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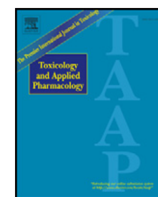

Modulation of keratinocyte expression of antioxidants by 4-hydroxynonenal, a lipid peroxidation end product

Ruijin Zheng [a], Diane E. Heck [b], Vladimir Mishin [a], Adrienne T. Black [a], Michael P. Shakarjian [b], Ah-Ng Tony Kong [a], Debra L. Laskin [a], Jeffrey D. Laskin [c,*]

[a] *Pharmacology and Toxicology and Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA*
[b] *Environmental Health Science, New York Medical College, Valhalla, NY, USA*
[c] *Environmental and Occupational Medicine, Rutgers University-Robert Wood Johnson Medical School, Piscataway, NJ, USA*

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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 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.

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Introduction

The skin is highly sensitive to oxidative stress induced environmental insults such as ultraviolet light, gamma radiation and various chemical toxicants (Black et al., 2008b; Isoir et al., 2006; 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 (Hirao and Takahashi, 2005; Tanaka et al., 2001), 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 (Black et al., 2010; Dhar et al., 2002; Haarmann-Stemmann et al., 2012; Keum et al., 2006; Marrot et al., 2008). 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

* Corresponding author at: Department of Environmental and Occupational Medicine, Rutgers University-Robert Wood Johnson Medical School, 170 Hoes Lane, Piscataway, NJ, USA. Fax: +1 732 445 0119.
E-mail address: jlaskin@eohsi.rutgers.edu (J.D. Laskin).

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reaction products such as peroxynitrite and various electrophilic nitrolipids can also upregulate oxidative stress responsive genes (Freeman et al., 2008; Szabo et al., 2007).

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 a l., 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 (ECL) kit 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, PD98059, 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., 2013). 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 3000 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 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 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 9000 ×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 (3000 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

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