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Amelioration of UVB-induced oxidative stress and inflammation in *fat-1* transgenic mouse skin

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ABSTRACT

ω-3 polyunsaturated fatty acids (PUFAs), which are abundant in fish oils, are known to scavenge lipid peroxyl radicals and potentiate host immune defence. As UVB-induced oxidative stress and inflammation have been implicated in apoptotic cell death, this study was aimed to investigate the anti-inflammatory, anti-oxidative, and anti-apoptotic capacity of *fat-1* transgenic mice capable of converting ω-6 to ω-3 PUFAs. Wild-type (WT) C57BL/6 mice and *fat-1* mice were maintained on the AIN-93 diet supplemented with 10% safflower oil rich in ω-6 PUFAs for 5 weeks. The ω-3/ω-6 PUFA ratio was significantly higher in the dorsal skin of *fat-1* mice than that in the WT mice. Upon single exposure to UVB (5.0 kJ/m^2) radiation, *fat-1* mice showed inflammatory as well as oxidative tissue damage and the expression of pro-inflammatory enzymes, cyclooxygenases-2 and inducible nitric oxide synthase in the skin to a much lesser extent than the WT mice. The protection of *fat-1* mice from UVB-induced skin inflammation was associated with decreased phosphorylation of STAT3. Moreover, UVB-induced apoptosis was attenuated in *fat-1* mouse skin. In comparison to WT animals, higher levels of Nrf2 and its target proteins, such as heme oxygenase-1, NAD(P)H:quinone oxidoreductase-1 and thioredoxin-1, were found in the skin of *fat-1* mice.

1. Introduction

Ultraviolet B (UVB) radiation is an important environmental insults responsible for the pathogenesis of skin aging and photocarcinogenesis [1,2]. Multiple lines of evidence suggest that UVB exerts its detrimental effect mainly through generation of reactive oxygen species (ROS) [3,4] causing oxidative modification of cellular macromolecules and accumulation of lipid peroxidation products, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) [5–7]. The UVB-induced oxidative stress also triggers epidermal inflammation through inappropriate modulation of intracellular signaling pathways, thereby predisposing skin cells to photocarcinogenesis [2,8].

Two representatives of pro-inflammatory enzymes, cyclooxygenase-2 (COX-2) [9,10] and inducible nitric oxide synthase (iNOS) [11] are overexpressed aberrantly in the epidermis upon exposure to UVB radiation. The expression of COX-2 [12] and iNOS [13.14] is regulated by an eukaryotic transcription factor, signal transducer and activator of transcription 3 (STAT3). Previous studies have demonstrated that UVB irradiation activates STAT3 in mouse skin [15]. Moreover, the aberrant expression of COX-2 and iNOS in UVB-irradiated cultured cells as well as in mouse skin *in vivo* also results, at least in part, from the inappropriate amplification of phosphoinositide-3-kinase (PI3K)-Akt signaling [16,17].

While ROS can induce inflammation and oxidative cell death, cellular redox homeostasis is maintained by the constitutive induction of cytoprotective genes that encode distinct set of anti-oxidative and other cytoprotective proteins such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1) and thioredoxin-1 (TXN1) [18]. HO-1 and NQO1 knockout mice were more prone to skin tumor development [19,20]. The robust induction of aforementioned cytoprotective proteins is known to be mediated by nuclear factor erythroid-related factor 2 (Nrf2) [21,22]. Thus, one of the potential strategies to prevent photo-inflammation and apoptosis is to augment the Nrf2-mediated induction of anti-oxidant gene expression.

ω-6 and ω-3 polyunsaturated fatty acids (PUFAs), in general,

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elicit opposing effects. While ω-6 PUFAs are proinflammatory, ω-3 PUFAs have anti-inflammatory properties. Thus, the ω-6/ω-3 PUFA ratio in the cellular lipid profile is essential for determining the risk for many inflammation-associated disorders [23–26]. Transgenic *fat-1* mice expressing the *fat-1* gene derived from *Caenorhabditis elegans*, are capable of producing ω-3 PUFAs from ω-6 PUFAs and thereby maintain a balanced ratio of ω-6/ω-3 PUFAs in their tissues and organs [27]. Our recent studies have demonstrated that endogenous ω-3 PUFA production in hairless *fat-1* mice and topically applied docosahexaenoic acid protect against photocarcinogenesis. In the present study, we investigated the short-term effects of an enhanced ω-3 PUFA tissue status in *fat-1* mice on UVB-induced oxidative stress, acute skin inflammation and apoptosis.

2. Materials and methods

2.1. Animals and diets

Male transgenic *fat-1* mice [27] were backcrossed to female mice with wild-type (WT) C57BL/6 background (Charles River Laboratories, Wilmington, MA). Animals were kept in standard cages under specific pathogen-free conditions. Each cage housed age- and weight-matched mice, combining one WT and one *fat-1* transgenic mouse. They were fed a special diet (10% safflower oil), high in ω-6 and low in ω-3 PUFAs. All studies were approved by the Institutional Animal Care and Use Committee at Seoul National University. The offsprings were genotyped for *fat-1*$^{+/-}$ heterozygosity using the Tissue-Direct™ PCR Kit (Lamda Biotech, Inc., St. Louis, MO). The genotyping primers of *fat-1* were as follows: *fat-1*, 5′-CTG CAC CAC GCC TTC ACC AAC C-3' (forward) and 5′-ACA CAG CAG CAG ATT CCA GAG ATT-3' (reverse). In this study, transgenic *fat-1* mice used were female heterozygous.

2.2. Preparation and maintenance of mouse embryonic fibroblasts (MEFs)

fat-1 transgenic and WT mice were maintained and housed in a climate-controlled quarters (24 ± 1 °C at 50% humidity) with a 12-h light/12-h dark cycle. They were given *ad libitum* access to 10% safflower oil diets and water. Male *fat-1* and female WT mice were paired and the pregnancies were monitored. Embryos were obtained at the day 13.5 after paring and used to prepare fibroblasts after removing head, heart and legs. The tails of the embryos were used to confirm the *fat-1* genotype by PCR, and the embryo bodies were minced into small pieces and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY) and kept at 37 °C with 5% CO_2.

2.3. Source of UVB radiation

The dorsal hair of haired mice was trimmed 2 days before exposure to UVB radiation. The UVB radiation source was a 5 × 8 Watt tube, which emits an energy spectrum with high fluency in the UVB region (with a peak at 312 nm). A Biolink BLX-312 UV crosslinker (Vilbert Lourmat, Marne-la-Valle´e, France) was used to irradiate mouse skin (5.0 kJ/m^2 dose of UVB) and MEFs (20 mJ/cm^2 dose of UVB).

2.4. Hematoxylin and eosin (H&E) staining and the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The skin was removed from euthanized mice at 24 h after UVB exposure. Sections of harvested mouse skin were fixed with 10% neutral buffered formalin and embedded in paraffin. Mounted sections (4 μm) were stained with H&E for light microscopy. Apoptotic cells were detected in paraffin-embedded skin sections by the TUNEL assay using the ApopTag® peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA). The apoptotic cells were visualized by light microscopy.

2.5. Immunohistochemical analysis

The dissected skin was prepared for measuring the expression patterns of MDA-modified protein, P-STAT3 (Tyr^{705}) and Nrf2. Four-μm sections of 10% formalin-fixed, paraffin-embedded tissues were cut on silanized glass slides, deparaffinized three times with xylene and dehydrated in graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with proteins modified with MDA (JaICA, Nikken SEIL Co. Ltd., Shizuoka, Japan), COX-2 (Cayman Chemical Co., Ann Arbor, MI), P-STAT3, caspase-3 (Cell Signaling Technology, Beverly, MA) and Nrf2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20, and then developed using horseradish peroxidase-conjugated secondary antibodies (rabbit or mouse; Dako, Glostrup, Denmark). The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin.

2.6. Tissue lysis and protein extraction

The UVB irradiated dorsal skin was collected, and fat was removed on ice to get the epidermis. Collected epidermis was homogenized in an ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris–HCl (pH 7.4), 20 mM ethylene glycol tetra-acetic acid, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 and protease inhibitors, 1 mM phenylmethyl sulfonylfluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA)-free cocktail tablet] followed by periodical vortex for 30 min at 0 °C. In other studies, embryonic fibroblasts were incubated with linoleic acid (LA), AA (Cayman Chemical Co., Ann Arbor, MI) or dimethyl sulfoxide (DMSO; Amresco, Solon, OH) for indicated durations. The treated cells were harvested, washed with phosphate buffered saline and suspended in the lysis buffer as mentioned above for 1 h on ice. The lysates were centrifuged at 14,800 *g* for 15 min at 4 °C. The aliquots collected from the supernatant containing protein were stored at −70 °C until use.

2.7. Fractionation of cytosolic and nuclear extracts

Scraped dorsal skin of mice was homogenized in 800 μL of hypotonic buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA and 0.1 mM PMSF]. To the homogenates was added 80 μL of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 15 min at 14,800 *g*. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed once with 500 μL of buffer A plus 50 μL of 10% NP-40, centrifuged, resuspended in 200 μL of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 20% glycerol] and centrifuged for 15 min at 14,800 *g*. The supernatant containing nuclear proteins was collected and stored at −70 °C until use.