reported in humans and animal models with SM (Tewari-Singh et al., 2013, 2014a,b). Our findings from the present study build a foundation where NM-induced skin injuries in SKH-1 hairless mouse model could be used to further understand the role of identified pathways and molecules in vesicant-induced skin injury responses and to effectively develop their therapeutic interventions.

2. Materials and methods

2.1. Materials and chemicals

NM was obtained commercially from Sigma–Aldrich Chemical Co. (St. Louis, MO). Primary antibodies for phosphorylated extracellular signal-regulated kinase [ERK1/2 (Thr202/ Tyr204)], c-Jun-NH2 kinase [JNK (Thr183/ Tyr185)], p38 (Thr180/ Tyr182), phosphoinositide-dependent kinase-1 [PDK1 (Ser241)], Akt (Ser473), p53 (Ser15), H2A.X (Ser139), ERK1/2, JNK, p38, Akt p53, MMP9 and tumor necrosis factor alpha (TNF α) were purchased from Cell Signaling Technology (Beverly, MA). Anti-4-hydroxynonenal (4-HNE) rabbit polyclonal antibody was kind gift from Dr. Dennis Petersen (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Denver). Anti-COX2

antibody and anti-5,5, dimethyl-2-(8-octanoic acid)-1-pyrroline N-oxide (DMPO) nitron polyclonal antiserum were purchased from Cayman Chemicals (Ann Arbor, MI). Antibody for iNOS was from Abcam (Cambridge, MA). Anti- β -actin antibody was purchased from Sigma-Aldrich Co. (St. Louis, MO). Phosphorylated anti-cJun anti-cFos-Rad 51, non-phosphorylated anti-cJun anti-cFos antibodies and consensus sequences of double stranded AP1 oligonucleotides were from Santa Cruz Biotechnology (Dallas, TX). Anti-mouse IgG HRP-conjugated secondary antibody was obtained from GE Healthcare Bio-Sciences (Pittsburgh, PA) and anti-rabbit IgG HRP-conjugated secondary antibody was obtained from Cell Signaling Technology (Beverly, MA). Goat anti-mouse IgG and anti-rabbit IgG secondary antibody labeled with either IRDye 800CW or IRDye 680LT were purchased from LI-COR Biosciences (Lincoln, NE). OxyBlot protein oxidation detection kit was purchased from Chemicon International (Temecula, CA). Protein assay kit was obtained from Bio-Rad laboratory (Hercules, CA) and enhanced chemiluminescence western blot detection reagents were purchased from GE Healthcare Bio-Sciences.

2.2. Animals and NM exposure

Male SKH-1 hairless mice (4–5 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed and acclimatized for one week under standard conditions at the Center of Laboratory Animal Care (CLAC), University of Colorado Denver, CO. Studies were carried out according to the specified protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado Denver. Acetone alone or NM (3.2 mg/mouse) prepared in acetone, was applied topically on to the dorsal surface of the mouse skin under a chemical and biological safety fume hood, and all the required personal protective equipment were used during the experimental treatments with NM as described previously (Tewari-Singh et al., 2009). For the time-response study, mice ($n = 5$ /group) were exposed topically to NM (3.2 mg in 200 μ l acetone/mouse) for 12, 24, 72 or 120 h, as described earlier (Jain et al., 2014a,b; Tewari-Singh et al., 2014a,b). At the end of each desired treatment time, mice were euthanized and the dorsal skin samples were collected and snap frozen in liquid nitrogen.

2.3. Preparation of tissue lysates and western blot analysis

Subcutaneous fatty tissue was removed from skin samples, and then either whole skin tissue extract or cytosolic and nuclear extracts were prepared and total protein was estimated by Lowry's method using Bio-Rad DC protein assay kit (Bio-Rad laboratories, Hercules, CA). Equal amounts of protein (60–80 μ g) from the desired samples was resolved on Tris-glycine gel (8–12%), transferred on to nitrocellulose membranes and blocked with 5% nonfat dry milk or Odyssey blocking buffer for 1 h. Thereafter, membranes were incubated with the appropriate primary antibodies overnight at 4 °C and then washed. Further, the membranes were incubated with HRP-conjugated or IR dye-conjugated secondary antibodies for 1 h at room temperature. The membranes were subjected to either enhanced chemiluminescence detection or analyzed under an Odyssey Infrared Imager (LI-COR Biosciences Lincoln, NE). The same membrane was re-probed with anti- β -actin antibody as loading control. All the autoradiograms and detected bands were scanned using adobe photoshop 6.0 (adobe Systems, Inc., San Jose, CA). Results obtained were quantified via densito-metric analysis of protein bands using the Image J Program (NIH, Bethesda, MD). The western blot analysis

for each molecule was carried out in 2–3 NM exposed animals from 5 exposed animals per group and results shown are representative of 2–3 animals in the study group.

2.4. Electrophoretic mobility shift assay (EMSA)

As described earlier, AP1 DNA binding activity was measured by EMSA (Gu et al., 2007). Briefly, nuclear extracts were prepared from control and NM-exposed skin tissues, and EMSA was carried out using AP1 consensus oligonucleotides radiolabeled with [γ - 32 P]ATP in the presence of T4 polynucleotide kinase as per the manufacturer's protocol (Promega, Madison, WI). Separation of labeled probe from free [γ - 32 P] ATP was completed using G-25 Sephadex column. Nuclear extract (10 μ g) was incubated with gel shift binding buffer followed by 32 P-labeled AP1 probe for 20 min at 37 °C. On native polyacrylamide gel, DNA-protein complexes were separated in EMSA-buffer by electrophoresis at 150 V/40 mA for 1 h at 25 °C, which was followed by gel drying and autoradiography.

2.5. Western blot analysis for protein oxidation

Oxidative modification of proteins in the skin tissue samples was analyzed via western blot analysis using OxyBlot protein oxidation detection kit as published earlier (Pal et al., 2009). Briefly, 20 μ g of protein samples were denatured and derivatized by 2,4-dinitrophenylhydrazine (DNPH) or control solution, and thereafter the reaction was stopped by adding the neutralizing solution. Both the derivatized sample and control were then loaded on the 12% SDS-polyacrylamide gel for electrophoresis, and blotted onto a nitrocellulose membrane. The membrane was blocked, and incubated overnight at 4 °C with the corresponding primary antibody (1:150 dilution) in non-fat dry milk as published earlier, followed by incubating it with HRP-conjugated secondary antibody (1:300 dilution) for 1 h at room temperature. Thereafter, membrane was washed in PBS-T and subjected to enhanced chemiluminescence detection.

2.6. Immunohistochemistry (IHC) for DNA oxidation in mouse skin

IHC of skin sections for the detection of DNA oxidation was carried out as reported earlier (Tewari-Singh et al., 2012). Briefly, 5 μ m skin sections were incubated overnight with mouse monoclonal anti-8-oxo-2-deoxyguanosine (8-OHdG; JalCA, Japan) antibody in PBS in humidity chamber at 4 °C after blocking of the endogenous peroxide activity. The N-Universal negative control rabbit IgG antibody (DAKO, Carpinteria, CA) was used as a negative control. After PBS wash, sections were incubated with appropriate biotinylated secondary antibody for 1 h, then incubated with HRP-conjugated streptavidin for 1 h followed by DAB for 5 min. Sections were counterstained with hematoxylin followed by dehydration and mounting for microscopic observation. The brown-colored DAB positive nuclei were counted in 10 randomly selected fields ($\times 400$ magnification), and the IHC data were analyzed statistically using the SigmaStat software version 2.03 (Jandel Scientific Corp., San Raphael, CA). Data are presented as mean $\pm$ SEM and were evaluated via one way ANOVA followed by the Bonferroni *t*-test for multiple comparisons. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Topical application of NM caused an increase in H2A.X and p53 phosphorylation as well as total p53 accumulation

Following 12–120 h of its topical exposure, NM caused a strong increase in H2A.X phosphorylation at ser139 compared to control in a time-dependent manner which was maximum at 12–24 h, though later time-point of 72 and 120 h also showed a strong effect (Fig. 1A). Furthermore, we observed NM-induced biphasic response patterns for both p53 phosphorylation at ser15 and its accumulation. As shown in Fig. 1B, NM caused a 3.4 fold increase in p53 phosphorylation at 24 h, which decreased to control level at 72 h but induced again to 3.7 fold at 120 h compared to vehicle control group. Consistent with these observations for p53 phosphorylation, p53 accumulation also increased and showed a similar biphasic response pattern after NM exposure (Fig. 1B).

3.2. Topical application of NM caused an increase in MAPKs phosphorylation

MAPKs are also pro-inflammatory mediators, which are activated in response to various stimuli including genotoxic stress (Zhong et al., 2005). Accordingly, we next assessed the involvement of MAPKs (ERK1/2, JNK and p38) in NM-induced inflammatory and blistering responses in SKH-1 hairless mouse skin, as reported recently by us (Jain et al., 2014a,b; Tewari-Singh et al., 2013). Our results showed that NM exposure caused a maximum increase in the phosphorylation of ERK1/2 followed by p38 and JNK1/2 (Fig. 2). As shown in Fig. 2A, ERK1/2 phosphorylation was highest (10.5 fold compared to control) at 12 h post-NM exposure and then dropped to 4.0 and 2.0 fold at 24 and 72 h post-exposure, respectively, followed by another surge to 4.0 fold at 120 h. Though an NM-induced biphasic response was observed, the JNK1/2 phosphorylation following NM exposure was evidenced only starting at 24 h (~3 fold) with maximum effect at 120 h (~5 fold) post-exposure compared to vehicle controls (Fig. 2B). NM-induced increase in p38 phosphorylation also exhibited a comparable bi-phasic response pattern with maximum effect of 4.5–4.9 fold induction at 24 and 120 h, respectively, with a dip in levels at 72 h post NM-exposure, compared to controls (Fig. 2C). Overall, these findings suggested that NM exposure caused a biphasic response towards all three studied MAPKs phosphorylation, similar to p53 phosphorylation and accumulation as shown in Fig. 1. Also notably, most of the NM-caused increases in MAPKs phosphorylation were not due to an increase in their total levels except some increase in total JNK1/2 at 120 h (Fig. 2).

3.3. Topical application of NM caused an increase in Akt and PDK1 phosphorylation

Regulated by a variety of extracellular signals, growth factors, cytokines and oxidative stress, serine-threonine kinase Akt (also known as protein kinase B) is activated by PDK and is involved in the regulation of cell survival and apoptosis (Romashkova and Makarov, 1999). Hence, we next investigated the involvement of Akt and its upstream kinase PDK1 in NM-mediated signaling in SKH-1 hairless mouse skin. As shown in Fig. 3A, when compared to control, a time-dependent increase in the phosphorylation of PDK1 at Ser241 was observed following NM exposure, with ~3–4 fold increase at 72–120 h. Consistent with these results, NM exposure also induced the phosphorylation of Akt at Ser473 and increased total Akt levels; however, the effect was stronger and biphasic (Fig. 3B).

3.4. Topical application of NM caused an increase in the DNA binding activity of AP1 as well as the phosphorylation and expression of cFos and cJun

A number of factors including chemical stresses activate MAPKs and Akt cascades that enhance AP1 activity (Zhong et al., 2005). Since NM exposure induced the phosphorylation of MAPKs and Akt we next evaluated the effect of NM exposure on their downstream target AP1 transcription factor and its subunits cJun and cFos. As shown in Fig. 4A, compared to vehicle control, a strong increase in AP1 DNA binding activity was observed at 12 h post-NM exposure that decreased at 24 h and was absent at 72 h, but increased much strongly again at 120 h. We next analyzed the phosphorylation and total protein levels of cFos and cJun subunits of AP1 complex. As shown in Fig. 4B and C, NM exposure caused an increase in the phosphorylation of both cFos and cJun with similar patterns, but the level of induction was much higher for cJun phosphorylation (~2–7 fold) compared to cFos (~2–3 fold). Interestingly, NM exposure also caused a strong increase (~4–6 fold) in the total cFos levels, but a marginal decrease in total cJun protein (Fig. 4B and C).

3.5. Topical application of NM caused an increase in the expression of inflammatory and proteolytic mediators

Following NM exposure in mice, histopathological studies have shown a robust inflammatory response and microvesication (Jain et al., 2014a,b; Tewari-Singh et al., 2014a,b). Since activation of pro-inflammatory mediators is mostly associated with activation of various signaling pathways including MAPK/Akt-AP1, which we found activated by NM in the present study, we next examined the effect of NM on the expression of pro-inflammatory molecules (COX2 and iNOS), cytokine TNF α , and protease MMP9, which are also increased after SM exposure (Shakarjian et al., 2010). NM exposure caused a very strong and time-dependent increase (19–39 fold compared to control) in COX2 levels in the mouse skin (Fig. 5A). Similarly, we found a strong increase in iNOS levels in NM exposed skin tissue compared to vehicle control. NM exposure caused a 21 fold induction in iNOS levels at 24 h post exposure, which decreased thereafter at 72 and 120 h post-exposure (Fig. 5B). We next assessed the expression of TNF α , a cytokine involved in inflammation, apoptosis, and immune system development, which could also have a role in the activation of signaling pathways as well as production of inflammatory and proteolytic mediators (Arroyo et al., 1999; Chatterjee et al., 2003; Wormser et al., 2005). Our results showed that NM exposure also increases TNF α levels in a bi-phasic manner, where a time-dependent increase compared to control was observed up to 24 h (10 fold) followed by a decline (7 fold) at 72 h and again an increase (12 fold) at 120 h post-NM exposure (Fig. 5C). Gelatinases, especially MMPs that could come from infiltrating neutrophils, have the ability to degrade basement membrane components and interrupt the epidermal-dermal junction. These also play an important role in vesicant-related inflammatory and immune responses (Parks et al., 2004; Ries et al., 2009; Shakarjian et al., 2010). Consistent with this and earlier reported finding that NM exposure causes microvesication, we found a robust increase (10–20 fold) in MMP9 levels, compared to control, following NM exposure at all-time points (Fig. 5D).

3.6. Topical application of NM caused an increase in lipid peroxidation, DMPO nitron protein adduct formation and protein oxidation, and DNA oxidation

Vesicating agents are bifunctional alkylating agents that can react rapidly with the cellular macromolecules, including DNA, RNA and proteins (Jowsey et al., 2009). This can induce the depletion of cellular glutathione (GSH) and antioxidant enzymes, subsequently causing accumulation of reactive oxygen species (ROS), which could cause lipid peroxidation, protein oxidation, and oxidative DNA damage (Armstrong et al., 2002; Laskin et al., 2010; Paromov et al., 2007). Since oxidative stress could be one of the factors causing the activation of MAPKs observed here with NM, we next examined whether NM exposure resulted in oxidative stress-related damage to biomolecules, which could be activating the signaling cascades resulting in the inflammatory and blistering responses. Following NM exposure, a distinct increase in 4-HNE adduct formation was evidenced mainly at 12 and 24 h after NM exposure (Fig. 6A), suggesting oxidative stress resulting from lipid peroxidation.

It has been reported that protein oxidation and protein adduct formation could contribute to vesicant toxicity (Pal et al., 2009). Using an improved spin trap immunoassay that measures protein radical adducts in biological systems (Vayalil et al., 2004), our results showed that NM exposure caused an increase in band-specific anti-DMPO staining (red arrows show the band size where an increase at any of the time points post NM-exposure was observed) mainly at 12 and 24 h post-exposure (Fig. 6B). Furthermore, immunoblot detection of carbonyl groups, a method broadly used as biomarker of oxidative stress (Levine and Stadtman, 2001), was used to detect protein oxidation. Results from this analysis also showed that NM exposure causes an increase in protein oxidation as evidenced by stronger carbonyl group bands relative to vehicle control (red arrows; Fig. 6C).

Based on above results showing lipid and protein oxidation by NM, we next assessed whether NM also induced, an oxidized nucleoside of DNA (nuclear and mitochondrial), is the commonly detected and studied DNA lesion following oxidative stress (Inturi et al., 2011; Loft et al., 1992; Valavanidis et al., 2009). Hence, in this study, 8-OHdG staining was assessed in skin tissue samples from control and 12–72 h NM exposed mice, where NM exposure indeed caused an increase (~2 fold compared to control) in DNA oxidation at all-time points examined (Fig. 6D; blue arrows, 8-OHdG positive cells).

3.7. Topical application of NM caused an increase in Rad51, key protein in homologous recombination repair pathway

Our previous study in skin epidermal keratinocytes in cell culture has shown that homologous recombination repair (HRR) is the key pathway involved in DNA damage repair following NM exposure (Tewari-Singh et al., 2014a; Tewari-Singh et al., 2014a,b). Based on our findings above measured by 8-OHdG staining showing that NM exposure caused a strong DNA damage in mouse skin, we next assessed the activation of HRR, a DNA damage repair pathway. Rad51 is a significant component of double strand DNA damage repair by homologous recombination (Lu et al., 2005), and indeed, NM exposure resulted in ~5 fold increase in Rad51 levels compared to control at 12 h of its exposure, which decreased to almost basal levels at 72 h and then increased (4.5 fold compared to control) again at 120 h post-NM exposure, showing a biphasic response (Fig. 7).

4. Discussion

Vesicant exposure damages DNA causing both single and double strand breaks (DSBs). This is reported as a significant lesion that could be a direct event due to its alkylating properties or via oxidative stress and GSH depletion (Jowsey et al., 2009; Kehe and Szinicz, 2005; Mukhopadhyay et al., 2006; Paromov et al., 2007; Ruff and Dillman, 2007; Tewari-Singh et al., 2011). The resulting DNA damage activates DNA damage sensors like tumor suppressor p53, histone variant H2A.X and cell cycle checkpoint kinases, to repair damaged DNA (Jowsey et al., 2009; Tewari-Singh et al., 2014a,b). Notably, p53 is known to promote genomic stability via ATM/ATR pathway and cell cycle arrest to allow the repair of DNA damage or to cause cell death (Lane, 1992). Reported findings have shown DNA damage, p53 and ATM/ATR activation, cell cycle arrest in S and G2 M phases, and apoptotic death by NM and other SM analogs (Inturi et al., 2011; Jowsey et al., 2009; Kehe et al., 2009; Tewari-Singh et al., 2010, 2014a,b)(Jowsey and Blain, 2015; Ruff and Dillman, 2007) J. Results from this study, showing an increase in the phosphorylation of both H2A.X and p53 and accumulation of p53 following NM exposure, indicate DNA damage. Together, these earlier findings and those observed in present study clearly suggest that DNA damage and p53 activation could be an early and important event following NM exposure of murine skin, which is similar to some earlier studies with SM (Black et al., 2010a; Inturi et al., 2013; Jowsey et al., 2009; Kehe et al., 2009; Shakarjian et al., 2010).

A role of HRR pathway in the repair and protection against SM and NM-induced DNA damage is earlier by us and others (Jowsey et al., 2010; Tewari-Singh et al., 2014a,b). Rad51 forms nuclear foci at the site of DSBs during HRR repair and is dependent on the presence of BRCA2. The present in vivo study also shows that DNA DSBs are formed following NM exposure (indicated by H2A.X phosphorylation) and that Rad51 is involved in NM-induced DNA damage response. Hence, this study builds a foundation where our in vivo model with NM could be used to further understand the role of DNA damage and related p53 activation, and Rad51 and HRR pathway in vesicant-induced DNA repair, which would help develop novel agents for the therapeutic intervention of vesicants-induced skin injuries.

NM exposure resulting in an increase in the phosphorylation of MAPKs ERK1/2, JNK1/2 and p38 in mouse skin, as observed in the present study, supports their role in vesicant-induced skin injury, as reported in earlier studies with SM in keratinocytes and CEES in keratinocytes and mouse skin (Black et al., 2010b; Dillman et al., 2004; Pal et al., 2009; Rebholz et al., 2008). Since many of the downstream targets of MAPKs activation lead to the production and action on inflammatory mediators, our results suggest that activation of MAPKs by NM in mouse skin plausibly plays a major role in NM-induced increase in COX2, iNOS, TNF α and MMP9 levels, resulting in inflammatory and vesicating responses similar to those by SM (Rosenthal et al., 1998). MAPK/ERK1/2 signaling is also associated as a positive ATM-dependent regulator of HRR DNA damage repair pathway (Golding et al., 2007; Mayr et al., 2002), suggesting that its activation might also play an important role in vesicant-induced HRR pathway activation; however, it warrants further studies in future. Akt plays an essential role in processes associated with responses to stress and growth factor signaling, and survival and apoptosis (Gu et al., 2005; Romashkova and Makarov, 1999). Our results indicate that increased phosphorylation of PDK1 might be the key factor for the

NM-induced Akt activation, and further supports the role of Akt in NM-induced skin injury. Both MAPK and Akt pathways could cause the activation of various transcription factors such as AP1 and NF- κ B (Romashkova and Makarov, 1999; Zhong et al., 2005). In present study, NM exposure of murine skin did cause a significant activation of AP1 suggesting its role in vesicant-induced skin injury responses. Regarding NF- κ B, its significant activation was not observed with NM exposure of murine skin in the current study (data not shown), which is consistent with a previous report indicating that NF- κ B may not be an important player in SM-induced inflammatory response (Ruff and Dillman, 2010). However, further studies are needed in future to more clearly examine the role of these and other transcription factors in vesicant-induced skin injury.

NM-induced increase in pro-inflammatory cytokine TNF α levels observed in mouse skin in this study indicates a significant role of TNF α in vesicant-induced increase in inflammatory mediators and inflammatory response, which could be via oxidative stress causing the activation MAPK-AP1 pathway. In addition to the direct role of MAPKs in TNF α production (Dillman et al., 2004; Ruff and Dillman, 2010), free radical-mediated TNF α cascade could play a major role in the activation of AP1 via MAPKs (Chatterjee et al., 2003; Mukhopadhyay et al., 2006) in vesicant-induced skin injury. The findings in the current study further support earlier reports on the role of TNF α in SM-mediated skin injury and that induction of TNF α could be due to ROS generation (Wormser et al., 2005). However, the mechanism of TNF α induction following vesicant exposure needs to be further elucidated in future.

Similar to SM, NM exposure of mouse skin results in marked inflammatory response where infiltration of inflammatory cells including mast cells, macrophages and neutrophils associated with myeloperoxidase (MPO) activity has been observed (Jain et al., 2014a,b; Millard et al., 1997). The influx of inflammatory cells at the site of injury and role of other inflammatory mediators like COX2 has been reported in SM-induced skin injury (Shakarjian et al., 2010). The present study with NM further supports the role of COX2 in mediating vesicant-induced inflammatory responses.

In addition to inflammatory mediators, extensive research has been carried out to elucidate the role matrix-degrading proteases play in skin blister formation following SM exposure. Studies have shown that gelatinases, especially MMP9, are most upregulated after SM exposure (Shakarjian et al., 2006, 2010). The present study, where a robust increase in MMP9 levels following NM exposure was observed, further supports the role of MMP9 in vesicant (including NM)-mediated vesication. AP1 is reported to cause transcriptional activation of MMPs (Angel et al., 2001; Shakarjian et al., 2006), hence, the observed NM-induced increase in MMP9 could be mediated via MAPK/Akt-AP1 pathway, which may also be due to an increase in TNF α that is known to up regulate the expression of MMPs.

Apart from causing damage to cellular macromolecules via its alkylating properties, SM induced ROS can result in lipid peroxidation, protein and DNA oxidation, and activation of signaling pathways, leading to pro-inflammatory gene expression and inflammatory responses (Brimfield et al., 2009; Inturi et al., 2011; Korkmaz et al., 2006; Pal et al., 2009; Ruff and Dillman, 2007). We also found in this study that NM exposure results in significant

4-HNE adduct formation indicating lipid peroxidation, protein modifications and adduct formation, and oxidative DNA damage. The observed oxidative damage by NM in present study could be due to a localized ROS production in the mitochondria and cells, or by the infiltration of neutrophils that can further release ROS. Increases in NM-induced neutrophils, macrophages and MPO activity has also been reported, and an important role of MPO in NM-induced skin injury has recently been established by us employing MPO knock-out mice (Jain et al., 2014a; Jain et al., 2014a,b). Apart from oxidative stress, nitrosative stress involving nitric oxide (NO), a potent oxidizing agent, has also been implicated in vesicant toxicity (Korkmaz et al., 2006; Shakarjian et al., 2010). NO is formed via NO synthesizing enzymes including iNOS, which can be regulated by MAPK-AP1 pathway (Steinritz et al., 2009). An up regulation in iNOS is also observed in response to TNF α , and iNOS inhibition is shown to reduce SM-induced inflammation, suggesting that iNOS up regulation could be one of the contributors to SM toxicity (Nyska et al., 2001; Shakarjian et al., 2010). Therefore, in this study, NM-induced increase in iNOS, which is an important inflammatory mediator, could also be due to the activation of MAPK pathway and/or stimulation of TNF α . Overall, both oxidative and nitrosative stresses could be an initial step in NM or SM-induced skin toxicity; indeed, the use of several antioxidants and iNOS inhibitors in reducing vesicant-induced skin injury does signify their role in skin injury from vesicating agents (Laskin et al., 2010; Nyska et al., 2001; Paromov et al., 2007; Shakarjian et al., 2010; Tewari-Singh et al., 2014a,b).

In summary and consistent with above discussions, our findings employing SKH-1 hairless mouse skin suggest the involvement of oxidative stress and TNF α in the activation of MAPK and Akt followed by AP1 signaling to stimulate the production of inflammatory and proteolytic mediators in vesicating agent NM-induced skin inflammation and microvesication, together with involvement of DNA damage-HRR pathway in NM-induced skin injuries including cell death (Fig. 8). The bi-phasic responses of DNA damage, MAPKs, Akt and inflammatory mediators was observed with NM for the first time in our study. Though there could be a brief recovery phase, the mechanism of action of vesicating agents is complex and further studies to elucidate this bi-phasic effect are required. These outcomes are important additions to the literature elucidating for the first time the mechanistic events of NM-induced clinical and histopathological sequelae of skin injuries, which are comparable to those reported in humans and animal models with SM. Furthermore, the mechanistic findings from the present study build a foundation to effectively develop therapeutic interventions against the identified pathways and molecules to counter vesicant-induced skin injury responses.

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Abbreviations

4-HNE	4-hydroxynonenal
AP-1	activator protein 1
DMPO	5,5-dimethyl-2-(8-octanoic acid)-1-pyrroline N-oxide
DNPH	2,4-dinitrophenylhydrazine
ERK	extracellular signal-regulated kinase
JNK	Jun-N terminal kinase
MAPK	mitogen-activated protein kinase
8-OHdG	8-oxo-2-deoxyguanosine
ROS	reactive oxygen species
NM	nitrogen mustard
COX-2	cyclooxygenase-2
MMP-9	matrix metalloproteinase-9
iNOS	inducible nitric oxide synthase
TNF-α	tumor necrosis factor- α

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HIGHLIGHTS

- Nitrogen mustard (NM) exposure in mice causes DNA damage and oxidative stress.
- NM exposure caused mainly a bi-phasic response in the activation of MAPKs and Akt.
- NM-induced induction of MAPKs led to the activation of transcription factor AP1.
- NM triggered an increase in the expression of COX-2, iNOS and MMP-9.
- Activation of above pathways by NM caused skin injury response in mice.

Fig. 1. Effect of NM on the phosphorylation of H2A.X and p53, and accumulation of p53 in SKH-1 hairless mouse skin

Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone. Dorsal skin of mice was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and western blot analysis was carried out for H2A.X (A), and p53 phosphorylation and total p53 (B) as detailed under 'Materials and Methods' Section 2. Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Results obtained were quantified by densitometric analysis of representative immunoblots for H2A.X ser139 and p53 ser15 phosphorylation and total p53 (A and B). Results shown are representative of 2–3 animals in the study group. VC, vehicle (acetone) control; NM, nitrogen mustard.

Fig. 2. Effect of NM on the phosphorylation of MAPK signaling molecules in SKH-1 hairless mouse skin

Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and subjected to western blot analysis as detailed under 'Materials and Methods' Section 2. The membranes were probed for phospho- and total ERK1/2 (A), JNK1/2 (B) and p38 (C). Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Results obtained were quantified by densitometric analysis of representative immunoblots for phospho and total ERK1/2, JNK1/2 and p38.

Results shown are representative of 2–3 animals in the study group. VC, vehicle (acetone) control; NM, nitrogen mustard.

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Fig. 3. Effect of NM on the phosphorylation of PDK1 and Akt in SKH-1 hairless mouse skin Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and subjected to western blot analysis as detailed under 'Materials and Methods' Section 2. The membranes were probed for phospho-PDK1 (A) and phospho- and total Akt (B). Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Results obtained were quantified by densitometric analysis of representative immunoblots for phospho-PDK1, phospho-Akt and total Akt. Results shown are representative of 2–3 animals in the study group. VC: vehicle (acetone) control; NM: nitrogen mustard.

Fig. 4. Effect of NM on AP1 activation and the expression of cFos and cJun in SKH-1 hairless mouse skin

Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and nuclear lysates were subjected to DNA binding activity by EMSA (A), and western immunoblotting (B and C) as detailed under 'Materials and Methods' Section 2. EMSA was carried out using AP1 oligo (A), and western blot analysis was done for phospho- and total cFos and cJun (B and C). Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Western blot results were quantified by densitometric analysis of representative immunoblots for phospho-cFos total cFos phospho-cJun and total cJun. Results shown are representative of 2–3 animals in the study group.

Fig. 5. Effect of NM on inflammatory and proteolytic mediators in SKH-1 hairless mouse skin Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and subjected to western blot analysis as detailed under 'Materials and Methods' Section 2. The membranes were probed for COX2 (A), iNOS (B), TNF α (C) and MMP9 (D), and protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Results obtained were quantified by densitometric analysis of representative immunoblots for COX2, iNOS, TNF α and MMP9. Results shown are representative of 2–3 animals in the study group.

Fig. 6. Effect of NM on lipid peroxidation, protein adduct formation, protein oxidation and DNA oxidation in SKH-1 hairless mouse skin

Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared as detailed under 'Materials and Methods' Section 2. Lipid peroxidation in the mouse skin tissue was analyzed by western blot analysis of 4-HNE-adducted protein modifications by subjecting the skin tissue lysates to SDS-PAGE and immunoblotting with anti-4-HNE antibody as detailed under the 'Materials and Methods' Section 2 (A). Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Protein adduct formation in the mouse skin tissue was analyzed by western blot analysis of DMPO nitron protein adduct formation by subjecting the skin tissue lysates to SDS-PAGE and immunoblotting with anti-DMPO antibody as detailed under the 'Materials and Methods' Section 2 (B). Protein loading was checked by stripping and re-probing the membrane with β -actin antibody. Protein oxidation was analyzed by Western blot analysis using anti-DNPH antibody, as detailed under 'Materials and Methods' Section 2 (C). Protein loading was checked by stripping and re-probing the membranes with β -actin antibody. The results in A–C are representative of 2–3 animals in the study group. DNA oxidation in mouse skin was analyzed by IHC staining of 8-OHdG in skin tissue (D). The brown colored DAB positive nuclei were 8-OHdG positive stained nuclei and were counted in 10 randomly selected fields (400 $\times$ magnification). * p < 0.01 compared to vehicle control group (n = 3–4);

red arrows, band size where an increase in DMPO-adduct formation or protein oxidation at any of the time points post NM-exposure was observed; blue arrows, 8-OHdG positive cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Fig. 7. Effect of topical NM exposure on HRR pathway molecules Rad51 in SKH-1 hairless mouse skin

Mice were exposed topically to either 3.2 mg NM in acetone or acetone alone and dorsal skin was collected at 12, 24, 72 and 120 h time points after exposure. Skin tissue lysates were prepared and subjected to western blot analysis as detailed under 'Materials and Methods' Section 2. The membranes were probed for Rad51, and protein loading was checked by stripping and re-probing the membranes with β -actin antibody. Results obtained were quantified by densitometric analysis of representative immunoblots. Results shown are representative of 2–3 animals in the study group.

Fig. 8. Schematic representation of possible mechanisms of NM-induced skin injury