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Modulation of keratinocyte expression of antioxidants by 4-hydroxynonenal, a lipid peroxidation end product

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Abstract

4-Hydroxynonenal (4-HNE) is a lipid peroxidation end product generated in response to oxidative stress in the skin. Keratinocytes contain an array of antioxidant enzymes which protect against oxidative stress. In these studies, we characterized 4-HNE-induced changes in antioxidant expression in mouse keratinocytes. Treatment of primary mouse keratinocytes and PAM 212 keratinocytes with 4-HNE increased mRNA expression for heme oxygenase-1 (HO-1), catalase, NADPH:quinone oxidoreductase (NQO1) and glutathione S-transferase (GST) A1-2, GSTA3 and GSTA4. In both cell types, HO-1 was the most sensitive, increasing 86-98 fold within 6 h. Further characterization of the effects of 4-HNE on HO-1 demonstrated concentration- and time-dependent increases in mRNA and protein expression which were maximum after 6 h with 30 μM. 4-HNE stimulated keratinocyte Erk1/2, JNK and p38 MAP kinases, as well as PI3 kinase. Inhibition of these enzymes suppressed 4-HNE-induced HO-1 mRNA and protein expression. 4-HNE also activated Nrf2 by inducing its translocation to the nucleus. 4-HNE was markedly less effective in inducing HO-1 mRNA and protein in keratinocytes from Nrf2−/− mice, when compared to wild type mice, indicating that Nrf2 also regulates 4-HNE-induced signaling. Western blot analysis of caveolar membrane fractions isolated by sucrose density centrifugation demonstrated that 4-HNE-induced HO-1 is localized in keratinocyte caveolae. Treatment of the cells with methyl-β-cyclodextrin, which disrupts caveolar structure, suppressed 4-HNE-induced HO-1. These findings indicate that 4-HNE modulates expression of antioxidant enzymes in

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Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

keratinocytes, and that this can occur by different mechanisms. Changes in expression of keratinocyte antioxidants may be important in protecting the skin from oxidative stress.

Keywords

heme oxygenase-1; caveolae; Nrf2; skin; antioxidants; stress proteins

Introduction

The skin is highly sensitive to oxidative stress induced environmental insults such as ultraviolet light, gamma radiation and various chemical toxicants (Isoir *et al.*, 2006; Black *et al.*, 2008b; Laskin *et al.*, 2010). Oxidative stress is associated with the generation of excessive amounts of highly toxic intermediates including superoxide anion, hydrogen peroxide and hydroxyl radicals (Halliwell and Whiteman, 2004). These reactive oxygen species (ROS) can initiate lipid peroxidation, a process that generates α,β-unsaturated hydroxyalkenals (Niki, 2009). One of these electrophilic species is 4-hydroxynonenal (4-HNE), a relatively abundant aldehyde that forms Michael adducts with nucleophilic sites in DNA, lipids and proteins (LoPachin *et al.*, 2009). 4-HNE is mutagenic and can disrupt cellular metabolic activity (Winczura *et al.*, 2012). It has been identified in sun damaged skin (Tanaka *et al.*, 2001; Hirao and Takahashi, 2005), radiation-induced dermatitis (Ning *et al.*, 2012), and in skin treated with chemicals such as the sulfur mustard analog 2-chloroethyl ethyl sulfide (Tewari-Singh *et al.*, 2012) and ozone (Valacchi *et al.*, 2003).

It is well recognized that oxidative stress initiates an adaptive response involving upregulation of stress response genes and antioxidants important in protecting cells from injury (Davies, 2000). Driving this process is activation of signaling molecules and transcription factors that control expression of these genes. In keratinocytes, these include mitogen activated protein (MAP) kinases and phospho-inositide-3-kinase (PI3K)/Akt, as well as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NF-κB (Dhar *et al.*, 2002; Keum *et al.*, 2006; Marrot *et al.*, 2008; Black *et al.*, 2010; Haarmann-Stemmann *et al.*, 2012). Molecules thought to be involved in mediating stress-induced alterations in adaptive response genes include lipid peroxidation products such as malondialdehyde, acrolein and 4-HNE (Pizzimenti *et al.*, 2013). It should be noted that nitric oxide synthase is induced during oxidative stress (Piantadosi and Suliman, 2012) and nitric oxide generated reaction products such as peroxynitrite and various electrophilic nitrolipids can also upregulate oxidative stress responsive genes (Szabo *et al.*, 2007; Freeman *et al.*, 2008).

Earlier studies by our laboratory showed that in keratinocytes, oxidative stress induced by UVB light and chemical toxicants results in upregulation of adaptive response proteins (Black *et al.*, 2008a). These include enzymes important in detoxifying or limiting the production of ROS such as superoxide dismutase (SOD), catalase and NAD(P)H quinone oxidoreductase 1 (NQO1) and the phase 2 enzymes heme oxygenase-1 (HO-1) and glutathione S-transferases (GST). The present studies were aimed at identifying antioxidants and stress proteins upregulated in mouse keratinocytes by 4-HNE and analyzing signaling pathways regulating this response. Coordinate regulation of expression of these adaptive

response proteins is likely to be important in controlling oxidative stress and tissue injury following exposure of the skin to xenobiotics.

Materials and Methods

Materials

Mouse monoclonal 4-HNE antibody was from R&D Systems (Minneapolis, MN) and rabbit polyclonal HO-1 antibody was from Enzo Life Sciences (Farmingdale, NY). Rabbit polyclonal caveolin-1 antibody, 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 DC (Detergent Compatible) 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). Reagents for MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) viability assays and M-MLV Reverse Transcriptase were from Promega (Madison, WI). SYBR Green Master Mix and other PCR reagents were purchased from Applied Biosystems (Foster City, CA). 4-HNE, PD 98059, SP600125 and wortmannin were from Calbiochem (La Jolla, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen Corp. (Carlsbad, CA). Mouse monoclonal β-actin antibody, SB203580, protease inhibitor cocktail (4-(2 aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin), methyl-β-cyclodextrin and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell culture and treatments

PAM 212 cells were obtained from Dr. Stuart Yuspa (National Institutes of Health) and maintained as previously described (Black *et al.*, 2008b). Cells were cultured in DMEM containing 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Primary mouse keratinocytes were isolated from the skin of newborn C57BL/6J wild type mice (The Jackson Laboratory, Bar Harbor, ME) or C57BL/6J Nrf2−/− mice (Khor *et al.*, 2006; Khor *et al.*, 2008) bred at the Rutgers University animal care facility. Keratinocytes were cultured following the procedure of Hager *et al.* (1999).

For experiments, keratinocytes were grown to 80~90% confluence in six well collagen IV-coated plates as previously described (Black *et al.*, 2008a). Cells were then treated with vehicle or increasing concentrations of 4-HNE (1-100 μM). At these concentrations, cell viability of PAM 212 cells and primary keratinocytes at 6 h was greater than 98% and 90%, respectively. For kinase inhibition experiments, cells were pretreated with p38 MAP kinase inhibitor SB203580 (10 μM), JNK kinase inhibitor SP600125 (20 μM), ERK1/2 kinase inhibitor PD98059 (10 μM) or phosphatidylinositide 3-(PI3K) kinase inhibitor wortmannin (0.1 μM) for 3 h prior to treatment with 4-HNE or vehicle control. Cells were analyzed 6 h later for mRNA and protein expression by real time PCR and western blotting, respectively. For caveolae inhibition experiments, PAM 212 keratinocytes were pre-incubated with control or 5 mM of methyl-β-cyclodextrin for 3 h. After washing with PBS, cells were treated with 4-HNE for 6 h and analyzed for protein expression by western blotting.

Western blotting

Western blotting was performed as previously described (Zheng *et al.*, 2013b). Briefly, cells were lysed by the addition of 300 μl SDS lysis buffer (10 mM Tris-base, pH 7.6, supplemented with 1% SDS and the protease inhibitor cocktail), transferred into 1.5 ml Eppendorf microcentrifuge tubes, sonicated on ice and centrifuged (300 × g, 10 min at 4° C). Total protein in supernatants was determined by the DC protein assay kit using bovine serum albumin as the standard. Lysates (15 μg protein/well) were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blocked in Tris buffer supplemented with 5% milk at room temperature. After 1 h, the blots were incubated overnight at 4°C with primary antibodies, washed with tTBS (Tris-buffered saline supplement with 0.1% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies. After 1 h at room temperature, proteins were visualized by ECL chemiluminescence.

Real-time PCR

Total RNA was isolated from the cells using Tri Reagent (Sigma). cDNA was synthesized using M-MLV reverse transcriptase. The cDNA was diluted 1:10 in RNase-DNase-free water for PCR analysis. For each gene, a standard curve was created from serial dilutions of cDNA mixtures of all 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 for detection of the fluorescent signal and the standard curve method was used for relative quantitative analysis. The primer sequences for the genes were generated using Primer Express software (Applied Biosystems) and oligonucleotides synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The house keeping gene β-actin was used to normalize all values. The forward (5'-3') and reverse (5'-3') primers used are listed in Table 1.

Analysis of 4-HNE uptake and metabolism

4-HNE uptake and metabolism experiments were performed as described earlier (Siems *et al.*, 1997). Briefly, keratinocytes were treated with 100 μM 4-HNE in 1.5 ml of serum-free culture medium. After 5-120 min, cells were washed and metabolism terminated by the addition of 1 ml of 70% perchloric acid in PBS. The cells were then removed from the dishes using a scrapper, transferred to 1.5 ml Eppendorf tubes and centrifuged at 3,000 g for 10 min at 4 °C. Supernatants were analyzed by HPLC (JASCO corporation, Tokyo, Japan) fitted with a JASCO 2075 plus UV detector and a Phenomenex 5μ C18 column (250 × 2.00 mm) using a mobile phase consisting of 70% 50 mM potassium phosphate buffer (pH 2.7) and 30% acetonitrile at a flow rate of 0.25 ml/min. 4-HNE (retention time = 20 min) was detected at 224 nm. In some experiments, 4-HNE metabolism was analyzed in cell lysates prepared by sonicating a suspension of 5×10^6 cells in 1 ml PBS on ice. Lysates were then centrifuged at 9,000 × g for 20 min at 4°C. Clear supernatants (15 μg protein/ml) were incubated with 100 μM 4-HNE in the absence or presence of 1 mM NADH or NADPH. Reactions were stopped by the addition of 0.1 ml 70% perchloric acid in PBS. Samples were then centrifuged (3,000 g for 10 min) and clear supernatants analyzed by HPLC.

Isolation of caveolae

Lipid rafts containing caveolae were prepared from PAM 212 cells as described by Smart *et al.* (1995). Briefly, treated cells were scraped into 5 ml ice-cold sucrose buffer (0.25 M sucrose, 1 mM EDTA, and 20 mM Tris, pH 7.8) and centrifuged at 1400 × g for 5 min at 4°C. Cell pellets were then suspended in 1 ml sucrose buffer and homogenized with 20 strokes in a Dounce homogenizer. Lysates were transferred to Eppendorf tubes and centrifuged for 10 min at 1000 × g at 4°C. Supernatants were collected and the homogenization process repeated with cell pellets. The supernatants were combined and then carefully layered on top of 8 ml of a 30% Percoll solution in sucrose buffer. The gradient was then centrifuged for 60 min at 84,000 × g in a Ti 70 rotor using an L7-55 Beckman ultracentrifuge (Brea, CA) to separate caveolae containing plasma membrane fractions. These were collected and stored at −70°C until analysis.

Statistical analysis

Data were evaluated using the two-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

4-HNE metabolism in keratinocytes

In initial experiments we analyzed the uptake and degradation of 4-HNE in PAM 212 keratinocytes. 4-HNE was found to accumulate in the cells within 15 min of treatment with 100 μM 4-HNE; rapid degradation of the reactive aldehyde was evident thereafter (Fig. 1, panels A and B). Using lysates from a human keratinocyte cell line, previous studies showed that 4-HNE was degraded in an NADH-dependent pathway (Aldini *et al.*, 2003; Aldini *et al.*, 2007). In contrast, we found that 4-HNE was rapidly degraded in PAM 212 cell lysates in the absence of pyridine nucleotides. However, both NADH and NADPH stimulated 4-HNE degradation (Fig. 1, panel C). The α,β-unsaturated bond of 4-HNE is known to form protein adducts by reacting with cysteine, histidine and lysine residues through Michael additions, processes thought to initiate the biological activity of reactive lipid peroxidation products (LoPachin *et al.*, 2009). In intact keratinocytes, 4-HNE treatment resulted in the formation of 4-HNE-protein adducts (Fig. 1, panel D). Within 15-30 min of treatment with 100 μM 4-HNE, two prominent bands (Mr = 43,000 and 75,000) were evident in western blots. With increasing periods of time, additional proteins of higher and lower molecular weights were also modified by 4-HNE.

4-HNE induces antioxidant proteins

Earlier studies showed that stress resulting from the reaction of lipid peroxidation products with cellular components upregulates oxidative stress-related genes including HO-1(Basu-Modak *et al.*, 1996). Consistent with these findings, we observed that 4-HNE readily induced HO-1 mRNA and protein in both PAM 212 cells and primary keratinocytes from wild type mice, as determined by real time PCR and Western blotting, respectively (Table 2 and Fig. 2). The effects of 4-HNE were concentration- and time-dependent. In both cell types, maximal increases in HO-1 mRNA expression (approximately 85-90-fold) were noted

with 30 μM 4-HNE after 6 h. Increases in protein expression were maximal with 10-30 μM 4-HNE after 6-12 h (Fig. 2). We next determined if 4-HNE upregulated other antioxidants in the cells. We found that 4-HNE (30 μM) induced expression of mRNA for NQO1, as well as the glutathione S-transferases, GSTA1-2, GSTA3 and GSTA4 (Table 2). Whereas increased expression of NQO-1 was 4-5 fold after 6 h and 24 h incubation with 4-HNE, in both PAM 212 cells and primary keratinocytes, increases in GSTA1-2 (30-60-fold) were only observed after 24 h. 4-HNE also upregulated GSTA3 and GSTA4 approximately 2-8 fold. Catalase was also upregulated by 4-HNE approximately 3 fold, but only in primary keratinocytes after 24 h. 4-HNE had no significant effects on SOD, GSTM1 or GSTP1.

Signaling pathways regulating 4-HNE-induced alterations in HO-1 expression

MAP kinase and PI3K/Akt signaling are known to be important in regulating antioxidant expression. We next characterized these signaling pathways in 4-HNE treated PAM 212 keratinocytes. 4-HNE was found to activate p38, Erk1/2, JNK MAP kinase activity, as well as Akt in the cells as measured by phosphorylation of these proteins (Fig. 3, upper panel). No significant changes were apparent in total expression of the MAP kinases or Akt. We next used inhibitors of the MAP kinases and PI3 kinase to evaluate their role in regulating HO-1 expression. SB203580, a p38 kinase inhibitor, SP600125, a JNK inhibitor, PD98059, an ERK1/2 kinase inhibitor and wortmanin, a PI3K inhibitor, were found to suppress 4-HNE-induced HO-1 mRNA and protein expression (Fig. 3, center and lower panels). The highest concentration of 4-HNE (30 μM) was able to overcome inhibition of HO-1 protein expression by the kinase inhibitors.

Role of caveolae in 4-HNE-induced expression of HO-1

We previously showed that plasma membrane caveolae play a role in the regulation of electrophilic nitro fatty acid-induced expression of stress proteins and antioxidants in keratinocytes (Zheng *et al.*, 2013a). In further studies we determined if caveolae regulate 4-HNE-induced expression of HO-1. Caveolar fractions, but not non-caveolar fractions of PAM 212 keratinocytes, were found to contain caveolin-1, the major structural protein in caveolae (Fig. 4, upper panel). 4-HNE upregulated HO-1 expression in both caveolar and non-caveolar fractions of the cells. The MAP kinase and Akt inhibitors were effective in suppressing expression of HO-1 in both caveolar and non-caveolar fractions (Fig. 4, center panel). Inhibitors of JNK and p38 were the most effective in blocking caveolar HO-1 expression. Treatment of keratinocytes with methyl-β-cyclodextrin, which depletes cholesterol and disrupts caveolae, similarly reduced 4-HNE-induced HO-1 expression (Fig. 4, lower panel).

Role of Nrf2 in 4-HNE-induced expression of HO-1

The transcription factor Nrf2 is known to play a role in the regulation of expression of antioxidants in response to 4-HNE (Huang *et al.*, 2012). Nrf2 forms a complex with Keap1, which functions to retain Nrf2 in the cytoplasm (Li and Kong, 2009). Binding of 4-HNE to Keap1 allows Nrf2 to translocate to the nucleus where it regulates antioxidant gene expression (Levonen *et al.*, 2007; Kobayashi *et al.*, 2009). In PAM 212 cells, 4-HNE was found to stimulate nuclear localization of Nrf2 in a concentration- and time-dependent

manner (Fig. 5, upper panel). To assess the role of Nrf2 in 4-HNE-induced expression of HO-1, we used keratinocytes from Nrf2−/− mice. We found that 4-HNE was significantly less effective in upregulating HO-1 mRNA expression in keratinocytes from Nrf2−/− mice than keratinocytes from wild type mice (Fig 5, lower panel).

Discussion

4-HNE is a highly reactive aldehyde that can modify cellular components and induce cytotoxicity. Metabolic degradation of 4-HNE limits its cytotoxic activity and protects against tissue injury (O'Brien *et al.*, 2005). The present studies demonstrate that 4-HNE is readily taken up by mouse keratinocytes; intracellular levels were maximal within 15 min of treatment, declining rapidly thereafter. Decreases in intracellular keratinocyte 4-HNE are consistent with its metabolism. In this regard, earlier studies showed both oxidative and reductive metabolism of 4-HNE in human keratinocytes, as well as the formation of GSH reaction products (Aldini *et al.*, 2003; Aldini *et al.*, 2007). A number of enzymes have been identified that metabolize 4-HNE, including alcohol dehydrogenase, aldehyde dehydrogenase, aldo-keto reductases, cytochrome P450's and glutathione S-transferases (Hartley *et al.*, 1995; Srivastava *et al.*, 2000; Amunom *et al.*, 2011). Glutathione S-transferases conjugate 4-HNE to glutathione, a process that promotes its extracellular transport (Tjalkens *et al.*, 1999). Glutathione conjugates and related metabolites generated by enzymatic oxidation/reduction of these conjugates have been identified in culture medium from human keratinocytes treated with 4-HNE (Aldini *et al.*, 2007). Similarly, we observed rapid degradation of 4-HNE in lysates of mouse keratinocytes. Of note, this process was stimulated by both NADH and NADPH. These pyridine nucleotides are cofactors for enzymes that degrade 4-HNE including alcohol dehydrogenase and aldehyde dehydrogenase which have been identified in human keratinocytes (Alary *et al.*, 2003; Aldini *et al.*, 2003). A considerable fraction of 4-HNE metabolism in mouse keratinocytes was found to be pyridine nucleotide-independent. Enzymes mediating this process in keratinocytes remain to be determined.

We also found that 4-HNE formed protein adducts in keratinocytes which was maximal 30 and 60 min post treatment. This occurred despite rapid degradation of 4-HNE. It has previously been reported that approximately 3-6% of 4-HNE administered to rat hepatocytes forms protein adducts (Siems and Grune, 2003); similar levels of 4-HNE protein adducts have been noted in human keratinocytes (Tanaka *et al.*, 2001). Although many 4-HNE modified proteins have been identified and characterized in different tissues (Petersen and Doorn, 2004; Jacobs and Marnett, 2010), including several involved in the regulation of signal transduction (Nakashima *et al.*, 2003), the identity of these proteins in mouse keratinocytes and their role in mediating adaptive responses remains to be determined.

Treatment of both primary keratinocytes and PAM 212 keratinocytes with 4-HNE was associated with upregulation of key stress proteins including HO-1, NQO-1, catalase, GSTA1-2, GSTA3 and GSTA4, which play important roles in antioxidant defense. Most prominent were changes in expression of HO-1 and GSTA1-2. HO-1 is the rate limiting enzyme in the degradation of heme; it confers protection against oxidative stress by inhibiting the formation of reactive oxygen species and suppressing inflammation

(Gozzelino *et al.*, 2010). GST's such as GSTA1-2 are important in the conjugation of electrophilic compounds to reduced glutathione (Board and Menon, 2013). Three major GST gene families, alpha (GSTA), mu (GSTM) and pi (GSTP) have been identified and each has preferred substrates. GSTA enzymes, and to a lesser extent, GSTP enzymes, remove lipid peroxidation products including 4-HNE (Hayes *et al.*, 2005). Our observation that there were marked increases in GSTA1-2 are consistent with the identification of 4-HNE-glutathione conjugates and related metabolites in human keratinocytes (Aldini *et al.*, 2003). They are in accord with findings that oxidative stressors in the skin such as UVB, as well as electrophilic nitrolipids formed during nitrosative stress, upregulate keratinocyte GSTA1-2 (Black *et al.*, 2008b; Zheng *et al.*, 2013b). In contrast, GSTM1 and GSTP1, which remove DNA and protein oxidation products (Hayes *et al.*, 2005), were unaffected by 4-HNE suggesting that they do not play a role in protecting the cells against this reactive aldehyde. Of note were our findings that following 4-HNE treatment of keratinocytes, the time for maximal induction of HO-1 expression was 6 h, while maximal induction of GSTA1-2 was 24 h. These differences likely represent distinct signaling pathways and/or transcription factors for these genes that are activated by 4-HNE. Increases in GSTA3 and GSTA4 were remarkably less relative to GSTA1-2, and were variable with respect to maximal induction times. This is consistent with findings that distinct signaling pathways control expression of the different GSTA isoforms (Hayes *et al.*, 2005).

4-HNE was also found to modulate expression of NQO1 and catalase. Whereas increases in NQO1 were evident after 6 h and 24 h in both keratinocyte populations, only a small increase in catalase was observed in primary keratinocytes after 24 h. NQO1 functions as an antioxidant by catalyzing the two-electron reduction of quinones and related electrochemically active compounds, an enzymatic process that limits formation of reactive oxygen species resulting from chemical redox cycling. NQO1 also directly scavenges superoxide anion (Siegel *et al.*, 2004). Catalase is important in degrading hydrogen peroxide and increases in expression of this enzyme, as well as NQO1 are important in limiting oxidative stress. Mechanisms mediating selective increases in catalase in primary keratinocytes require further investigation.

A question remains as to the mechanism by which 4-HNE upregulates expression of adaptive response proteins in keratinocytes. Several signaling molecules have been identified that control their expression, most notably, MAP kinases and PI3K/Akt (Yang *et al.*, 2003; Usatyuk and Natarajan, 2004). We previously demonstrated that 4-HNE activates both of these signal transduction pathways in corneal epithelial cells (Zheng *et al.*, 2013b); herein we report that 4-HNE has similar effects on keratinocytes. The fact that inhibitors of these enzymes suppressed 4-HNE-induced expression of HO-1 in keratinocytes demonstrates their functional significance of these cells. Generally similar findings on signaling pathways mediating the actions of 4-HNE have been reported in rat and human liver cells and bovine lung microvascular endothelial cells (Moneypenny and Gallagher, 2005; Usatyuk *et al.*, 2006; Sampey *et al.*, 2007). The mechanism by which MAP kinase signaling cascades are activated by 4-HNE is not known. In human bronchial epithelial cells, 4-HNE down modulates the protein-tyrosine phosphatase SH2 domain containing phosphatase-1 (SHP-1) which negatively regulates JNK activity (Rinna and Forman, 2008).

This may be important in the action of 4-HNE in mouse keratinocytes. Recent studies have also demonstrated that activation of PI3K/Akt signaling by 4-HNE in hepatocytes occurs via modification and inhibition of PTEN, a regulatory protein that suppresses Akt activity (Shearn *et al.*, 2011). A similar pathway may be involved in 4-HNE-induced PI3K/Akt signaling in keratinocytes.

Nrf2/Keap-1 is an electrophilic stress signaling pathway active in keratinocytes that has been reported to regulate expression of adaptive response genes including HO-1, NQO1 and various GST's (Marrot *et al.*, 2008; Piao *et al.*, 2012). Earlier studies have shown that electrophilic substrates, including 4-HNE, modulate Nrf2 and antioxidant expression (Chen *et al.*, 2005; Zhang and Forman, 2009). In the skin, Nrf2 is thought to be important in controlling inflammation during wound healing, responses to photodamage, and chemical carcinogenesis (Braun *et al.*, 2002; Xu *et al.*, 2006; Schafer *et al.*, 2010; Saw *et al.*, 2011). Our studies demonstrate that 4-HNE activates Nrf2 in keratinocytes, as measured by nuclear localization of the protein; moreover, this process is coordinate with induction of HO-1. Findings that loss of Nrf2 markedly reduces 4-HNE-induced HO-1 expression indicate that the Nrf2/Keap-1 mediates, at least in part, the stress response pathway in keratinocytes. These data also indicate that 4-HNE utilizes more than one pathway to control HO-1 induction.

Caveolae are specialized flask-shaped glycosphingolipid-containing membrane structures controlling many cellular functions including vesicular transport, cholesterol homeostasis, inflammation, apoptosis and proliferation (Razani *et al.*, 2002). The major structural component of caveolae is caveolin-1, a protein known to be expressed in basal keratinocytes in mouse and human skin (Capozza *et al.*, 2003; Sando *et al.*, 2003). Caveolin-1 controls epidermal proliferation and differentiation and aberrant caveolin-1 expression is common in diseases such as systemic sclerosis and psoriasis (Campbell and Gumbleton, 2000; Manetti *et al.*, 2012). We found that 4-HNE-induced HO-1 was localized in both caveolar and non-caveolar fractions of keratinocytes. Previous studies have described compartmentalization of HO-1 in caveolae in rat pulmonary artery endothelial cells and mouse mesangial cells induced by endotoxin and in mouse keratinocytes treated with electrophilic nitrofatty acids (Jung *et al.*, 2003; Kim *et al.*, 2004; Zheng *et al.*, 2013b). The function of caveolar localized HO-1 is unknown. Caveolin-1 is known to directly interact with HO-1 and modulate its activity (Taira *et al.*, 2011). Caveolin-1 also interacts with caveolar toll-like receptor-4 (TLR4), an endotoxin receptor important in negatively regulating proinflammatory signaling including cytokine production (Wang *et al.*, 2009; Mirza *et al.*, 2010). These investigators further showed that this process is enhanced when HO-1 traffics to caveolae. It is possible that caveolar-associated HO-1 also contributes to the antiinflammatory activity of this antioxidant in keratinocytes by suppressing proinflammatory signaling. Cytoplasmic HO-1 resides in the endoplasmic reticulum where it presumably functions as an antioxidant. Of note was our observation that MAP kinases and PI3K/Akt are not selective and suppress localization of HO-1 in both caveolar and cytoplasmic fractions of keratinocytes. These data are consistent with studies showing that p38 MAP kinase signaling can control endotoxin-induced production of HO-1 and its localization in mouse macrophage caveolae (Wang *et al.*, 2006). Taken together, these data suggest that MAP kinase and PI3K/Akt signaling are

important in the control of the synthesis of HO-1, but not trafficking of this antioxidant to caveolae.

A novel aspect of our work is the observation that not only is HO-1 localized in keratinocyte caveolae, but it was also regulated by these structures. Thus, disruption of caveolae by cholesterol depletion suppressed 4-HNE-induced HO-1 expression. These results are in accord with our previous studies showing that disruption of caveolae in mouse keratinocytes reduced 9- and 10-nitrooleic acid-induced expression of HO-1, hsp 27 and hsp70 (Zheng *et al.*, 2013a). These data provide additional support for the idea that caveolae are important in negatively controlling inflammation. The factors that contribute to caveolae regulation of antioxidant expression in keratinocytes are not known. It is possible that 4-HNE initiates signaling via caveolae. In this regard, many growth factors and cytokines initiate signaling via caveolae-associated receptors and these may be targets for 4-HNE (Pike, 2005; Harvey and Calaghan, 2012)

In summary, our findings support the idea that 4-HNE functions as a signaling molecule in keratinocytes, upregulating adaptive response genes that are crucial for protecting cells against oxidative stress. Multiple signaling pathways controlling expression of antioxidants in keratinocytes were identified including MAP kinase and PI3K signaling and activation of Nrf2. It is well recognized that signaling pathways in addition to these have been identified that control expression of genes such as HO-1; thus, it is likely that more than one pathway is involved in regulating keratinocyte antioxidant expression in response to 4-HNE. An emerging literature also implicates stress protein trafficking to caveolae as a control point in inflammation (Chidlow and Sessa, 2010). Our data support this idea as electrophilic species generated during oxidative stress are able to drive antioxidants into caveolae. Further studies are needed to more precisely understand the role of adaptive response genes and caveolae in protecting the skin against oxidative stress.

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Highlights

- Lipid peroxidation generates 4-hydroxynonenal, a reactive aldehyde.
- 4-HNE induces antioxidant proteins in mouse keratinocytes.
- Induction of antioxidant proteins is regulated via MAP kinases, Nrf2 and caveolae.
- 4-HNE is an effective signaling molecule in keratinocytes.

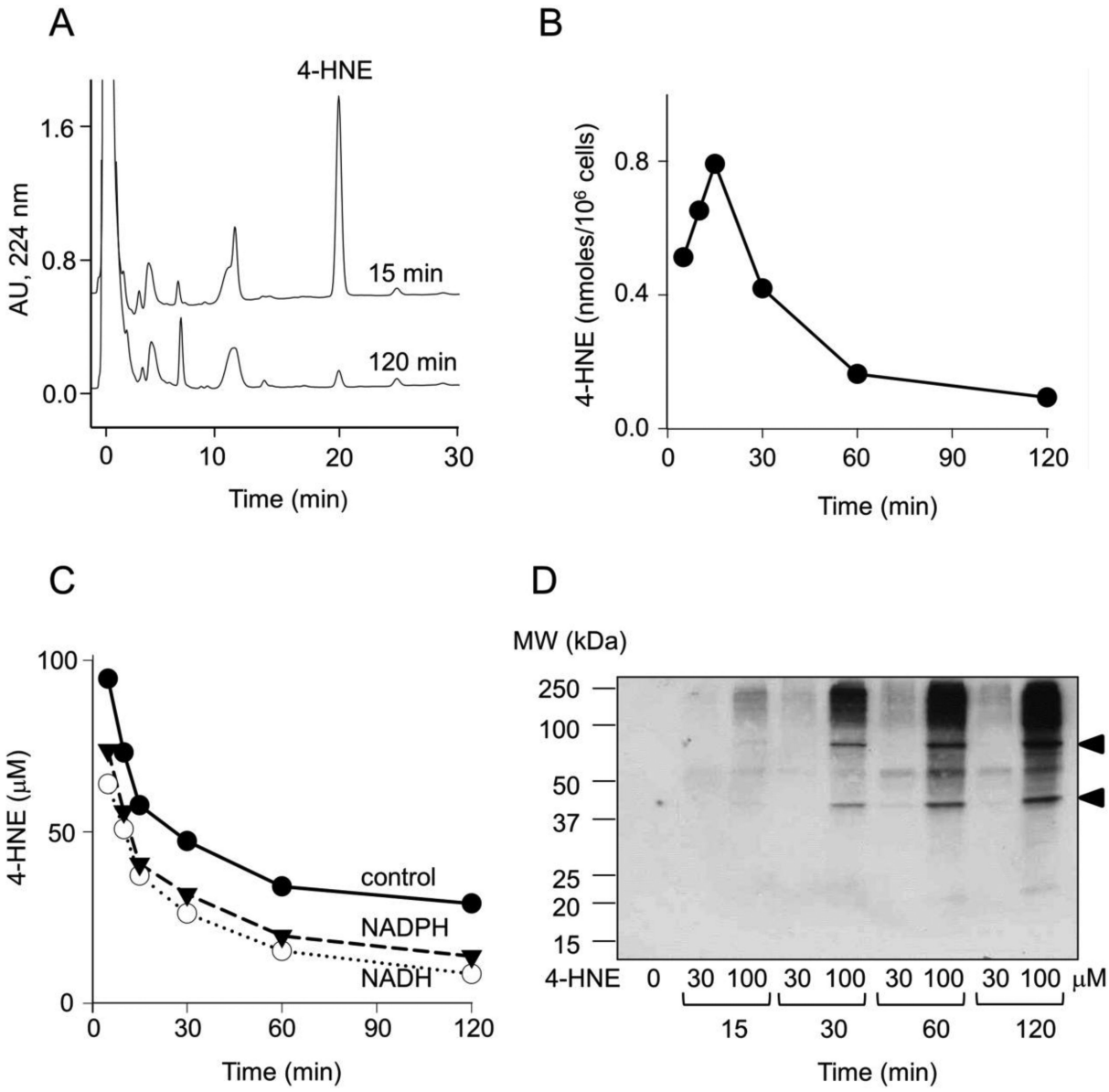

Figure 1. Metabolism of 4-HNE in PAM 212 keratinocytes

Cells were treated with 30 or 100 μM 4-HNE for increasing periods of time up to 120 min. For analysis of 4-HNE, cells were washed and 4-HNE extracted as described in the Materials and Methods. Panel A. Representative HPLC tracing of 4-HNE extracted from cells 15 min and 120 min post treatment with 100 μM 4-HNE. Panel B. Time course of 4-HNE accumulation and degradation in cells treated with 100 μM 4-HNE. Panel C. Time-course of 4-HNE metabolism in S9 fractions of mouse keratinocytes. S9 fractions of PAM 212 cells were incubated with 100 μM 4-HNE in the absence and presence of 1 mM NADH or NADPH. At the indicated times, 4-HNE was extracted and quantified by HPLC. Panel D. Time-dependent formation of 4-HNE-protein adducts in intact cells treated with control, 30, or 100 μM 4-HNE. For this analysis, treated cells were lysed and analyzed by Western

blotting using a monoclonal antibody to 4-HNE. Arrowheads show the appearance of modified proteins with molecular weights of 43 and 75 kDa.

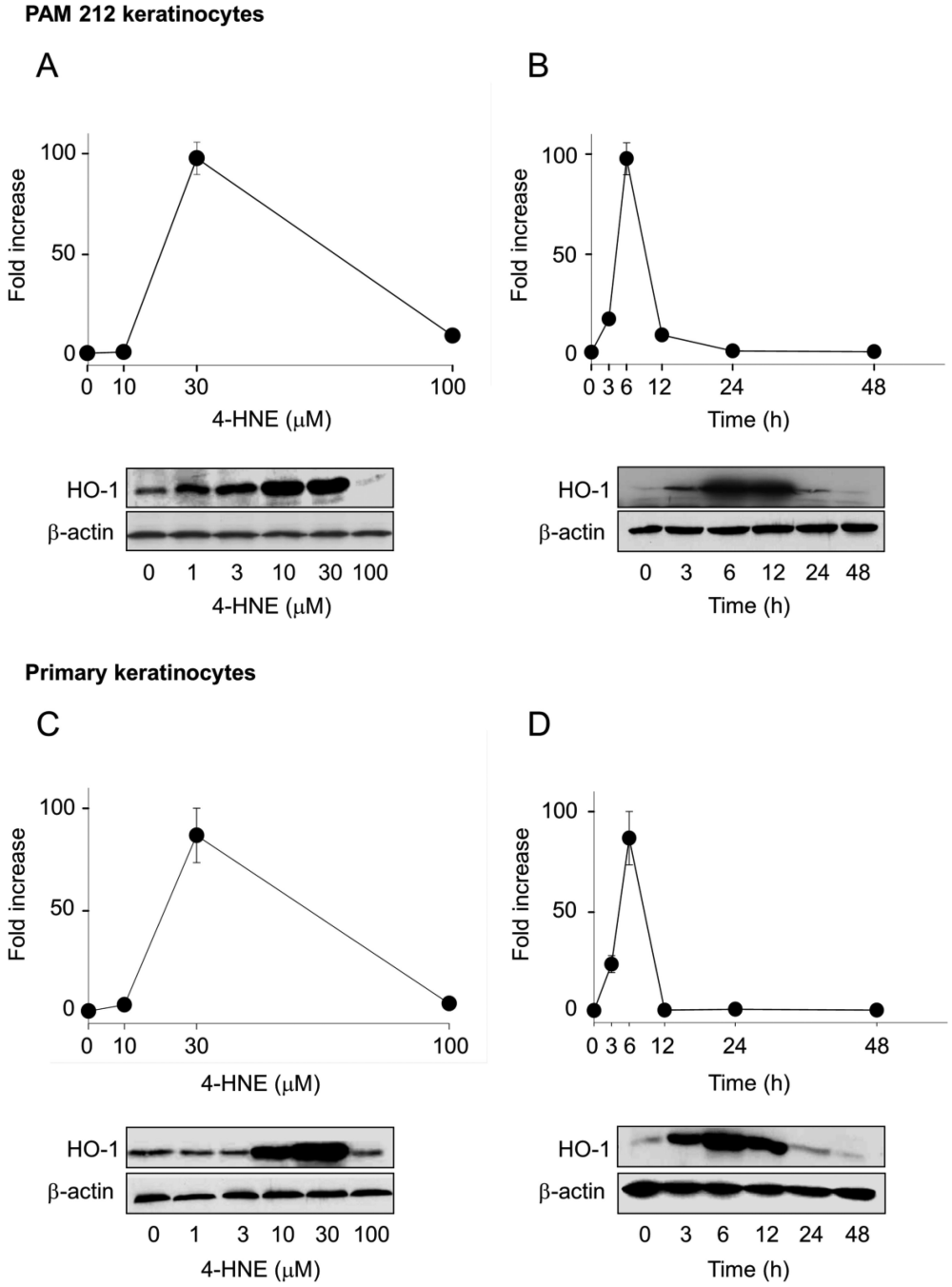

Figure 2. Effects of 4-HNE on HO-1 expression in keratinocytes

PAM 212 cells (panels A and B) or primary cultures of mouse keratinocytes (panels C and D) were treated with increasing concentrations of 4-HNE for 6 h (panels A and C) or with 30 μM 4-HNE for increasing periods of time (panels B and D). Cells were then analyzed for HO-1 mRNA and protein expression by real time PCR and Western blotting, respectively. In each panel, the upper figures show HO-1 mRNA expression, while the lower panels show Western blots for HO-1 protein expression. Expression of β-actin is shown in each of the

western blots as a control. Data for mRNA are expressed as fold increase relative to control. Bars represent the mean ± SE (n = 3).

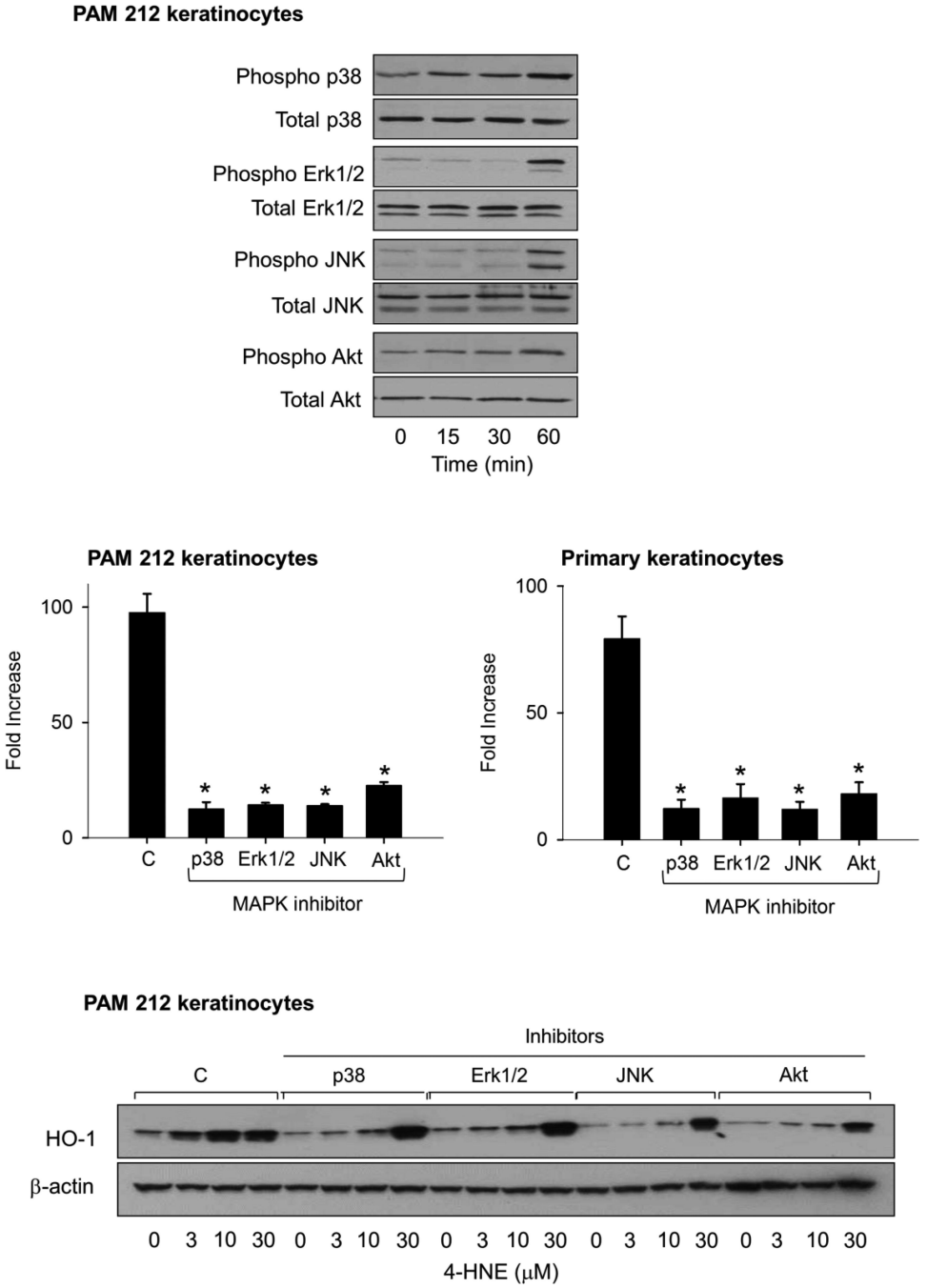

Figure 3. Role of MAP kinase and PI3K/Akt signaling in 4-HNE-induced HO-1 expression in keratinocytes

Upper panel. Effects of 4-HNE on expression of MAP kinases and PI3K/Akt. PAM 212 keratinocytes were treated with vehicle control or 30 μM 4-HNE for 15, 30 or 60 min. Cell lysates were prepared and analyzed for total and phosphorylated p38, JNK, Erk1/2, or PI3K/Akt by western blotting. Center panel. Effects of MAP kinase and PI3K/Akt inhibitors on 4-HNE-induced HO-1 mRNA expression. PAM 212 cells and primary mouse keratinocytes were pre-incubated for 3 h with inhibitors of p38 kinase (SB203580, 10 μM), JNK (SP600125, 20 μM), Erk1/2 (PD98059, 10 μM) or PI3K (wortmannin, 0.1 μM) and

then with 30 μM 4-HNE for additional 6 h. Expression of HO-1 mRNA was analyzed by real-time PCR and is expressed as fold-increase relative to vehicle control. Each bar represents the mean ± SE (n = 3). *Significantly different from control ($p < 0.05$). Lower panel. Effects of MAP kinase and PI3K/Akt inhibitors on 4-HNE-induced HO-1 protein expression in PAM 212 keratinocytes. Total cell lysates were prepared from control and treated cells and analyzed for HO-1 protein expression by Western blotting.

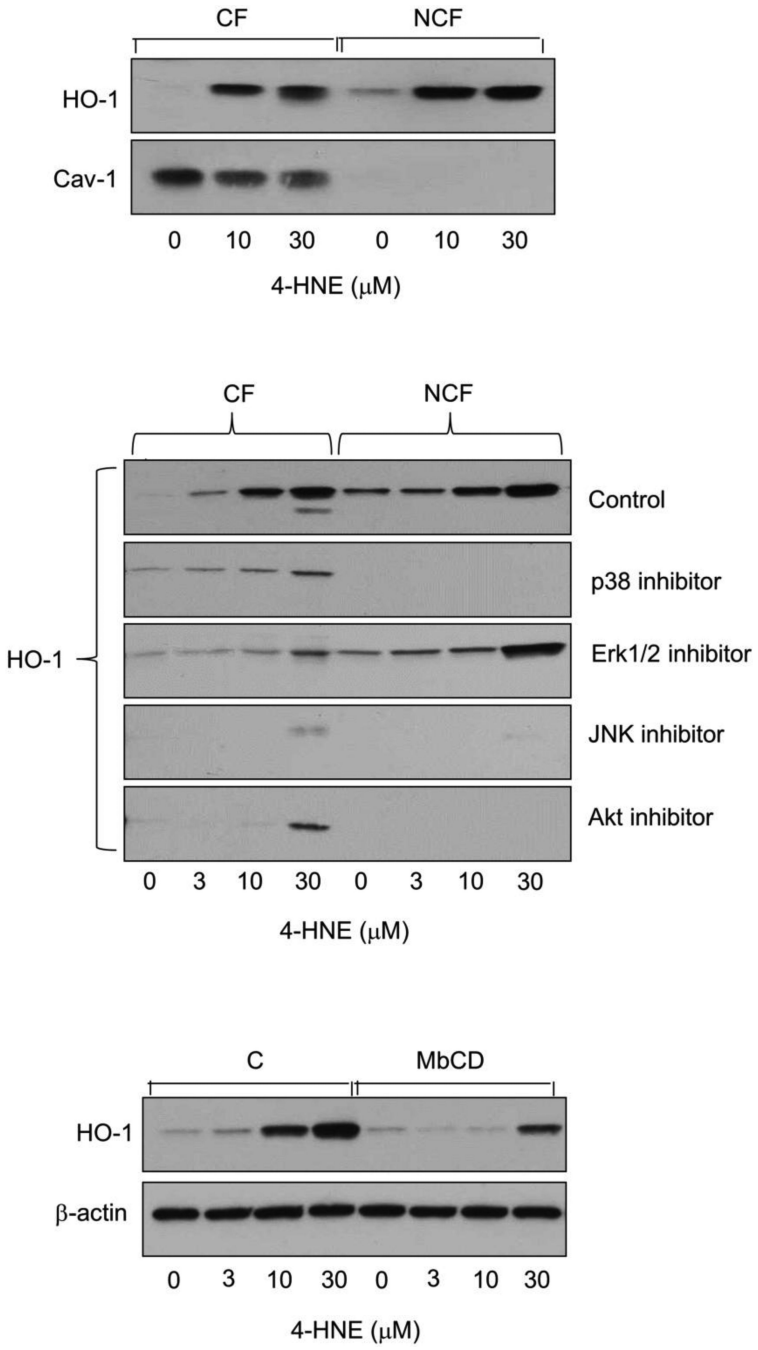

Figure 4. Localization of 4-HNE-induced HO-1 in caveolae

Upper panel. Distribution of HO-1 in caveolar fractions (CF) and non-caveolar fractions (NCF) of PAM 212 cells. Keratinocytes were treated with 0, 10 or 30 μM 4-HNE. After 6 h, caveolar and non-caveolar fractions were prepared and analyzed for HO-1 and caveolin-1 (Cav-1) protein expression by western blotting. Center panel. Effects of kinase inhibitors on expression of HO-1 in caveolar and non-caveolar fractions of PAM 212 cells. Keratinocytes were pre-incubated with MAP kinase and PI3K inhibitors and then 4-HNE (0, 3, 10 or 30 μM) as described in the legend to Figure 3. After an additional 6 h, caveolar and non-

caveolar fractions were prepared and analyzed for HO-1 protein by western blotting. Lower panel. Effects of cholesterol depletion on HO-1 expression. Cells were pretreated with 5 mM methyl-β-cyclodextrin (MbCD) or control (C) for 3 h and then with 4-HNE for 6 h. Total cell lysates were then prepared and analyzed for HO-1 protein expression by western blotting.

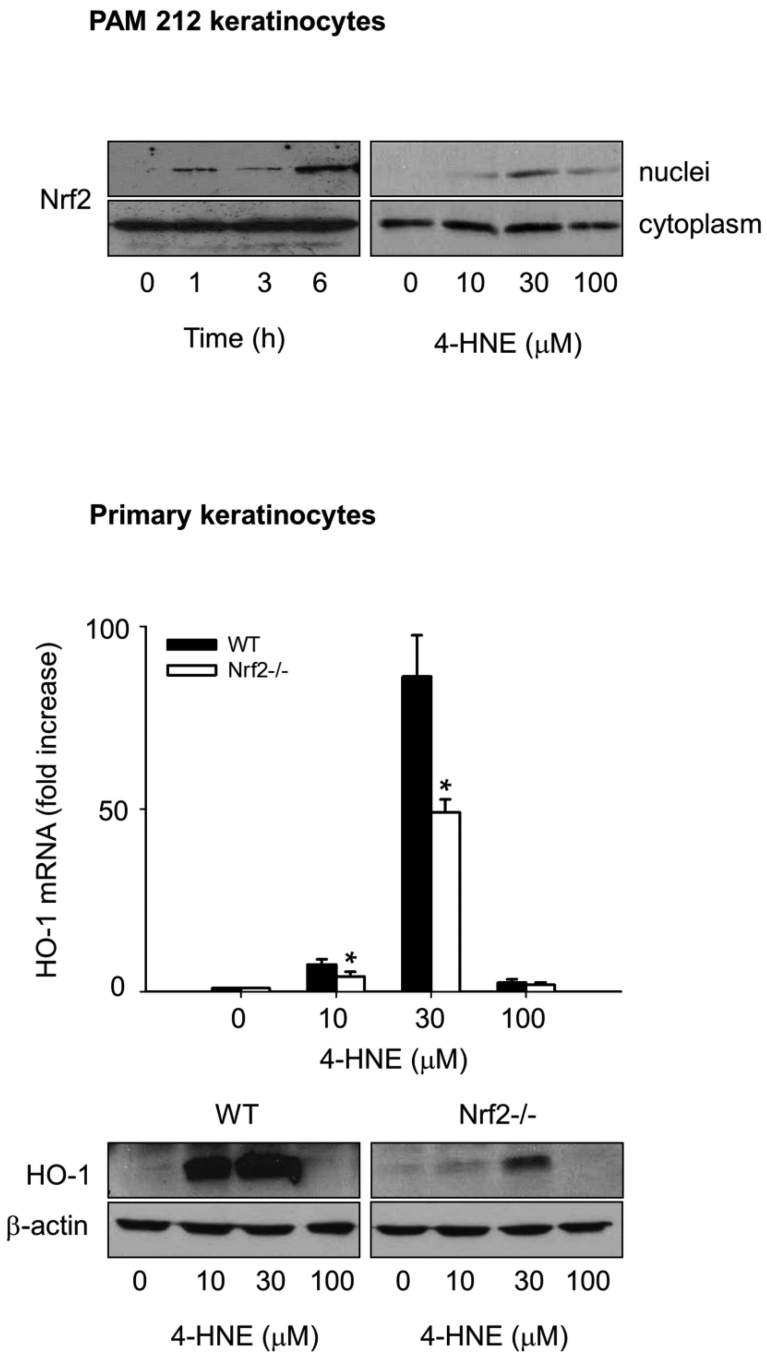

Figure 5. Role of Nrf2 in 4-HNE-induced HO-1 expression in mouse keratinocytes

Keratinocytes were incubated with control or 4-HNE and analyzed for Nrf2 or HO-1 expression. Upper panel. Effects of 4-HNE on nuclear localization of Nrf2. PAM 212 cells were treated with 30 μM 4-HNE for 0, 1, 3 and 6 h or 0, 10, 30 and 100 μM 4-HNE for 3 h. Nuclear and cytoplasmic fractions of the cells were then prepared and Nrf2 expression analyzed by western blotting. Lower panel. Effects of Nrf2 expression on 4-HNE-induced HO-1 expression. Primary keratinocytes from wild type and Nrf2−/− mice were treated with 0, 10, 30 and 100 μM 4-HNE for 6 h and expression of HO-1 mRNA (upper panel) and

protein (lower panel) analyzed by real-time PCR and Western blotting, respectively, as described in the Materials and Methods. HO-1 mRNA expression is presented as fold-increase relative to control. Each bar represents the mean ± SE (n = 3).

Table 1

Real-time PCR primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	TCA CCC ACA CTG TGC CCA TCT ACG A	GGA TGC CAC AGG ATT CCA TAC CCA
HO-1	ACC AGG GCA TCA AAA ACT TG	GCC CTG AAG CTT TTT GTC AG
GSTA1-2	CAG AGT CCG GAA GAT TTG GA	CAA GGC AGT CTT GGC TTC TC
GSTA3	GCA AGC CTT GCC AAG ATC AA	GGC AGG GAA GTA ACG GTT CC
GSTA4	CCC TTG GTT GAA ATC GAT GG	GAG GAT GGC CCT GGT CTG T
NQO1	ACG CCT GAG CCC AGA TAT TG	TCT GCA GCT TCC AGC TTC TTG
Catalase	ACC AGG GCA TCA AAA ACT TG	GCC CTG AAG CTT TTT GTC AG
GSTM1	CCT ACA TGA AGA GTA GCC GCT ACA T	TAG TGA GTG CCC GTG TAG CAA
GSTP1	CCT TGG CCG CTC TTT GG	GGC CTT CAC GTA GTC ATT CTT ACC
SOD	ACC AGT GCA GGA CCT CAT TTT AA	TCT CCA ACA TGC CTC TCT TCA TC

Table 2

Effects of 4-HNE on antioxidant and stress-related gene expression

Gene	PAM 212 keratinocytes[1]		Primary keratinocytes	
	6 h	24 h	6 h	24 h
	Fold increase			
HO-1	97.7 ± 8.2*	9.7 ± 0.3*	86.3 ± 13.3*	1.7 ± 0.2
NQO1	5.0 ± 0.8*	4.5 ± 1.4*	5.1 ± 0.8*	4.8 ± 0.5*
SOD	1.9 ± 0.7	1.5 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
Catalase	1.5 ± 0.2	1.0 ± 0.0	1.5 ± 0.3	2.8 ± 0.3*
GSTA1-2	3.9 ± 0.9*	63.1 ± 15.8*	3.0 ± 0.6*	30.6 ± 3.0*
GSTA 3	2.9 ± 0.5*	1.0 ± 0.3	2.6 ± 0.2*	4.1 ± 0.2*
GSTA 4	8.5 ± 0.3*	1.4 ± 0.4	2.8 ± 1.0	2.6 ± 0.3*
GSTP1	1.7 ± 0.4	2.4 ± 0.6	0.9 ± 0.2	2.4 ± 0.4
GSTM1	0.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.3	1.9 ± 0.2

mRNA was extracted 6 h and 24 h later and analyzed for gene expression by real time PCR. Data are presented as fold change relative to control. Each value represents the mean ± SE (n = 3). Asterisks show values that are significantly different from control ($p < 0.05$).

[1] Keratinocytes were treated with vehicle control or 30 μM 4-HNE.