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The generation of 4-hydroxynonenal, an electrophilic lipid peroxidation end product, in rabbit cornea organ cultures treated with UVB light and nitrogen mustard

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Abstract

The cornea is highly sensitive to oxidative stress, a process that can lead to lipid peroxidation. Ultraviolet light B (UVB) and nitrogen mustard (mechlorethamine) are corneal toxicants known to induce oxidative stress. Using a rabbit air-lifted corneal organ culture model, the oxidative stress responses to these toxicants in the corneal epithelium was characterized. Treatment of the cornea with UVB (0.5 J/cm^2) or nitrogen mustard (100 nmol) resulted in the generation of 4-hydroxynonenal (4-HNE), a reactive lipid peroxidation end product. This was associated with increased expression of the antioxidant, heme oxygenase-1 (HO-1). In human corneal epithelial cells in culture, addition of 4-HNE or 9-nitrooleic acid, a reactive nitrolipid formed during nitrosative stress, caused a time-dependent induction of HO-1 mRNA and protein; maximal responses were evident after 10 hr with 30 μM 4-HNE or 6 hr with 10 μM 9-nitrooleic acid. 4-HNE and 9-nitrooleic acid were also found to activate Erk1/2, JNK and p38 MAP kinases, as well as phosphoinositide-3-kinase (PI3)/Akt. Inhibition of p38 blocked 4-HNE- and 9-nitrooleic acid-induced HO-1 expression. Inhibition of Erk1/2, and to a lesser extent, JNK and PI3K/Akt, suppressed only 4-HNE-induced HO-1, while inhibition of JNK and PI3K/Akt, but not Erk1/2, partly reduced 9-nitrooleic acid-induced HO-1. These data indicate that the actions of 4-HNE and 9-nitrooleic acid on corneal epithelial cells are distinct. The sensitivity of corneal epithelial cells to oxidative stress may be an important mechanism mediating tissue injury induced by UVB or nitrogen mustard.

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Keywords

cornea; nitrogen mustard; UVB; 4-hydroxynonenal; 9-nitrooleic acid; lipid peroxidation; nitrosative stress

Introduction

As the outermost layer of the eye, the cornea is highly sensitive to injury induced by environmental insults. These result in alterations in the cornea's structural integrity, which can impair vision (Lu *et al.*, 2001; Cejka *et al.*, 2010). The mechanisms by which environmental insults damage the cornea are not well understood. Our laboratories have been investigating the pathogenesis of corneal injury induced by ultraviolet B light (UVB) and vesicants, such as nitrogen mustard and sulfur mustard (Gordon *et al.*, 2010; Black *et al.*, 2011; Malaviya *et al.*, 2012). These agents induce oxidative and nitrosative stress which can cause aberrant epithelial cell growth and differentiation, and cytotoxicity via necrosis and apoptosis (Heck *et al.*, 2003; Laskin *et al.*, 2010). In the cornea, this results in opacities, ulcerative diseases and keratitis (Cejkova *et al.*, 2004; Kadar *et al.*, 2013).

An important consequence of oxidative and nitrosative stress is the generation of excessive amounts of toxic reactive intermediates, including superoxide anion, hydrogen peroxide, hydroxyl radicals and nitric oxide (Hughes, 2008). Recent studies have suggested that modification of lipids by these reactive oxygen and nitrogen species is one of the major contributors to tissue damage (Roberts *et al.*, 2010). For example, during oxidative stress, reactive oxygen species can initiate lipid peroxidation, a process that generates highly reactive electrophilic species such as α, β-unsaturated hydroxyalkenals (Niki, 2009). Additional reactive oxidants are also generated when superoxide anion chemically reacts with nitric oxide to produce intermediates such as peroxynitrite (Pacher *et al.*, 2007). Subsequent reactions of nitric oxide-derived oxidants with double bonds of fatty acids, particularly the highly abundant oleic, linoleic, and arachidonic acids, lead to the generation of nitro-fatty acids, such as 9- and 10-nitrooleic acid (Devasagayam *et al.*, 2004; Jain *et al.*, 2008). Both α, β-unsaturated hydroxyalkenals and the nitrooleic acids are known to directly modify structural components in cells via Michael additions leading to toxicity (Esterbauer *et al.*, 1991; Trostchansky and Rubbo, 2008). These lipid-derived intermediates have been identified as endogenous signaling molecules and their ability to cause inappropriate or altered cellular signal transduction can contribute to tissue injury (Niki, 2009).

The present studies were aimed at assessing the consequences of UVB- and vesicant-induced oxidative stress in the cornea. For these studies we used an air lifted rabbit cornea organ culture, a model system that has previously been used to characterize corneal wound healing (Gordon *et al.*, 2010). We found that exposure of the cornea to UVB or nitrogen mustard resulted in the appearance of 4-hydroxynonenal (4-HNE), a relatively abundant α, β-unsaturated hydroxyalkenal. This was associated with increased expression of the antioxidant heme oxygenase-1 (HO-1), a stress protein important in protecting the cornea from injury induced by oxidative and nitrosative stress (Neil *et al.*, 1995; Patil *et al.*, 2008). Mechanisms by which 4-HNE and a related nitro-fatty acid derived mediator, 9-nitrooleic

acid, induce HO-1 were analyzed using human corneal epithelial cells in culture. Our data demonstrate that reactive lipids formed during the pathogenesis of corneal injury are important in regulating the cytotoxic actions of UVB and nitrogen mustard. Results from these studies provide additional support for the key contribution of oxidative stress and lipid peroxidation to the actions of xenobiotics in the cornea.

Materials and Methods

Reagents

Alexa Fluor 488 conjugated goat anti-mouse IGg, ProLong® Gold antifade reagent, 4′-6-diamidino-2-phenylindole (DAPI), Dulbecco's Modified Eagle's Medium (DMEM), keratinocyte serum-free (KSF) medium, epidermal growth factor, bovine pituitary extract and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). Mouse monoclonal 4-HNE antibody was from R&D Systems (Minneapolis, MN), rabbit anti-HO-1 polyclonal antibody from Stressgen Biotechnology (Victoria, BC, Canada) and mouse monoclonal anti-HO-1 antibody from Abcam (Cambridge, MA). Rabbit polyclonal p38, phospho-p38, JNK, phospho-JNK, Erk1/2, phospho-Erk1/2, AKT and phospho-AKT antibodies were from Cell Signaling Technology (Beverly, MA). The Detergent Compatible (DC) protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA) and the Western Lightning enhanced chemiluminescence kit (ECL) from Perkin Elmer Life Sciences (Boston, MA). SYBR Green Master Mix and other PCR reagents were from Applied Biosystems (Foster City, CA). 4-HNE, PD 98059, SP600125 and Wortmannin were from Calbiochem (La Jolla, CA) and 9-nitrooleic acid from Cayman Chemical (Ann Arbor, MI). Nitrogen mustard (mechlorethamine), SB203580, protease inhibitor cocktail which contained 4-(2-aminoethyl) benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin, and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Rabbit cornea organ culture and treatments

Eyes from young adult New Zealand white rabbits were purchased from Pel-Freez (Rogers, AR). Preparation of corneal organ cultures has been described previously (Gordon *et al.*, 2010). Briefly, corneas with a 2 mm surrounding scleral rim were dissected from the rabbit eyes. The endothelial sides of the corneas were filled with 1.5% agar in DMEM, and after solidification, the cornea was inverted epithelial side up. DMEM supplemented with 1% non-essential amino acids, 0.01 mg/ml ciprofloxacin, and 0.1 mg/ml ascorbic acid was added up to the scleral rim. The corneas were incubated in a 5% CO_2 incubator at 37 ºC and routinely wetted with DMEM every 6 hr. Nitrogen mustard (100 nmol in PBS) or PBS control was applied directly onto one set of cornea in volumes no greater than 20 μl. After 1 hr, the corneas were washed and refed with fresh medium. Other corneas were exposed to 0.5 J/cm^2 UVB as previously described (Po, 2012). Control corneal organ cultures were placed under the light and covered with black vinyl to block UVB irradiation. At 3 hr and 6 hr post treatment, corneas exposed to nitrogen mustard or UVB were placed in optimal cutting temperature (OCT) medium and frozen until 10 μm sections were cut on a Microm HM505E cryostat. Cornea sections were then transferred to glass slides and stored at −80 ºC until analysis.

Immunofluorescence

Sections of corneas were fixed in cold methanol for 10 min, air dried, then rinsed with PBS and blocked with 5% goat serum at room temperature. After 60 min, sections were washed with PBS and incubated with a 1:100 dilution of HO-1 antibody or a 1:200 dilution of 4-HNE antibody in 1.5 % goat serum overnight at 4 ºC. Sections were then washed with PBS containing 0.2 % Tween-20 and incubated with a 1:1,000 dilution of goat anti-mouse Alexa-Fluor 488 secondary antibody for 1 hr at room temperature. The nuclei were counterstained with DAPI and the section treated with ProLong® Gold antifade reagent. Fluorescent images of corneas were captured with a ZEISS X-cite series 120Q fluorescence microscope (Thornwood, NY).

Cell cultures and treatments

The origin of the human corneal epithelial (HCE) cells has been described previously (Black *et al.*, 2011). Cells were maintained in keratinocyte serum-free (KSF) medium supplemented with 5% fetal bovine serum, 0.1% gentamicin, 0.05 μg/ml epidermal growth factor and 0.05 mg/ml bovine pituitary extract. For RNA and protein analysis, monolayers of cells were grown to 90% confluency in six well plates and treated with either vehicle control, 4-HNE (30 μM) or 9-nitrooleic acid (10 μM). For mRNA analysis, cells were washed with PBS and total RNA was extracted using Tri-Reagent. For protein analysis, cells were washed with PBS and 300 μl of lysis buffer (10 mM Tris-base, pH 7.6, supplemented with 1% SDS and protease inhibitor cocktail) was added to the culture dish wells; after transfer to 1.5 ml Eppendorf microcentrifuge tubes, the cells were sonicated on ice. Lysates were centrifuged (300 x g, 10 min at 4° C) and the supernatants prepared for western blotting analysis. Total protein concentrations in supernatants were determined using the DC protein assay kit with bovine serum albumin as the standard. For kinase inhibition experiments, cells were pretreated for 3 hr with p38 MAP kinase inhibitor SB203580 (10 μM), JNK kinase inhibitor SP600125 (20 μM), Erk1/2 kinase inhibitor PD98059 (10 μM) or PI3/Akt kinase inhibitor Wortmannin (0.1 μM). 4-HNE, 9-nitrooleic acid or vehicle control was then added to the medium for a 6 hr incubation. Cells were then lysed and analyzed for protein expression by western blotting.

Analysis of mRNA expression

Total RNA was converted to cDNA using M-MLV reverse transcriptase as previously described (Black *et al.*, 2011). For PCR analysis, a standard curve was generated from serial dilutions of cDNA mixtures of the samples. Real-time PCR was conducted on an ABI Prism 7900 Sequence Detection System using 96-well optical reaction plates. SYBR-Green was used as the fluorescent detection signal and the standard curve method was used for relative quantitative analysis. Primer sequences were generated using Primer Express software (Applied Biosystems) and the oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The house keeping gene, β-actin, was used to normalize the values. Forward and reverse (5′-3′) primer sequences were: HO-1, GCT CAA AAA GAT TGC CCA GA and GCG GTA GAG CTG CTT GAA CT; β-actin, AAA GAC CTG TAC GCC AAC AC and GTC ATA CTC CTG CTT GCT GAT.

Western blotting

Western blotting was performed as previously described (Black *et al.*, 2008). Briefly, cell lysates were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in Tris buffer supplemented with 5% milk for 60 min at room temperature. The blots were then incubated overnight at 4°C with primary antibodies, washed with tTBS (Tris-buffered saline supplement with 0.1% Tween 20), and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized by ECL chemiluminescence.

Analysis of 4-HNE metabolism

HCE cells (2×10^6 cells/ml) were suspended in Eppendorf tubes in 1 ml serum-free KSF medium and incubated with 100 μM 4-HNE at 37 °C. 4-HNE and metabolites were extracted from the samples after 0–180 min by the addition of an equal volume of acetonitrile/acetic acid (96:4, v/v) and rapid mixing. After centrifugation (1000 x g, 10 min at 4° C), acetonitrile/acetic acid-containing supernatants were analyzed by HPLC as described by Hartley et al (1995) with some modifications, using a Jasco HPLC system (Jasco Corporation, Tokyo, Japan). 4-HNE and its metabolites were separated on a Phenomenex 5 μ C18 column (250 × 2.00 mm) using a mobile phase consisting of 70% 50 mM potassium phosphate buffer (pH 2.7) and 30% acetonitrile (v/v). Samples were analyzed isocratically at a flow rate of 0.25 ml/min, monitoring the HPLC effluent at 224 nm.

Statistical analysis

Data were evaluated using the two-way ANOVA. $P < 0.05$ was considered statistically significant. All experiments were repeated two times.

Results

Effects of UVB or nitrogen mustard on the formation of 4-HNE-adducts and HO-1 expression in cultured rabbit corneas

Consistent with previous studies (Maumenee and Scholz, 1948; Koliopoulos and Margaritis, 1979), we found that UVB or nitrogen mustard caused significant damage to the epithelial layer of the cornea. Figure 1 (upper panel) shows the 5–7 layer epithelium of unexposed rabbit cornea. Treatment with UVB or nitrogen mustard resulted in a thickening of the epithelial layer with a downward hyperplasia within 3 hr (Fig. 1, center panels). At 6 hr post exposure, areas of separation appeared between the epithelial and stromal layers of the corneas (Fig. 1, lower panels).

As a highly reactive end product of lipid peroxidation, 4-HNE forms stable protein adducts with histidine, lysine, and cysteine side chains which can be used as biomarkers for oxidative tissue damage (Gutteridge, 1995). Using an antibody that detects 4-HNE-histidine adducts, we found that corneas treated with UVB (Fig. 2) or nitrogen mustard (Fig. 3) for 3 hr or 6 hr readily generated 4-HNE in a time related manner. 4-HNE-adducts were detected at the apical surface of the corneal epithelium and at the basal epithelial surface at the basement membrane (Figs. 2 and 3, middle and right panels). Greater amounts of 4-HNE

adducts were detected in intermediate areas of the epithelium in UVB or nitrogen mustard treated corneas with increasing periods of time. In contrast, minimal 4-HNE adducts were found in control corneas (Figs. 2 and 3, left panels).

HO-1 is an inducible enzyme synthesized in tissues in response to oxidative stress (Gozzelino *et al.*, 2010). Control corneas expressed low levels of HO-1 on their apical and basal epithelial surfaces (Figs. 4 and 5, left panels). A marked upregulation in HO-1 expression was observed 3 hr and 6 hr after UVB or nitrogen mustard treatment (Figs. 4 and 5, middle and right panels). HO-1 was expressed at the apical epithelial surface and at the basal surface above the basement membrane. HO-1 was also observed in the central cornea 6 hr post UVB or nitrogen mustard treatment. Of note was that upregulation of HO-1 was found to be associated with its appearance in the nuclei of the corneal epithelial cells.

Effects of 4-HNE on cultured corneal epithelial cells

We next used cultured human corneal epithelial cells to analyze the effects of 4-HNE on HO-1 expression. 4-HNE was readily taken up by the corneal epithelial cells, as reflected by its rapid disappearance from the medium; its half-life in the medium was approximately 25 min (Fig. 6, upper and middle panels). 4-HNE was detected inside the cells with levels declining rapidly after 5 min (Fig. 6, bottom panel). Potential metabolites of 4-HNE were also observed in the cells, as measured by the appearance of a broad shoulder on the 4-HNE HPLC peak. As observed with 4-HNE, these metabolites degraded over time. Figure 7 shows that 4-HNE treatment also caused a time-dependent appearance of protein adducts in the corneal epithelial cells. A prominent band with a molecular weight of 45 kDa appeared in the cells 15 min after 4-HNE treatment (Fig. 7). With increasing time (30–90 min), a 43 kDa molecular weight band appeared, as well as a number of relatively lower abundance higher molecular weight bands, ranging in molecular weight from 50 kDa to greater than 200 kDa.

Treatment of corneal epithelial cells with 4-HNE resulted in a time-dependent induction of HO-1 mRNA and protein (Fig. 8, left panels). A several-fold increase was evident 6–10 hr after treatment with 30 μM 4-HNE. We next compared the actions of 4-HNE with the nitrosative stress lipid mediator, 9-nitrooleic acid, which is known to be generated following nitration of oleic acid (Jain *et al.*, 2008). As observed with 4-HNE, 9-nitrooleic acid induced expression of mRNA and protein for HO-1 in human corneal epithelial cells (Fig. 8, right panels). However, the effects of 9-nitrooleic acid on HO-1 mRNA were transient; maximal levels were attained after 6 hr with 10 μM 9-nitrooleic acid.

In further studies we analyzed the role of MAP kinase and PI3/Akt signaling in 4-HNE- and 9-nitrooleic acid-induced HO-1 expression. Both fatty acid derived lipids were found to activate corneal ERK1/2, p38 and JNK MAP kinases, as measured by increases in the phosphorylated forms of these enzymes (Figs. 9 and 10). The effects of 4-HNE were time-dependent, reaching a maximum after 60–90 min with each enzyme. The stimulatory effects of 9-nitrooleic acid on p38 kinase were generally similar to 4-HNE. In contrast, JNK was only activated after 90 min, while ERK1/2 expression increased after 30 min and declined thereafter. Both 4-HNE and 9-nitrooleic acid also activated PI3/Akt signaling; these increases were time-dependent and maximal after 90 min with 4-HNE and 15 min with 9-

nitrooleic acid. To determine if 4-HNE and 9-nitrooleic acid-induced upregulation of HO-1 expression in corneal epithelial cells was dependent on MAP kinases and PI3/Akt, we used specific enzyme inhibitors. Treatment of the cells with the p38 MAP kinase inhibitor, SB203580, or the ERK-1/2 kinase inhibitor, PD98059, markedly suppressed 4-HNE-induced expression of HO-1. A JNK inhibitor, SP600125, and a PI3K/Akt inhibitor, wortmannin, only partially inhibited HO-1 expression. In contrast, the p38 kinase inhibitor only blocked 9-nitrooleic acid-induced expression of HO-1; partial inhibition of HO-1 expression was evident with the JNK and PI3K/Akt inhibitors. Minimal effects were observed on 9-nitrooleic acid-induced expression of HO-1 with the ERK1/2 inhibitor (Fig. 10).

Discussion

Earlier studies have shown that ocular exposures to UVB or vesicants results in damage to the corneal epithelium, which involves both acute and chronic keratopathies (Bergmanson, 1990; Ghabili *et al.*, 2010). Depending on dose, inflammation and edema are observed as well as increasing corneal thickness, and sloughing of epithelial cells. Detachment of epithelial cells is thought to be due to degradation of the basement membrane and basal cell hemidesmosome transmembraneous components, which are critical for the attachment of the epithelium to the basement membrane (Petrali *et al.*, 1997; McNutt *et al.*, 2012; Kadar *et al.*, 2013). Our laboratories have been characterizing the effects of ocular toxicants including UVB and vesicants on rabbit cornea using an air-lifted organ culture model (Gordon *et al.*, 2010). In this model, the corneal epithelium retains its typical differentiated phenotype with an intact basement membrane separating the epithelial and stromal cell layers (Foreman *et al.*, 1996; Gordon *et al.*, 2010). Using this organ culture system, the present studies demonstrate that both UVB and nitrogen mustard, a sulfur mustard analog, cause damage to the cornea. This is characterized by a thickening of the epithelium, largely due to epithelial hyperplasia, and localized separation of the epithelium from the basement membrane zone. These data are consistent with the effects of UVB or sulfur mustard on rabbit cornea *in vivo (*Petrali *et al.*, 1997; Cejka *et al.*, 2010) and demonstrate the utility of the cornea organ culture model for mechanistic studies on the action of xenobiotics.

Using UVB or nitrogen mustard which generates similar corneal damage in the rabbit organ cultures, we found evidence of 4-HNE production by corneal epithelium by immunofluorescence. This was confirmed by our identification of 4-HNE-modified proteins in the corneal epithelial cell cultures. These proteins were most abundant at the apical and basal surfaces of the corneal epithelia. The apical expression was not simply autofluorescence since the intensity of the signal was far greater than that of the unexposed control corneas. With time, 4-HNE modified proteins were also evident in other suprabasal layers of the corneal epithelium, although at reduced amounts relative to the apical and basal expression patterns. These data indicate that UVB- or nitrogen mustard-induced corneal toxicity is associated with oxidative stress and lipid peroxidation. This is consistent with earlier studies showing that these toxicants can generate superoxide anion and hydrogen peroxide (Korkmaz *et al.*, 2006; Black *et al.*, 2011). A similar induction of 4-HNE production has previously been described in mouse keratinocytes treated with UVB (Zhaorigetu *et al.*, 2003). Our observation that the 4-HNE was localized in cells near the

corneal surface is consistent with the fact that these cells are the first to encounter the toxicants, and likely receive the highest doses resulting in significant oxidative stress. The reason for increased expression in the basal epithelial cells following treatment with UVB or nitrogen mustard is not known, although it is known that both agents have a profound effect here. In the skin, basal keratinocytes are a major target for both nitrogen mustard and sulfur mustard, possibly due to their ability to stimulate the production of proteases that degrade the basement membrane (Papirmeister, 1991). In this regard, sulfur mustard has been reported to induce a variety of basement membrane degrading enzymes in the skin including elastase, tryptase, calpain, matrix metalloproteinase-2 and matrix metalloproteinase-9 (Shakarjian *et al.*, 2010). Matrix metalloproteinases are also known to be induced in the cornea in different diseases and these or related proteases may also contribute to UVB- and nitrogen mustard-induced injury in basal corneal epithelial cells (Sakimoto and Sawa, 2012).

HO-1 is an important adaptive response protein induced in cells subject to oxidative stress. It functions to protect cells by inhibiting apoptosis and inflammation (Gozzelino *et al.*, 2010). We found that areas of 4-HNE production in the cornea after UVB or nitrogen mustard treatment were associated with increased expression of HO-1. These data are consistent with earlier studies demonstrating that corneal injury in mice results in increased HO-1 expression and activity, which contributes to reduced inflammation and increased wound healing (Patil *et al.*, 2008), and that UVB is an effective inducer of HO-1 in human corneal epithelial cells in culture (Black *et al.*, 2011). Both nitrogen mustard and sulfur mustard have also been reported to upregulate HO-1 *in vivo* (Malaviya *et al.*, 2010; Malaviya *et al.*, 2012). The ability of UVB or nitrogen mustard to modulate corneal epithelial cell expression of antioxidants such as HO-1 is likely to be important in regulating inflammation and protecting the cornea against oxidative stress. Of interest were our findings that following UVB or nitrogen mustard treatment, HO-1 translocated into the nucleus of the corneal epithelial cells. Earlier studies demonstrated that nuclear HO-1 can serve in the regulation of gene transcription and may be involved in cell growth regulation and tumor progression (Elguero *et al.*, 2012; Gandini *et al.*, 2012). In the cornea, nuclear HO-1 may serve to regulate expression of genes important in controlling the resolution of inflammation and wound healing. Further studies are needed to determine the precise functions of nuclear HO-1 in regulating corneal responses to UVB or nitrogen mustard.

4-HNE is known to modulate HO-1 expression in many cell types (Zhang and Forman, 2009; Ishikado *et al.*, 2010). To investigate its mechanism of action in the cornea, a human corneal epithelial cell line known to be sensitive to UVB was used (Black *et al.*, 2011). In these cells, UVB regulates HO-1 expression via MAP kinase signaling. We found that human corneal epithelial cells readily take up 4-HNE, and that the 4-HNE was effective in inducing HO-1 mRNA and protein. These data indicate that one level of HO-1 regulation is mRNA expression. In addition to reactive oxygen species, many tissues generate reactive nitrogen species during oxidative stress. Earlier studies have shown that exposure of skin to UVB, or lung to nitrogen mustard, leads to oxidative, as well as nitrosative stress (Malaviya *et al.*, 2012; Terra *et al.*, 2012). In human corneal epithelial cells and in rabbit cornea, UVB has been shown to stimulate expression of nitric oxide synthases (Cejkova *et al.*, 2005; Black *et al.*, 2011). One consequence of nitrosative stress is nitration of unsaturated fatty

acids. Generated in nitric oxide-dependent oxidative reactions, several of these lipid products are electrophilic fatty acid nitroalkenes and include nitrolinoleic acid and nitrooleic acid derivatives. Like 4-HNE, these nitro-fatty acids react via Michael additions and form adducts with many cellular components; their reaction with signaling proteins can regulate their function and control gene expression (Freeman *et al.*, 2008). Of interest was our finding that 9-nitrooleic acid, a relatively abundant nitro-fatty acid, was comparable to 4-HNE in inducing HO-1 mRNA and protein expression in corneal epithelial cells, although the kinetics of the responses to the two reactive lipids were distinct. Thus, induction of HO-1 mRNA and protein by 4-HNE was more rapid and persistent when compared to 9-nitrooleic acid. Differences in the activities of these lipid mediators in inducing HO-1 in corneal epithelial cells may be due to their relative reactivity with signaling molecules important for controlling expression of stress response genes (Schwobel *et al.*, 2010) (see further below), and/or differences in their uptake and metabolism. Importantly, our data suggest that nitrosative stress and subsequent generation of reactive nitrolipids plays a role in regulating an adaptive response in corneal epithelial cells. Further studies are needed to determine if nitro-fatty acids such as 9-nitrooleic acid can be generated in the cornea after treatment with stressors such as UVB and nitrogen mustard.

Reactive aldehydes including 4-HNE activate MAP kinase and PI3K/Akt signaling cascades, key processes regulating HO-1 expression (Schmitz *et al.*, 2002; Salinas *et al.*, 2003; Iles *et al.*, 2005). Both 4-HNE and 9-nitrooleic acid were found to activate these pathways in corneal epithelial cells; 4-HNE was more effective than 9-nitrooleic acid, a finding consistent with its increased ability to induce HO-1. Studies with kinase inhibitors demonstrated that p38 MAP kinase was the most active in regulating 4-HNE- and 9-nitrooleic acid-induced HO-1 expression. Erk1/2, and to a lesser extent, JNK and PI3K/Akt, were also involved in 4-HNE-induced HO-1 expression, while JNK and PI3K/Akt, but not Erk1/2, were partly effective in regulating the activity of 9-nitrooleic acid. These data indicate 4-HNE and 9-nitrooleic acid modulate HO-1 expression by distinct mechanisms. It is well recognized that additional signaling pathways control HO-1 expression including Nrf2/Keap-1 and NF-κB (Nguyen *et al.*, 2003; Wijayanti *et al.*, 2004). In this regard, 4-HNE has been shown to activate Nrf2 and NF-κB (Ruef *et al.*, 2001; Kansanen *et al.*, 2011). It remains to be determined if 4-HNE and 9-nitrooleic acid also control the activity of Nrf2 and NF-κB in corneal epithelial cells, and consequent HO-1 expression. In earlier studies using human corneal epithelial cells, we found that UVB-induced expression of HO-1 mRNA was associated with activation of Erk1/2 JNK and p38 MAP kinases (Black *et al.*, 2011). In contrast to 4-HNE and 9-nitrooleic acid, the effects of UVB were mediated by JNK and not p38 MAP kinase. These data indicate that mechanisms regulating HO-1 by 4-HNE and 9-nitrooleic acid are also distinct from UVB.

Of interest were our findings that 4-HNE was rapidly metabolized by the corneal epithelial cells. This is evidenced by 1) its rapid disappearance from the medium of treated cells, 2) its rapid intracellular metabolism, 3) the appearance of both intra- and extracellular metabolites, and 4) the formation of 4-HNE-protein adducts. Metabolism of 4-HNE and related reactive aldehydes to inactive products is an important mechanism for protecting cells from oxidative stress. Several pathways mediate 4-HNE metabolism including oxidation to 4-hydroxy-2-

nonenoic acid by aldehyde dehydrogenase, reduction to non-2-ene-1,4-diol by alcohol dehydrogenase, and conjugation by glutathione-S-transferases (Canuto *et al.*, 1994; Spycher *et al.*, 1996; Tjalkens *et al.*, 1998). The cornea contains both aldehyde dehydrogenases and alcohol dehydrogenases; of interest is the fact that aldehyde dehydrogenase is a highly abundant corneal crystallin, a structural protein important in maintaining clarity of the cornea (Cooper *et al.*, 1993; Chen *et al.*, 2013). The metabolism of 4-HNE by isoforms of this enzyme including ALDH3A1 in humans, and most other mammals, and ALDH1A1 in rabbits, has been well characterized (Chen *et al.*, 2013). Metabolism of 4-HNE by glutathione-S-transferases in human and bovine corneas has also been reported (Srivastava *et al.*, 1994; Singhal *et al.*, 1995). At the present time, the precise identity of the 4-HNE-derived metabolites, as well as the enzymes mediating 4-HNE metabolism in the corneal epithelial cells used in our studies are not known.

As described above, 4-HNE forms protein adducts via Michael additions across its carbon-carbon double bond (Esterbauer *et al.*, 1991). In cultured corneal epithelial cells, a number of proteins with molecular weights ranging from 43 to 300 kDa were modified by 4-NHE. That adducts can be formed in corneal cells is consistent with our findings of 4-HNE-protein adducts in the epithelium of rabbit cornea in organ culture following treatment with UVB or nitrogen mustard. The identity of the modified proteins in either intact cornea or the corneal epithelial cell cultures is not known. 4-HNE can directly or indirectly modify signaling proteins controlling expression of adaptive response genes and these may be important in regulating expression of HO-1 (Leonarduzzi *et al.*, 2004; Siow *et al.*, 2007; Rudolph and Freeman, 2009; Huang *et al.*, 2012). Further studies are needed to identify proteins modified by 4-HNE in the cornea and to determine their role in mediating injury induced by UVB or nitrogen mustard.

In summary, our data demonstrate that in the rabbit cornea organ culture model, injury following exposure to UVB or nitrogen mustard results in oxidative stress, as exemplified by the generation of 4-HNE modified proteins and expression of HO-1. Using corneal epithelial cell cultures, MAP kinase and PI3K/Akt signaling were found to be important mechanisms by which 4-HNE modulates expression of HO-1. Similar results were observed in corneal cell cultures with 9-nitrooleic acid, a lipid-derived product resulting from nitrosative stress, although 4-HNE and 9-nitrooleic acid appear to act by distinct mechanisms. Earlier studies have shown that oxidative and nitrosative stress induced by UVB or mustards contribute to toxicity in target tissues. Both stress processes can arise by a variety of mechanisms in injured tissues including increases in enzymes that generate reactive oxygen and reactive nitrogen species, disruption of mitochondrial function, and changes in intracellular antioxidants such as sulfhydryl-containing amino acids, small molecular weight peptides such as glutathione, and various antioxidant enzymes (Niki, 2009). A more precise definition of the mechanisms by which UVB or nitrogen mustard induce oxidative and nitrosative stress in the cornea is needed as is the role of antioxidants in protecting against tissue injury.

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Highlights

- UVB or nitrogen mustard causes rabbit corneal epithelial injury.
- 4-hydroxynonenal (4-HNE) was formed and heme oxygenase-1 (HO-1) was increased.
- 4-HNE induced HO-1 mRNA and protein expression in human corneal epithelial cells.
- The induction of HO-1 by 4-HNE was through MAP kinase activation.

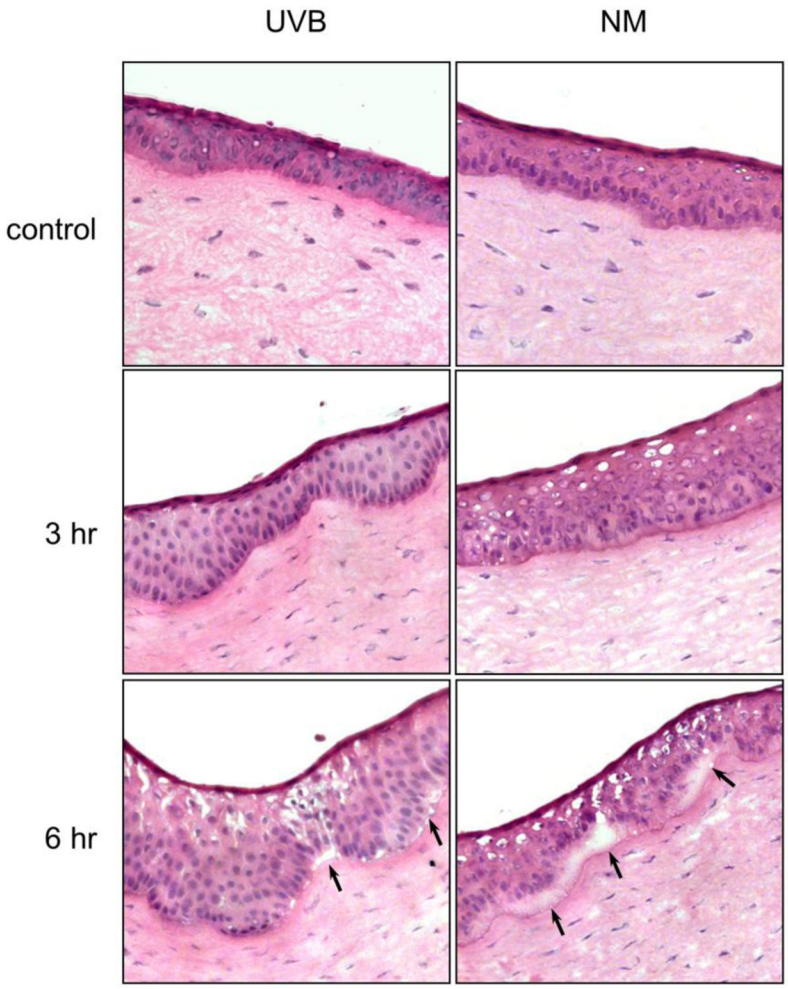

Figure 1. Morphology of rabbit corneas treated with UVB or nitrogen mustard

Cornea organ cultures were exposed to control, UVB (0.5 J/cm^2) or nitrogen mustard (NM, 100 nmol). Black arrows indicate areas of separation of epithelium from the stroma following UVB or NM treatment. After 3 hr and 6 hr, histological sections were prepared and stained with hematoxylin and eosin. Original magnification x 400

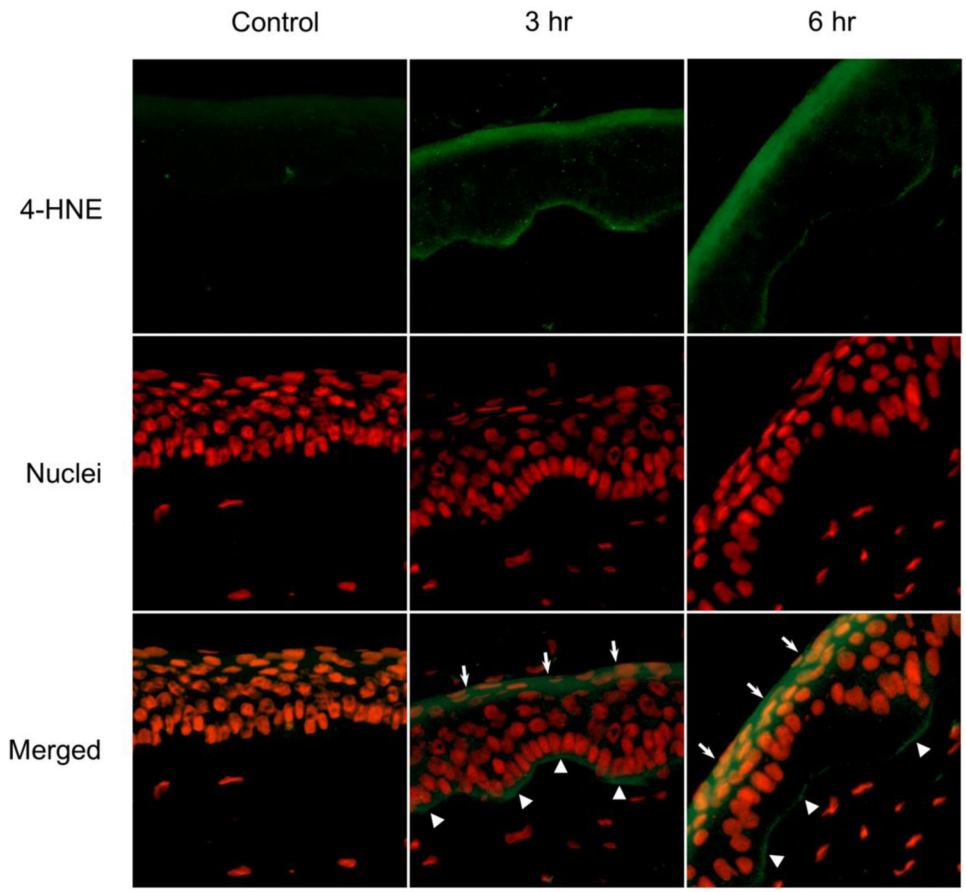

Figure 2. Effects of UVB on 4-HNE formation in rabbit corneas

Cornea organ cultures were exposed to control or UVB (0.5 J/cm^2). After 3 hr and 6 hr, histological sections were prepared and the central portions of the corneas were analyzed for 4-HNE using mouse monoclonal 4-HNE primary antibody and Alexa-Fluor 488 labeled secondary antibody. Nuclei were visualized using DAPI staining. White arrows and arrowheads indicate areas of 4HNE adduct formation on the apical epithelial surface and basal epithelial surface, respectively. Original magnification x 400

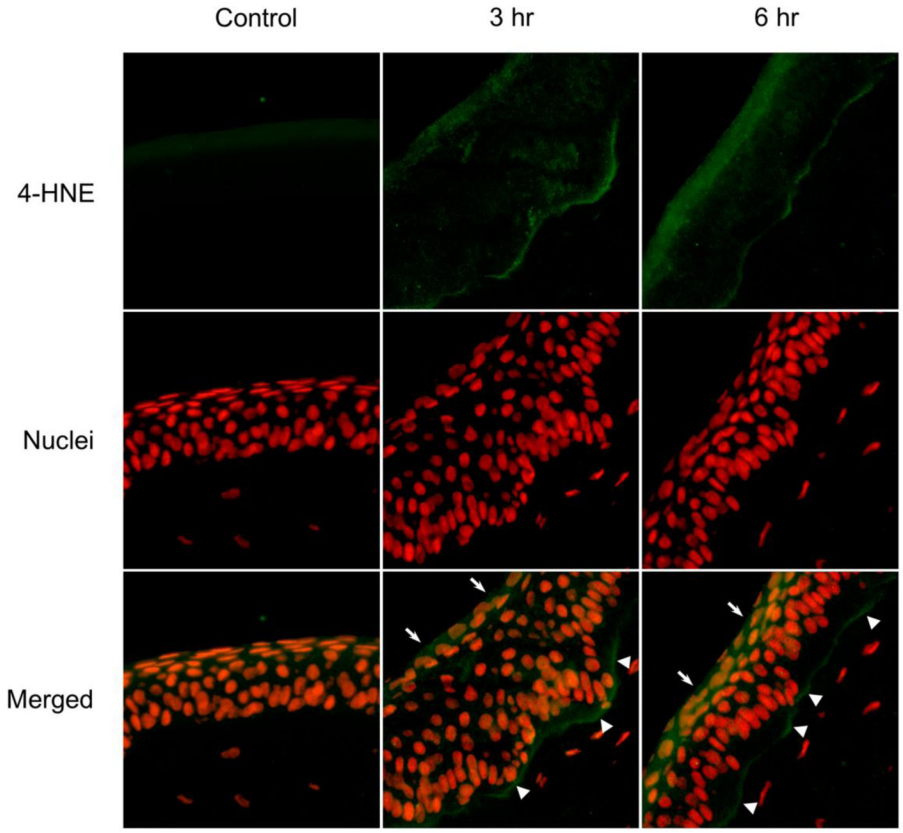

Figure 3. Effects of nitrogen mustard on 4-HNE formation in rabbit corneas

Cornea organ cultures were treated with control or 100 nmol nitrogen mustard. After 3 hr and 6 hr, histological sections were prepared and the central portions of the corneas were analyzed for 4-HNE using mouse monoclonal primary 4-HNE antibody and Alexa-Fluor 488-labeled secondary antibody. Nuclei were visualized using DAPI staining. White arrows and arrowheads indicate areas of 4-HNE adduct formation on the apical epithelial surface and basal epithelial surface, respectively. Original magnification x 400

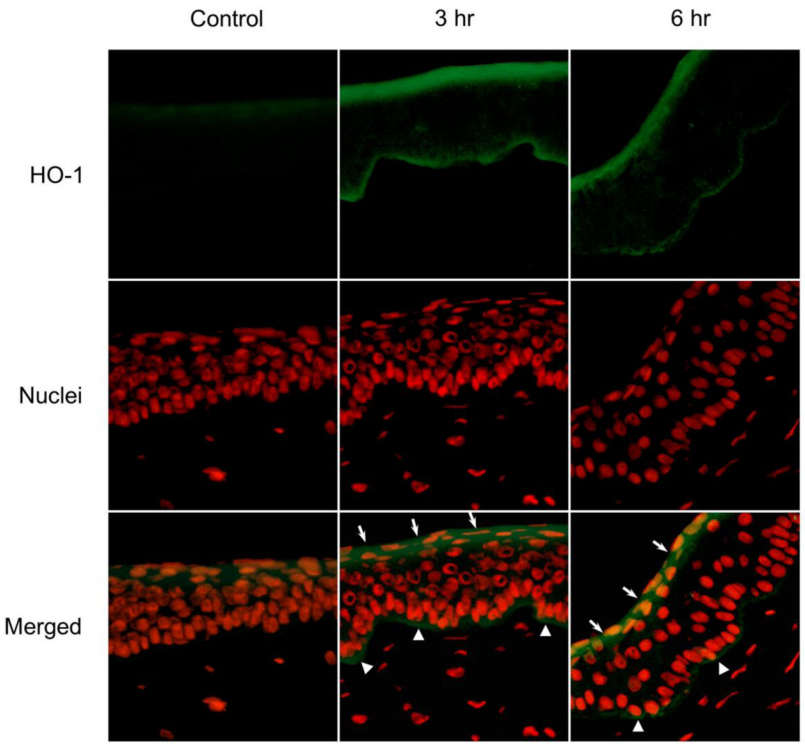

Figure 4. Effects of UVB on HO-1 expression in rabbit corneas

Cornea organ cultures were exposed to control or UVB (0.5 J/cm^2). After 3 hr and 6 hr, histological sections were prepared and the central portions of corneas analyzed for HO-1 expression using mouse monoclonal primary HO-1 antibody and Alexa-Flour 488-labeled secondary antibody. Nuclei were visualized using DAPI staining. White arrows and arrowheads indicate areas of HO-1 formation on the apical epithelial surface and basal epithelial surface, respectively. Original magnification x 400

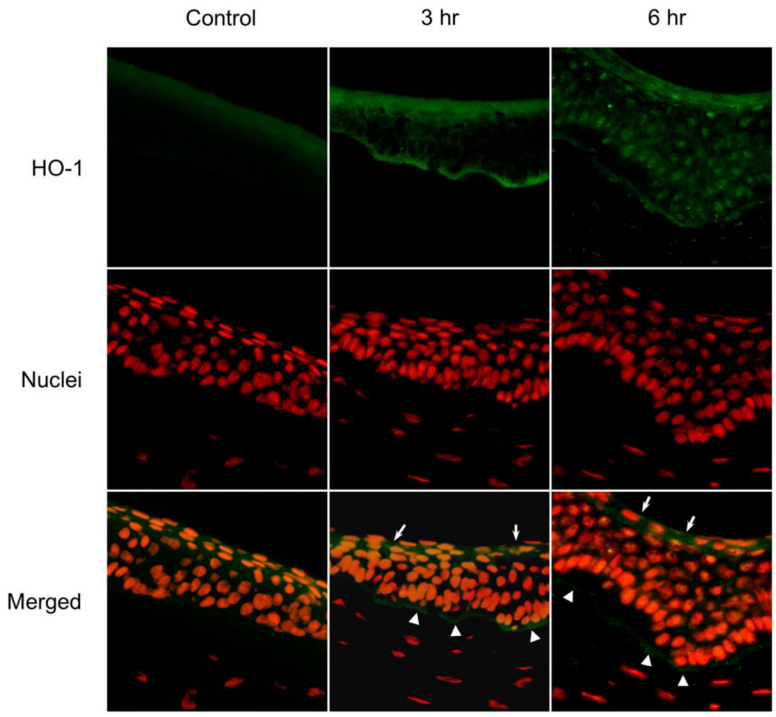

Figure 5. Effects of nitrogen mustard on HO-1 expression in rabbit corneas

Cornea organ cultures were treated with control or 100 nmol nitrogen mustard. After 3 hr and 6 hr, histological sections were prepared and the central portions of corneas analyzed for HO-1 expression using mouse monoclonal HO-1 antibody and Alexa-Flour 488-labeled secondary antibody. Nuclei were visualized using DAPI staining. White arrows and arrowheads indicate areas of HO-1 formation on the apical epithelial surface and basal epithelial surface, respectively. Original magnification x 400

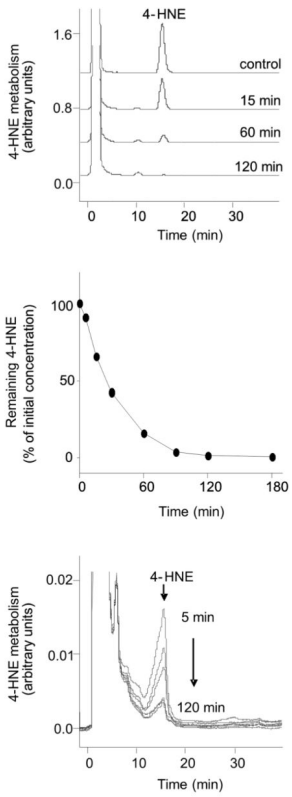

Figure 6. 4-HNE metabolism in human corneal epithelial cells

Cell suspensions (2×10^6/ml) were incubated with 100 μM 4-HNE. At the indicated time points, reactions were stopped by the addition of an equal volume of acetonitrile/acetic acid (96:4, v/v). Top panel: HPLC analysis of 4-HNE metabolism. After stopping the reaction, cells were pelleted and clear supernatants analyzed by HPLC. Middle panel: Kinetics of 4-HNE metabolism. Bottom panel: Cells were extracted and analyzed by HPLC after treatment with 100 μM 4-HNE for 5, 15, 30, 60 and 120 min.

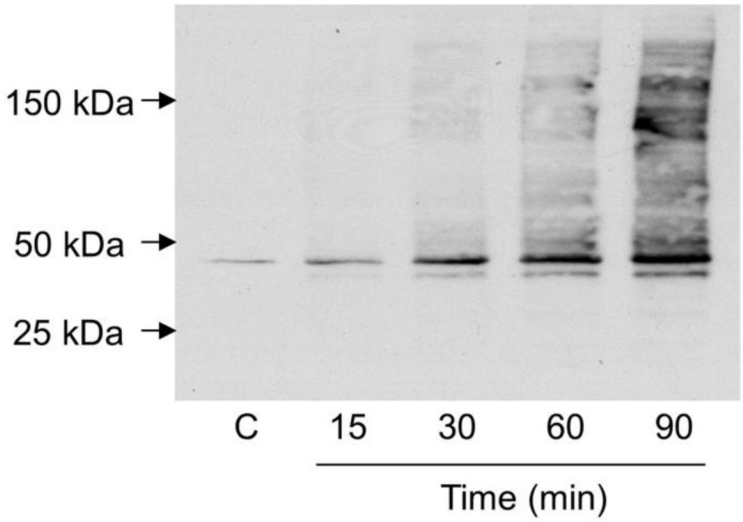

Figure 7. Formation of 4-HNE-protein adducts in human corneal epithelial cells

Cells were treated with vehicle control (C) or 4-HNE (30 μM) for 0, 15, 30, 60 and 90 min. Cell lysates were then prepared, and protein analyzed by western blotting using mouse monoclonal antibody to 4-HNE. Each lane contained 10 μg of corneal epithelial protein.

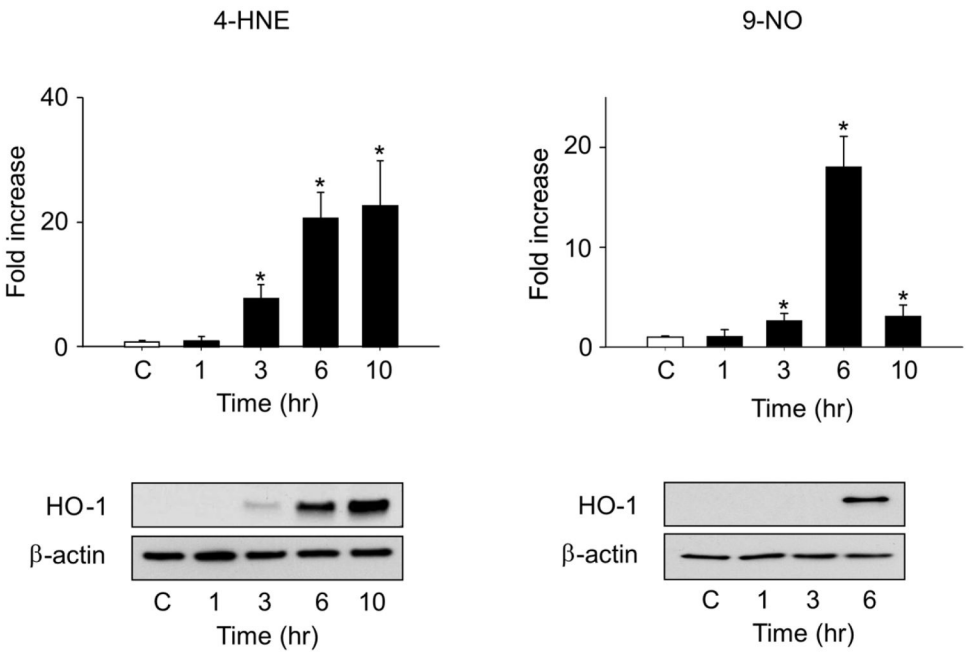

Figure 8. Effects of electrophilic lipid peroxidation products on HO-1 expression in human corneal epithelial cells

Cells were treated with vehicle control (C), 30 μM 4-HNE (left panels) or 10 μM 9-nitrooleic acid (9-NO, right panels). mRNA or protein was extracted from the cells at the indicated times and analyzed by real time PCR (upper panels) and western blotting (lower panels), respectively. β-actin was used as a protein loading control. Data are presented as the mean ± SE (n = 3). *Significantly different from control ($P < 0.05$).

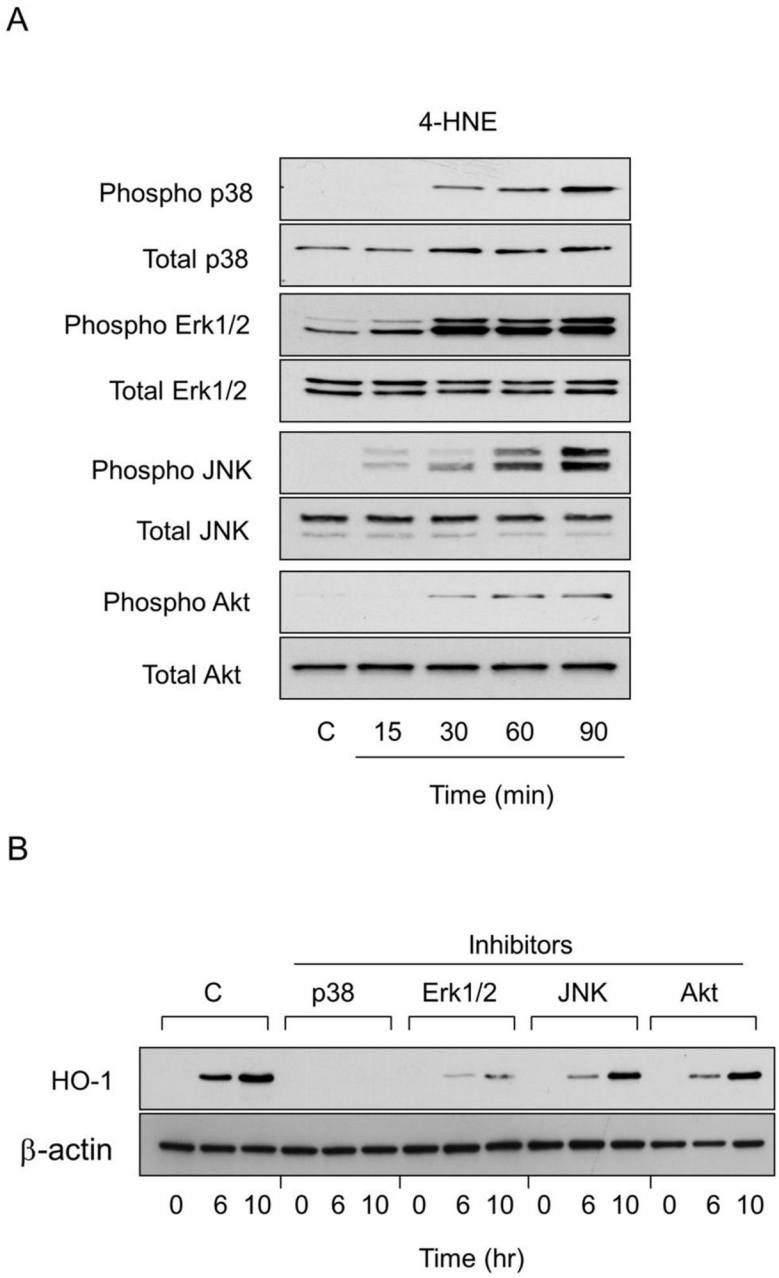

Figure 9. Role of MAP kinases and PI3/Akt kinase signaling in 4-HNE-induced HO-1 expression in human corneal epithelial cells

Panel A: Effects of 4-HNE on expression of MAP kinases and PI3K/Akt kinase. Cells were treated with control (C) or 30 μM 4HNE for 15, 30, 60 and 90 min. Cell lysates were prepared and analyzed for total and phosphorylated p38, JNK, Erk1/2, or PI3/Akt by western blotting. Panel B: Effects of MAP kinase and PI3K/Akt inhibitors on 4-HNE-induced HO-1 expression. Cells were pre-incubated with inhibitors to p38 (SB203580,10 μM), JNK (SP600125, 20 μM), Erk1/2 (PD98059, 10 μM) or PI3K (wortmannin, 0.1 μM)

for 3 hr and then treated with 30 μM 4-HNE for additional 6 hr or 10 hr. Total cell lysates were prepared and analyzed for HO-1 protein expression by western blotting.

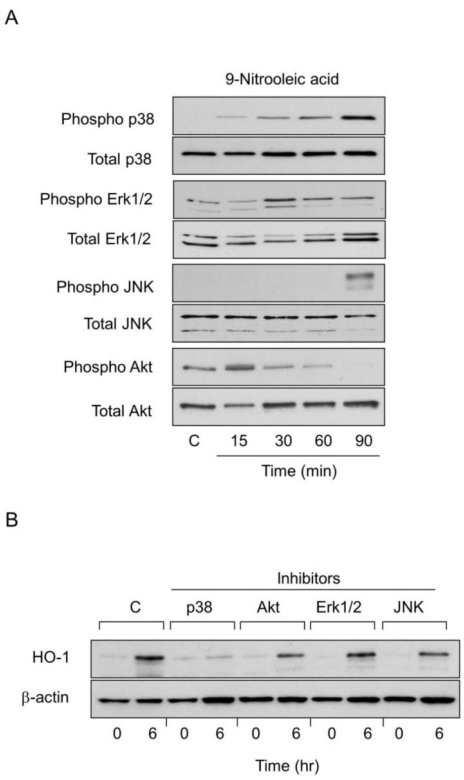

Figure 10. Role of MAP kinases and PI3/Akt kinase signaling in 9-nitrooleic acid-induced HO-1 expression in human corneal epithelial cells

Panel A: Effects of 9-nitrooleic acid on expression of MAP kinases and PI3K/Akt kinases. Cells were treated with control (C) or 10 μM 9-nitrooleic acid for 15, 30, 60 and 90 min. Cell lysates were prepared and analyzed for total and phosphorylated p38, JNK, Erk1/2, or PI3/Akt using western blotting. Panel B: Effects of MAP kinase and PI3/Akt inhibitors on 9-nitrooleic acid-induced HO-1 expression. Human epithelial cells were pre-incubated with inhibitors to p38 (SB203580,10 μM), JNK (SP600125, 20 μM), Erk1/2 (PD98059, 10 μM) or PI3/Akt kinase (wortmannin, 0.1 μM) for 3 hr and then treated with 10 μM 9-nitrooleic acid for additional 6 hr. Total cell lysates were then prepared and analyzed for HO-1 protein expression by western blotting.