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Original Contribution

Skin cells and tissue are capable of using L-ergothioneine as an integral component of their antioxidant defense system

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ABSTRACT

The cellular defense system against harmful levels of reactive oxygen species consists of antioxidant enzymatic activities and small nonenzymatic molecules. L-Ergothioneine has long been recognized as a potent and stable low-molecular-weight antioxidant that humans consume with diet and that accumulates in cells normally subjected to high levels of oxidative stress. As L-ergothioneine is plasma membrane-impermeative, its protective function is restricted to cells that express the L-ergothioneine-specific receptor/transporter OCTN1. Here we report for the first time that both as resident skin cells and in culture, epidermal keratinocytes synthesize OCTN1, which enables them to internalize and accumulate L-ergothioneine. This accumulation confers upon the cells an increased antioxidant potential. Consequently, it reduces the levels of reactive oxygen species and DNA, protein, and lipid damage in keratinocytes subjected to solar-simulating UV oxidative stress. Our results suggest that L-ergothioneine not only prevents oxidative damage but also may enable DNA repair in the UV-irradiated cells. The diminished oxidative damage to cellular constituents limits the apoptotic response and results in increased cell viability. The cells' ability to take up, accumulate, and utilize the potent antioxidant L-ergothioneine positions this naturally occurring amino acid and its receptor/transporter as an integral part of the antioxidative defense system of the skin.

Exposure of skin cells to solar UV irradiation inevitably causes DNA, protein, and lipid damage, which, through complex feed-forward and feedback interactions, compromises the energy status and the metabolic and nuclear activities of the cells, resulting ultimately in photoaging and/or photocarcinogenesis [see 1,2]. Major effectors of the UV damage are the reactive oxygen species generated in high amounts as a consequence of the interactions of UV light and intracellular molecules. Concerns about sun damage have focused attention on the skin's antioxidant defenses. To protect from the cytotoxic effects of reactive oxygen species (ROS) and to ensure their modulatory effects at lower physiological concentrations, the skin maintains a network of antioxidants that includes vitamins such as C and E, small molecules such as glutathione, and enzymes such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase. L-Ergothioneine (EGT) has long been recognized as an antioxidant amino acid that humans consume with plant food and is found distributed throughout the body [3,4]. The absence of toxicity, the broad spectrum of its antioxidant properties, its high water solubility, and its unusual stability at physiologic pH [see 4] make EGT a very attractive component of the antioxidant defense system [5]. However, unlike a number of water-soluble antioxidants (e.g., flavonoids, phenolic acid, and polyphenols), EGT is cell-membrane impermeative and requires a specific carrier to be internalized. Its function is restricted to only cells and tissues in which the EGT receptor/transporter OCTN1 is produced [6]. Here we provide evidence that skin cells and tissue express OCTN1. In particular, we demonstrate that EGT is accumulated in the epidermis and the epidermal keratinocytes and serves to protect them from solar-simulated (ss)UV damage by decreasing the levels of ROS, maintaining cell vitality, and eliminating the need for a massive apoptotic response. Our data support a conclusion that EGT and its receptor/transporter may represent integral components of the skin's antioxidant defense system.

Materials and methods

Antibodies

The following antibodies were used in this study: for OCTN1, goat anti-OCTN1 (C-13) (sc-19819; Santa Cruz Biotechnology, Santa Cruz,

Abbreviations: ROS, reactive oxygen species; EGT, L-ergothioneine; NHDF, normal human dermal fibroblasts; NHEK, normal human epidermal keratinocytes; ssUV, solar-simulating UV; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase.

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CA, USA); for PARP-1, rabbit anti-PARP (214/215) cleavage-site-specific antibody (44-698G; Biosource, Camarillo, CA, USA); for actin, monoclonal anti-actin (clone AC-40; Sigma, Saint Louis, MO, USA); Alexa Fluor 488-conjugated donkey anti-goat (A11056; Santa Cruz Biotechnology); and HRP-conjugated goat anti-rabbit (31462; Pierce, Rockford, IL, USA).

Cell culture

Neonatal normal human dermal fibroblasts (NHDF) were grown in fibroblast basal medium supplemented with 2% fetal bovine serum, insulin, recombinant human fibroblast growth factor-B, gentamicin, and amphotericin (Lonza, Allendale, NJ, USA).

Neonatal normal human epidermal keratinocytes (NHEK) were grown in EpiLife basal medium containing 60 μM calcium chloride, supplemented with 0.2% v/v bovine pituitary extract, 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin, 0.2 ng/ml human epidermal growth factor, and gentamicin/amphotericin (Cascade Biologics, Portland, OR, USA).

The cells were grown in a humidified 5% CO_2 incubator at 37°C.

Skin organ cultures

The studies were conducted following procedures that are in accordance with the principles of the Helsinki Declaration. Adult tissue samples were provided by the Cooperative Human Tissue Network. The samples were obtained during scheduled surgery after the patients had given informed consent. Neonatal human foreskin samples were obtained after circumcisions with the informed consent of the parents. Upon arrival, the adult skin tissue was washed several times with phosphate-buffered saline (PBS); the subcutaneous fat was dissected and discarded. The skin was equilibrated overnight in a 5% CO_2 incubator at 37°C in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, 0.1 μM hydrocortisone, and penicillin/streptomycin (GIBCO, Invitrogen, Carlsbad, CA, USA). Where indicated, EGT was present in the medium during this period. The foreskin samples were similarly equilibrated in the medium for 1 h. Skin and foreskin samples were then washed in PBS, frozen in Neg50 frozen section medium (Richard-Allan Scientific, Kalamazoo, MI, USA), and kept at −70°C until sectioned.

UV irradiation

ssUV (UVA + UVB) was delivered with a Sun 340 (UVA340) sun lamp with a UV spectrum of 295–390 nm that closely resembles the UV spectrum of sunlight through the mid-UVA range [7]. The radiation was monitored using a Model IL1400A radiometer/photometer and a UVA 28504/W 11803 probe (International Light, Newburyport, MA, USA). The cells were washed once with PBS and irradiated with ssUV in PBS or in the appropriate cell culture medium without additives and phenol red (basal medium). After irradiation, the cells were incubated in medium supplemented with additives (complete medium) with or without phenol red. The skin organ cultures were irradiated floating in 5 ml PBS with epidermal side facing the UV source. Where indicated, EGT was present in the medium during irradiation. Immediately after irradiation, the PBS was replaced with fresh complete DMEM with or without EGT. The incubation proceeded with the epidermal side facing the medium, in the humidified CO_2 incubator for the desired period of time. The sham-irradiated controls were treated similarly but without UV irradiation.

OCTN1 immunolocalization in human skin tissue and cultured cells

NHEK, NHDF, and frozen sections (5 μm) of normal adult skin and neonatal foreskin were fixed with methanol/acetone (1/1) for 10 min at −20°C. They were then permeabilized with 0.2% Triton X-100 in PBS, blocked with 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min at room temperature, and then incubated with the primary antibody overnight at 4°C and subsequently with the secondary antibody for 1 h at room temperature. Slides were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, CA, USA). The skin sections were prepared and their quality was ascertained by hematoxylin/eosin staining following a standard protocol in a licensed pathology laboratory (McLean Laboratories, LLC, Smithtown, NY, USA).

Preparation of cell extracts and Western blot analysis

NHEK and NHDF total, nuclear, and cytoplasmic extracts were prepared and quantified as previously described [8]. Approximately 20 μg nuclear proteins was resolved on 4–12% NuPAGE Bis-Tris mini gels (Invitrogen) and electrophoretically transferred onto 0.2-μm nitrocellulose membranes (Invitrogen). The membranes were blocked with PBS/5% Carnation nonfat dry milk/0.5% Triton X-100 for 1 h at room temperature, incubated with primary antibody (in block solution containing 10% fetal bovine serum) overnight at 4°C and subsequently with the respective secondary antibodies for 1 h at room temperature, and developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Membranes reprobed with anti-actin antibody for protein loading normalization were first stripped with Restore Plus Western blot stripping buffer (Pierce, Thermo Scientific). Membrane images were captured with a Kodak 440CF Image Station running Kodak Molecular Imaging Software version 4.5.0.

Measurement of EGT accumulation

The cytoplasmic and nuclear extracts prepared from NHDF and NHEK were desalted and purified from proteins and nucleic acids using Microcon Centrifugal Filter Devices YM-10 with a protein cutoff at 10,000 Da and nucleic acids cutoff at 30/20 bases/base pairs (Amicon, Millipore, Bedford, MA, USA). EGT standards were prepared by spiking EGT in the range of 0 to 250 μM into equal volumes of extraction buffers and processing the samples over the Microcon devices. To assess for interference from cellular components, 100 μM EGT was also spiked into equal volumes containing 200 μg cytoplasmic or nuclear extracts and its detection after processing of the samples was compared to that in extraction buffer alone. Neither the cytoplasmic nor the nuclear environment showed any appreciable effect on the ability to detect EGT (data not shown). Equal volumes (20 μl) of standard and test samples were resolved on a Varian Meta S.1 5 ODS HPLC column (Waters, Milford, MA, USA) with a 95-5% water-acetonitrile gradient. The EGT peak (λ_{max} 256 nm) was eluted at 3.9 ± 0.2 min. To measure the EGT content in the epidermis of the skin organ cultures, the epidermis of approximately 1-cm^2 skin samples was heat-separated from the dermis [9], immediately frozen in liquid nitrogen, and ground to powder and a total cellular extract was prepared in PBS supplemented with 10 μl/ml protease (No. P8340) and phosphatase (No. P5726) inhibitors (Sigma). The epidermal extracts were processed for EGT detection as described. The EGT peak area was normalized to protein content determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Detection of ROS

The UV-dependent ROS burst was detected 3 h postirradiation using 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA)–a cell-permeative indicator for reactive oxygen species that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell (Molecular Probes, Invitrogen). NHEK (1×10^4/well) were seeded on eight-well chamber slides in complete keratinocyte medium. The next day, EGT was added and the cells were incubated for 24 h. The