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Original Contribution

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 μM 9-NO or 10-NO for 6 h upregulated mRNA expression of heat shock proteins (hsp's) 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, whereas 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 after 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 seem to be important in the localization of stress proteins in response to nitrofatty acids.

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 of 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], whereas 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

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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 (MAP) 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). Goat polyclonal cyclooxygenase-2 (Cox-2), and heat shock protein (hsp) antibodies, goat polyclonal hsp27 antibody and rabbit polyclonal hsp70 antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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, USA). Horseradish peroxidase-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, USA). The Western Lightning enhanced chemiluminescence (ECL) kit was from PerkinElmer Life Sciences (Boston, MA, USA). NE-PER nuclear and cytoplasmic extraction reagents were from Thermo Scientific (Rockford, IL, USA) and SYBR Green Master Mix and other PCR reagents from Applied Biosystems (Foster City, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen Corp. (Carlsbad, CA, USA). PD98059, and SP600125 were from Calbiochem (La Jolla, CA, USA). 9-NO and 10-NO were from Cayman Chemical (Ann Arbor, MI, USA). 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, USA).

Cell culture 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 μg/ml streptomycin. Cells were seeded into either six-well plates (1×10^6 cells/well) or 10-cm plates (5×10^6 cells/plate) and incubated at 37 °C in a 5% CO_2 incubator. After reaching ~90% confluence, cells were treated with vehicle control or increasing concentrations of freshly prepared 9-NO or 10-NO (5–25 μM). For protein analysis, treated cells, grown in six-well dishes, 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 then centrifuged (100*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 μM), the JNK kinase inhibitor SP600125 (20 μM), or the Erk1/2 kinase inhibitor PD98059 (10 μM) for 3 h. Nitrooleic acid or vehicle control was then added to the medium. After 6 h, the cells were removed from the plates and centrifuged at 1000*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 μl packed volume) were resuspended in 200 μl ice-cold cytoplasmic extraction reagent (Thermo Scientific) in Eppendorf centrifuge vials and centrifuged for 5 min at 16,000*g*. Supernatants were immediately transferred to clean prechilled tubes and the nuclear fractions extracted from the pellets by adding 100 μl 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 phosphate-buffered saline, 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*g* at 4 °C. Supernatants were collected and the homogenization process was repeated with cell pellets. After the supernatants were combined, 2 ml was carefully layered on top of 8 ml of a 30% Percoll solution in sucrose buffer and centrifuged for 30 min at 84,000*g* in a Ti 70 rotor using an L7-55 Beckman ultracentrifuge (Brea, CA, USA) 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 caveolar and noncaveolar fractions were quantified using the DC protein assay kit with bovine serum albumin as the standard [21]. Samples (15 μg/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), ß-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 Tris-buffered saline supplemented 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.

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, USA) 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 (Coralville, IA, USA). A mouse β-actin housekeeping 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. A $p < 0.05$ was considered statistically significant.