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Regulation of keratinocyte expression of stress proteins and antioxidants by the electrophilic nitrofatty acids 9- and 10-nitrooleic acid

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Abstract

Nitric oxide and various by-products including nitrite contribute to tissue injury by forming novel intermediates via redox-mediated nitration reactions. Nitration of unsaturated fatty acids generates electrophilic nitrofatty acids such as 9-nitrooleic acid (9-NO) and 10-nitrooleic acid (10-NO), which are known to initiate intracellular signaling pathways. In these studies, we characterized nitrofatty acid-induced signaling and stress protein expression in mouse keratinocytes. Treatment of keratinocytes with 5–25 μ M 9-NO or 10-NO for 6 h upregulated mRNA expression of heat shock proteins (hsp) 27 and 70; primary antioxidants, heme oxygenase-1 (HO-1) and catalase; secondary antioxidants glutathione-S-transferase (GST) A1/2, GSTA3 and GSTA4; and Cox-2, a key enzyme in prostaglandin biosynthesis. The greatest responses were evident with HO-1, hsp27 and hsp70. In keratinocytes, 9-NO activated JNK and p38 MAP kinases. JNK inhibition suppressed 9-NO induced HO-1, hsp27 and hsp70 mRNA and protein expression, while p38 MAP kinase inhibition suppressed HO-1. In contrast, inhibition of constitutive expression of ERK1/2 suppressed only hsp70 indicating that 9-NO modulates expression of stress proteins by distinct mechanisms. 9-NO and 10-NO also upregulated expression of caveolin-1, the major structural component of caveolae. Western blot analysis of caveolar membrane fractions isolated by sucrose density centrifugation revealed that HO-1, hsp27 and hsp70 were localized within caveolae following nitrofatty acid treatment of keratinocytes suggesting a link between induction of stress response proteins and caveolin-1 expression. These data indicate that nitrofatty acids are effective signaling molecules in keratinocytes. Moreover, caveolae appear to be important in the localization of stress proteins in response to nitrofatty acids.

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Keywords

Nitrooleic acid; Skin; Nitric oxide; Heat shock proteins; Heme oxygenase-1; Free radicals

Introduction

It is becoming increasingly apparent that nitration products of unsaturated fatty acids represent an important class of endogenous biological mediators [1, 2]. Generated in nitric oxide-dependent oxidative reactions, several of these lipid products are electrophilic fatty acid nitroalkenes including nitrooleic acid and nitrolinoleic acid derivatives [3]. These fatty acids can react via Michael additions across carbon-carbon double bonds forming adducts with many cellular components, most notably, proteins [4]. By reacting with signaling proteins, nitrooleic acids and nitrolinoleic acids can regulate their function and control gene expression [5]. Electrophilic nitrofatty acids are formed in cells under conditions of nitrosative stress; they have been reported to inhibit expression of inflammatory genes and upregulate expression adaptive response genes, many of which are important in protecting cells against stress-induced injury and tissue damage [6]. Beneficial effects of nitrofatty acids have been described in several animal models of cardiovascular, inflammatory and metabolic diseases [7–9].

Earlier studies by our laboratory showed that mouse and human keratinocytes upregulate inducible nitric oxide synthase and generate nitric oxide in response to inflammatory mediators. We also demonstrated that nitric oxide is important in the control of wound healing [10]. Nitric oxide also controls keratinocyte proliferation [11], while in human skin, it plays a key role in regulating cellular responses in diseases states such as psoriasis [12, 13], as well as to infections, heat, ultraviolet light and wounding [14–16]. The aim of the present studies was to analyze the response of keratinocytes to the nitrofatty acids 9-nitrooleic acid (9-NO) and 10-nitrooleic acid (10-NO). We found that both nitrofatty acids upregulated expression of antioxidants and stress proteins. Moreover, some of these responses were regulated by mitogen activated protein kinases and caveolae. Coordinate regulation of expression of antioxidants and adaptive genes are likely to be important in mediating nitric oxide-induced inflammation and tissue injury.

Material and methods

Materials

Rabbit anti-heme oxygenase-1 (HO-1) polyclonal antibody was from Stressgen Biotechnology (Victoria, BC, Canada). Rabbit polyclonal caveolin-1 antibody, goat polyclonal cyclooxygenase-2 (COX-2) and heat shock protein (hsp) 27 antibodies, and rabbit polyclonal hsp70 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal heat shock factor-1 (HSF-1), caveolin-1, p38, phospho-p38, JNK, phospho-JNK, Erk1/2, and phospho-Erk1/2 antibodies were from Cell Signaling Technology (Beverly, MA). HRP conjugated goat anti-rabbit antibody, rabbit anti-goat secondary antibody, and the DC (Detergent Compatible) protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA). The Western Lightning enhanced chemiluminescence kit

(ECL) was from Perkin Elmer Life Sciences (Boston, MA). NE-PER nuclear and cytoplasmic extraction reagents were from Thermo Scientific (Rockford, IL) and SYBR Green Master Mix and other PCR reagents from Applied Biosystems (Foster City, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen Corp. (Carlsbad, CA). PD 98059 and SP600125 were from Calbiochem (La Jolla, CA). 9-NO and 10-NO were from Cayman Chemical (Ann Arbor, MI). SB203580, protease inhibitor cocktail containing 4-(2 aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin, methyl- β -cyclodextrin (MbCD), Tri Reagent and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell cultures and treatments

PAM212 keratinocytes were obtained and maintained as previously described [17]. The cells were originally prepared from primary keratinocytes isolated from BALB/c mice [18]. For all experiments, cells were cultured in DMEM containing 10% FBS supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were seeded into either 6-well plates (1×10^6 cells/well) or 10 cm plates (5×10^6 cells/plate) and incubated at 37°C in a 5% CO₂ incubator. After reaching ~90% confluence, cells were treated with vehicle control or increasing concentrations of freshly prepared 9-NO or 10-NO (5–25 μ M). For protein analysis, treated cells, grown in 6-well dishes, were lysed by the addition of 300 μ l 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 then centrifuged ($100 \times g$, 5 min at 4°C). Supernatants were then analyzed by Western blotting. Cells were prepared for mRNA analysis as previously described [19].

For kinase inhibition experiments, cells were pretreated with the p38 MAP kinase inhibitor, SB203580 (10 μ M), the JNK kinase inhibitor, SP600125 (20 μ M), or the ERK1/2 kinase inhibitor, PD98059 (10 μ M) for 3 hr. Nitrooleic acids or vehicle control was then added to the medium. After 6 hr, the cells were removed from the plates using a scraper and centrifuged at $1000 \times g$ for 10 min. Cells were then analyzed for mRNA and protein expression by real-time PCR and Western blotting, respectively. For analysis of cytoplasmic and nuclear expression of HSF-1, cell pellets (~20 μ l packed volume) were resuspended in 200 μ l ice-cold cytoplasmic extraction reagent (Thermo Scientific) in Eppendorf centrifuge vials and centrifuged for 5 min at $16,000 \times g$. Supernatants were immediately transferred to clean pre-chilled tubes and the nuclear fractions extracted from the pellets by adding 100 μ l ice-cold nuclear extraction reagent. Samples were stored at –70°C until analysis.

Isolation of caveolae

Caveolar fractions of cells were prepared as described by Smart et al. [20]. Briefly, treated cells were washed three times with PBS, scraped into 5 ml sucrose buffer (0.25 M sucrose, 1 mM EDTA, and 20 mM Tris, pH 7.8) and centrifuged at 1400 g for 5 min. 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 $1000 \times g$ at 4°C. Supernatants were collected and the homogenization process repeated with cell pellets. After combining the supernatants, 2 ml were carefully layered on top of 8 ml of

a 30% Percoll solution in sucrose buffer and centrifuged for 30 min at $84,000 \times g$ in a Ti 70 rotor using an L7–55 Beckman ultracentrifuge (Brea, CA) to separate caveolae containing plasma membrane fractions. Fractions were collected and stored at -70°C until analysis.

Western blotting

Protein concentrations of total cell lysate, nuclear and cytoplasmic fractions, and caveolae and non-caveolae fractions were quantified using the DC protein assay kit with bovine serum albumin as the standard [21]. Samples (15 $\mu\text{g}/\text{well}$) were then electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking in 5% milk in Tris buffer at room temperature, the blots were incubated overnight at 4°C with HO-1 antibody (1:1000), hsp27 antibody (1:200), hsp70 antibody (1:400), β -actin antibody (1:3000), caveolin-1 antibody (1:1000), Cox-2 antibody (1:500), HSF-1 antibody (1:500), or MAP kinase antibodies (1:1000), washed with tTBS (Tris-buffered saline supplement with 0.1% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies. After 1 hr at room temperature, proteins were visualized by ECL chemiluminescence.

Real-time PCR

Total RNA was isolated from the cells using the Tri Reagent as previously described by [19]. 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 generated from serial dilutions of cDNA mixtures of all the samples. Real-time PCR was conducted on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) 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 the oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). A mouse β -actin house-keeping gene was used to normalize all the values. The forward (5'-3') and reverse (5'-3') primers used are listed in Table 1.

Statistical analysis

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

Results

Effects of nitrooleic acids on antioxidants and stress proteins

9-NO and 10-NO were found to upregulate expression of HO-1, hsp27, hsp70, Cox-2, and the glutathione-S-transferases GSTA1-2, GSTA3 and GSTA4 in a generally similar manner in the range of 5–25 μM (Table 2). HO-1 (9–55 fold) was most responsive, followed by hsp70 (5–34 fold), hsp27 (9–20 fold), and Cox-2 (5–9 fold). GSTA1-2 (3–18 fold), GSTA3 (5–19 fold) and GSTA4 (3–19 fold) also showed similar upregulation in response to the nitrooleic acids. Catalase was only induced by 10 μM and 25 μM 10-NO (4–6 fold).

Both 9-NO and 10-NO also upregulated HO-1 protein in time- and concentration-dependent manners (Figure 1 and Figure 2, panel A); this was maximal 6 hr after treatment with 25 μ M nitrooleic acids. The unmodified fatty acid, oleic acid, which cannot undergo a Michael addition, had no effect on HO-1 (Figure 1, panel A). 9-NO and 10-NO also upregulated protein expression for Cox-2, hsp27, hsp70 (Figure 2). Whereas induction of HO-1, hsp27 and hsp70 was similar for 9-NO and 10-NO, Cox-2 expression was more responsive to 10-NO.

Previous work has shown that heat shock proteins and HO-1 are regulated by the transcription factor HSF-1 [22, 23]. We found that HSF-1 was largely localized in the cytoplasm of PAM212 keratinocytes. A marked increase in nuclear localization of HSF-1 was noted after treatment of the cells with 9-NO or 10-NO (Figure 3). These effects were time-dependent in the range of 0.5 ~ 2 hr.

Role of MAP kinase signaling in nitrooleic acid induced expression of HO-1, hsp27 and hsp70

We next analyzed the effects of the nitrofatty acids on MAP kinase activation in keratinocytes. We found that 10 μ M 9-NO induced p38 and JNK phosphorylation, with no effects of expression of total p38 or total JNK protein expression (Figure 4). In contrast, Erk1/2 kinase was constitutively activated in the cells and 9-NO did not change this activity. To evaluate the role of MAP kinases in induction of HO-1, hsp27 and hsp70 we used SB203580, a p38 kinase inhibitor, SP600125, a JNK inhibitor, and PD98059, an Erk1/2 inhibitor. JNK inhibition suppressed 9-NO-mediated increases in mRNA and protein expression for all three proteins, while p38 kinase inhibition only suppressed the induction of HO-1, and Erk1/2 inhibition only suppressed hsp70 (Figure 5).

Role of caveolae in nitrooleic acid-induced protein expression

Previous studies showed that caveolae regulate expression of adaptive response genes including hsp27 and hsp70 [24]. We found that caveolar fractions, but not non-caveolar fractions, of keratinocytes contained caveolin-1, the major structural protein in caveolae (Figure 6). Interestingly, treatment of the cells with 9-NO or 10-NO increased expression of caveolin-1 (Figure 6). Constitutive levels of hsp27 and hsp70, but not HO-1, were identified in non-caveolar fractions of control keratinocytes. Treatment with 9-NO and 10-NO selectively increased expression of hsp27 and hsp70 in caveolar fractions of the cells. In contrast, HO-1 was induced by 9-NO and 10-NO in both caveolar and non-caveolar fractions of the cells. When the cells were treated with an inhibitor that disrupts caveolae (MbCD), both control and induced expression of hsp27 and HO-1 were suppressed, while only control levels of hsp70 were expressed in inhibitor-treated cells. Neither 9-NO nor 10-NO altered expression of hsp70 in MbCD-treated cells.

Discussion

Cells adapt to stress by generating mediators that protect against injury and promote wound repair [25, 26]. It is well recognized that skin and skin-derived cells including keratinocytes can generate excessive amounts of nitric oxide following injury or in response to cytokines,

processes that can lead to nitrosative stress [27]. Nitrofatty acids are known to be generated in human tissues [28, 29] following nitrosative stress. By stimulating expression of adaptive proteins and antioxidants and/or inhibiting cytokine signaling, these reactive lipids function as antiinflammatory agents [1]. Sulfhydryl residues in proteins in different cell compartments including membranes, cytoplasm, mitochondria and nucleus that regulate signal transduction are highly susceptible to modification by nitrofatty acids and mediate gene expression changes [30]. The present studies demonstrate that keratinocytes respond to nitrofatty acids by synthesizing a number of adaptive proteins that are important in protecting cells against stressors. Thus, 9-NO or 10-NO effectively induce keratinocyte expression of heat shock proteins, antioxidant enzymes, enzymes that generate antioxidants, and enzymes that detoxify reactive oxygen species (e.g., HO-1, catalase and GST's), and Cox-2, which generates eicosanoids. These findings are consistent with a protective role of nitrofatty acids in keratinocytes.

In keratinocytes, HO-1 and catalase are upregulated by stressors that stimulate nitric oxide production including ultraviolet light, paraquat and heavy metals [19, 21, 31]. Similarly, we observed increased expression of HO-1, catalase and GST's following nitrofatty acid stimulation of keratinocytes. HO-1 is a key cellular antioxidant and in mouse skin, its expression has been shown to accelerate wound healing [32]. Nitrofatty acids have been reported to induce HO-1 in the vasculature of mice, a process that is thought to contribute to their ability to exert antiinflammatory activity and protect against vascular injury [2, 33] and we speculate that it plays a similar role in keratinocytes. Catalase detoxifies hydrogen peroxide, effectively reducing oxidative stress. Increased expression of catalase in keratinocytes and mouse skin has been shown to protect against hydrogen peroxide-induced damage as well as UVB-induced apoptosis [34–36]. In mouse skin catalase also stimulates proliferation of keratinocytes surrounding excisional dermal wounds [37]. We also found that 9-NO and 10-NO upregulated keratinocyte GSTA1-2, GSTA3 and GSTA4. These mediate the conjugation of glutathione to oxidized cellular macromolecules, facilitating their elimination and limiting tissue injury [38, 39]. In addition, GSTA enzymes terminate lipid peroxidation chain reactions by removing hydroperoxides and aldehydes generated during oxidative stress [38, 39]. Our findings are consistent with previous work showing that oxidative stressors including paraquat and UVB light effectively up regulate these GST's in mouse keratinocytes [19, 21]. These enzymes likely act in concert with HO-1 and catalase to limit oxidative and nitrosative stress and promote wound healing.

The nitrofatty acids were also found to upregulate keratinocyte expression of mRNA and protein for Cox-2. At present it is unclear whether this contributes to injury or repair as both pro- and antiinflammatory eicosanoids are generated via Cox-2 from prostaglandin (PG) H₂ [40, 41]. Of particular interest is the antiinflammatory eicosanoid 15-deoxy ^{12,14} PGJ₂ [42]. UVB light has been shown to stimulate production of PGJ₂ by mouse keratinocytes [42]. It remains to be determined if antiinflammatory prostaglandins are produced in keratinocytes following nitrofatty acid treatment, and the extent to which they play a role in ameliorating skin inflammation. However, as PGE₂ generated via Cox-2 is an important mediator of skin inflammation [42], one cannot exclude the possibility that nitrofatty acids also contribute to the proinflammatory activity of nitrosative stress.

Hsp's are molecular chaperones upregulated following oxidative stress [43]. They function to protect cells against injury and facilitate the resolution of inflammation and wound healing. In the skin, hsp's have also been shown to enhance tissue repair [44]. In previous studies, we demonstrated that hsp27 and hsp70 are rapidly induced in mouse and human keratinocytes by dermal vesicants which induce oxidative and nitrosative stress [24]. Similarly, we found that 9-NO and 10-NO effectively upregulated keratinocyte mRNA and protein for hsp27 and hsp70. Both hsp's are important in maintaining the integrity of proteins; they also function as antioxidants, and play key roles in protecting cells against apoptosis and cell damage [45–48]. Our data are consistent with earlier studies demonstrating that nitrofatty acids can upregulate hsp's and related proteins, providing further support for the idea that these proteins are important in protecting cells against nitrosative stress [49].

A question arises as to the mechanism by which nitrofatty acids modulate expression of antiinflammatory/adaptive response proteins in keratinocytes. Previous work from our laboratory and others has shown that expression of many of the stress related proteins are regulated, at least in part, by MAP kinase signaling [17, 50]. The present studies demonstrate that 9-NO activated JNK and p38 MAP kinases in keratinocytes. These data are in accord with reports showing increases in MAP kinase activity in response to other Michael acceptors, including 4-hydroxynonenal in lung microvascular endothelial cells and epithelial cells [51, 52]. Our findings that JNK inhibition suppressed 9-NO-induced HO-1, hsp27 and hsp70 expression provide support for a role of this MAP kinase in regulating the activity of nitrofatty acids. We also found that inhibition of p38 MAP kinase suppressed 9-NO-induced HO-1 expression, while ERK1/2 inhibition suppressed hsp70 expression. These data indicate that 9-NO regulates expression of adaptive response genes by distinct mechanisms. The intracellular signaling pathways leading to 9-NO activation of MAP kinase activity are not known. 9-NO may directly interact with the kinases to control their activity [4, 53] or it may trigger upstream signaling pathways that activate MAP kinase signaling [53].

It should be noted that additional regulatory pathways have been identified by which nitrofatty acids modulate gene expression. For example, the Nrf2/Keap-1 pathway is known to be important in mediating protection against electrophilic and oxidative stress by induction of phase 2 enzymes including the GST's [54]. In human endothelial cells, nitrofatty acids have been reported to act via Nrf2/Keap-1 signaling, which controls expression of adaptive response genes including HO-1, NQO1 and GSH biosynthetic enzymes [33, 49, 55, 56]. Nitrofatty acids also activate hypoxia inducible factor (HIF) signaling and regulate HIF-1 α target genes in human endothelial cells [57], as well as peroxisome proliferator-activating receptors [33, 58]. In contrast, nitrofatty acids inhibit LPS-induced NF- κ B signaling in mouse macrophages and aorta's, a process that may be important in controlling inflammation [7].

Another important pathway regulating expression of hsp's is the transcription factor HSF-1. Localized in the cytoplasm under physiological conditions, HSF-1 translocates to the nucleus under conditions of stress [59]. Trimerization and phosphorylation regulates the transcription of HSF-1 sensitive genes [60]. We found that mouse keratinocytes

constitutively express HSF-1 in the cytoplasm, and to a lesser extent, the nucleus. Treatment of keratinocytes with 9-NO or 10-NO reduced cytoplasmic expression of HSF-1, increasing its nuclear expression, suggesting that HSF-1 mediates, at least in part, the action of the nitrofatty acids. Cytoplasmic localization of HSF-1 is thought to be controlled by its association with hsp70 and hsp-90 [61, 62]. This is in accord with our findings of constitutive expression of hsp70 in the keratinocytes. Nitrofatty acids may function by binding to hsp70 or a related protein, a process that could cause it to dissociate from and activate HSF-1 [53]. Binding to hsp's has been described for other electrophiles including 4-hydroxynonenal which stimulate nuclear translocation of HSF-1 [63–65]. Further studies are needed to determine the relative contributions of HSF-1, MAP kinases and other signaling molecules in controlling the expression of adaptive stress response proteins in keratinocytes.

Caveolae are specialized membrane lipid rafts that function to control a variety of biochemical signaling molecules regulating growth and differentiation [66]. Cav-1 is the major structural protein in caveolae [67]. Basal keratinocytes in mouse and human skin strongly express Cav-1 [68, 69]. Basal cell proliferation is increased in mice lacking Cav-1, along with susceptibility to carcinogens [70–72]. Cav-1 $-/-$ mice are also more sensitive to phorbol ester-induced epidermal hyperplasia, as well as the development of papillomas in a two stage mouse skin carcinogenesis model [71]. Thus, Cav-1/caveolae play dynamic roles in regulating epidermal homeostasis and responses to environmental stimuli. Our data demonstrate that nitrofatty acids effectively induce Cav-1 protein in keratinocytes. Similar upregulation of Cav-1 has been described after treatment of keratinocytes with the vesicant, 2-chloroethyl ethyl sulfide [24]. Increased Cav-1 expression may be an important adaptive response that facilitates the sequestration of signaling molecules regulating production of inflammatory mediators. This may contribute to the antiinflammatory actions of the nitrofatty acids. Our data also show that low constitutive expression of hsp27 and hsp70 in keratinocytes is largely in non-caveolar fractions of the cells, with minimal constitutive expression of HO-1. Of note, increases in hsp27, hsp70 and HO-1 in response to nitrofatty acids were largely associated with caveolae. In this regard, earlier work has shown that cellular stressors can induce HO-1 or hsp70 expression in lipid rafts and caveolae in several cell types including mouse mesangial cells, human epithelial cells and rat endothelial cells [73–75]. The function of HO-1 and hsp's in caveolae is unknown. Caveolae contain not only HO-1, but also other heme degrading enzymes including biliverdin reductase [76]. It is possible that localization of HO-1 in caveolae is important in the generation of antioxidants that are important in protecting their structural integrity. Hsp's function to support the folding and transport of proteins [77], which may be important in protecting caveolae from nitrosative stress. Caveolae are also known to transport hsp's into the extracellular environment where they play a role in immune regulation [73, 78]. This may be important in controlling inflammatory reactions and protecting against stress-induced damage [78, 79]. Also of note is our finding that caveolae regulate nitrofatty acid-induced expression of stress proteins. Thus, suppression of caveolae by MbCD blocked 9-NO and 10-NO-induced increases in HO-1 and hsp's. Similar results have been described with vesicants and heat shock [24, 74]. Based on these data, a reduction in caveolae would be expected to reduce nitrofatty acid-induced antiinflammatory activity. In this regard, increased inflammatory cell

infiltration has been observed in mouse skin from Cav-1 $-/-$ mice following treatment with a phorbol ester tumor promoter [71], which is known to induce nitrosative stress [80–82].

In summary, our data show that concentrations of nitrofatty acids that occur under physiological or pathological conditions upregulate expression of genes that are important in regulating stress-induced damage in keratinocytes. That many of these genes are important in the control of inflammation is consistent with an emerging literature indicating that these electrophilic lipids are antiinflammatory, and that their production following nitrosative stress may be important in protecting against tissue injury. Our data also adds to the understanding of the signaling pathways by which nitrofatty acids regulate gene expression. A particularly novel aspect of our work is the identification of HO-1 and hsp's in keratinocyte caveolae, and their response to 9-NO and 10-NO. These data support the notion that caveolae are important in sequestering stress induced proteins, as well as regulating their expression. Taken together, these findings further support the idea that nitrofatty acids function as signaling molecules to regulate cellular responses to nitrosative stress.

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Highlights

- Nitric oxide-dependent reactions with lipids generate electrophilic nitrooleic acids.
- Electrophilic nitrooleic acids induce antioxidant proteins in mouse keratinocytes.
- Induction of antioxidant proteins was regulated in part via MAP kinases and caveolae.
- Nitrooleic acids are effective signaling molecules in keratinocytes.
- Nitrofatty acids may be important in regulating skin inflammation.

Figure 1. Effects of nitrooleic acids on HO-1 expression

Keratinocytes were treated with vehicle control (C), 10 μ M oleic acid (OA), or 10 μ M 9-NO for 6 hr (Panel A) or with control (C) or 10 μ M 9-NO or 10-NO for 0–6 hr (Panels B and C, respectively). Cell lysates were prepared and analyzed for HO-1 expression by western blotting. β -actin was used as a control. One representative blot from 3 experiments is shown

Figure 2. Effects of nitrooleic acids on stress-related gene expression

mRNA or protein was extracted from keratinocytes treated with 0, 5, 10 or 25 μ M nitrooleic acids for 6 hr. Samples were then analyzed by quantitative RT-PCR (upper panels) or western blotting (lower panels) for HO-1, Cox-2, hsp27 or hsp70 gene and protein expression. Bars represent the mean $\pm$ SE ($n = 3$). *Significantly different from control ($P < 0.05$). All increases in HO-1 and hsp27 by 9-NO and 10-NO were significant when compared to control ($p < 0.05$). Increases in Cox-2 were significant following treatment with 10 μ M 9-NO and 10 μ M and 25 μ M 10-NO. Increases in hsp70 were significant at all concentrations of 9-NO and 10 μ M and 25 μ M 10-NO.

Figure 3. Effects of nitrooleic acids on HSF-1 activation

Cells were treated with control (C), 10 μ M 9-NO, or 10 μ M 10-NO for 0.5, 1 or 2 hr. Nuclear and cytoplasmic fractions were prepared and analyzed for HSF-1 expression by western blotting. One representative blot from 3 experiments is shown.

Figure 4. Effects of 9-nitrooleic acid on MAP kinase activation

Keratinocytes were treated with control (C) or 10 μ M 9-NO for 5 min or 15 min. Cell lysates were prepared and analyzed for total or phosphorylated MAP kinases by western blotting. One representative blot from 3 experiments is shown.

Figure 5. Role of MAP kinase signaling in 9-NO-induced expression of HO-1 and hsp's
Keratinocytes were pre-incubated with inhibitors of p38 (SB203580, 10 μ M), JNK (SP600125, 20 μ M), or Erk1/2 (PD98059, 10 μ M) for 3 hr and then with 9-NO for additional 6 hr. Cell lysates were prepared and analyzed for mRNA (left panels) or protein expression (right panels) by real time PCR and western blotting, respectively. Bars represent the mean $\pm$ SE ($n = 3$). Changes in HO-1 mRNA expression were only significant with the p38 and JNK inhibitors ($p < 0.05$). Changes in hsp27 mRNA expression were only significant with the JNK inhibitor. Changes in mRNA expression of hsp70 were only significant with the JNK and Erk1/2 inhibitors.

Figure 6. Localization of nitrooleic acid-induced proteins in caveolae

Upper panels: Keratinocytes were treated with 0, 5 μ M or 10 μ M 9-NO or 10-NO. After 6 hr, caveolar fractions (CF) and non-caveolar fractions (NCF) were prepared and analyzed for protein expression by western blotting. Lower panels: Cells were pretreated with MbCD (5 mM) or control (C) for 30 min and then with nitrooleic acids. After 6 hr, total cell lysates were prepared and analyzed by western blotting. Representative blots from 3 separate experiments are shown.

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
Cox-2	CAT TCT TTG CCC AGC ACT TCA C	GAC CAG GCA CCA GAC CAA AGA C
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
Catalase	ACC AGG GCA TCA AAA ACT TG	GCC CTG AAG CTT TTT GTC AG
Hsp27	AAG GAA GGC GTG GTG GAG AT	TTC GTC CTG CCT TTC TTC GT
Hsp70	CAG CGA GGC TGA CAA GAA GAA	GGA GAT GAC CTC CTG GCA CT

Table 2

Effects of nitrooleic acids on gene expression in mouse keratinocytes.

	9-NO ¹			10-NO		
	5 μ M	10 μ M	25 μ M	5 μ M	10 μ M	25 μ M
HO-1	9.5 $\pm$ 4.2 *	54.0 $\pm$ 4.2 *	44.0 $\pm$ 4.6 *	2.5 $\pm$ 0.3 *	40.6 $\pm$ 0.2 *	77.4 $\pm$ 9.2 *
Hsp27	12.3 $\pm$ 1.3 *	18.1 $\pm$ 3.3 *	9.4 $\pm$ 0.9 *	10.5 $\pm$ 0.8 *	18.2 $\pm$ 2.1 *	19.2 $\pm$ 4.8 *
Hsp70	5.0 $\pm$ 0.5 *	6.0 $\pm$ 0.6 *	29.7 $\pm$ 4.6 *	1.5 $\pm$ 0.5 *	11.2 $\pm$ 3.1 *	33.4 $\pm$ 6.5 *
Cox-2	2.7 $\pm$ 0.9	5.0 $\pm$ 1.2 *	1.9 $\pm$ 0.2	0.9 $\pm$ 0.1	5.3 $\pm$ 1.5 *	9.4 $\pm$ 1.6 *
Catalase	1.8 $\pm$ 0.1	2.8 $\pm$ 0.7	1.7 $\pm$ 0.2	1.7 $\pm$ 0.6	5.7 $\pm$ 1.2 *	4.0 $\pm$ 1.4 *
GSTA1-2	2.1 $\pm$ 0.9	14.0 $\pm$ 3.4 *	3.7 $\pm$ 1.6 *	5.7 $\pm$ 0.7 *	17.9 $\pm$ 1.2 *	17.9 $\pm$ 1.1 *
GSTA 3	5.0 $\pm$ 0.2 *	9.6 $\pm$ 1.3 *	7.3 $\pm$ 1.1 *	5.4 $\pm$ 0.8 *	17.0 $\pm$ 3.4 *	18.3 $\pm$ 5.0 *
GSTA 4	2.9 $\pm$ 0.5 *	11.2 $\pm$ 2.0 *	6.8 $\pm$ 1.7 *	4.4 $\pm$ 0.7 *	18.7 $\pm$ 4.2 *	1.7 $\pm$ 0.1

¹ Keratinocytes were treated with control, 5 μ M, 10 μ M or 25 μ M 9-NO or 10-NO for 6 hr. mRNA was isolated and analyzed for gene expression by quantitative RT-PCR. Data are presented as fold change in gene expression relative to control. Values are means $\pm$ SE (n=3).

* Significantly (p< 0.05) different from control.