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Protective effect of triphlorethol-A against ultraviolet B-mediated damage of human keratinocytes

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ABSTRACT

Ultraviolet B (UVB) radiation on human skin induces pathophysiological processes such as oxidative stress and inflammation. In previous reports, the antioxidant effects of triphlorethol-A were shown to protect cells against hydrogen peroxide-induced cell damage and gamma ray-induced oxidative stress. In this study, the role of triphlorethol-A in protecting human keratinocytes (HaCaT) against UVB-induced cell damage was investigated. Triphlorethol-A-treated cells were irradiated with UVB (150 mJ/cm^2). Triphlorethol-A decreased UVB-induced intracellular ROS and restored the activities of antioxidant enzymes decreased by UVB radiation. Triphlorethol-A decreased UVB damage to cellular components, such as lipid membrane and DNA, restored cell viability and reduced UVB-induced apoptosis by inhibiting the mitochondria-mediated caspase pathway. Triphlorethol-A also reduced the UVB-induced loss of $\Delta\Psi_m$ and the active forms of caspase 9 and caspase 3. The anti-apoptotic effect of triphlorethol-A was found to involve the inhibition of c-Jun NH_2-terminal kinase, which was induced by UVB exposure. And triphlorethol-A showed an absorptive capacity at range of UVB.

These results suggest that triphlorethol-A protects human keratinocytes against UVB by enhancing the activities of the antioxidant system, inhibiting cellular damage and absorbing the UVB.

1. Introduction

Ultraviolet radiation, and in particular the middle wavelength (UVB, range 280–320 nm), elicits many adverse effects in the skin, including cancer [1], suppression of the immune system [2,3] and photoaging [4]. UVB damage is either direct or occurs indirectly through the production of reactive oxygen species (ROS) [5–7]. In healthy aerobic organisms, ROS production is balanced by antioxidant defense systems; however, when ROS are present in excess, antioxidant defense systems are overwhelmed, resulting in oxidative damage to DNA, lipids and proteins, as well as oxidative stress leading to inflammation, apoptosis and carcinogenesis [8,9].

The enzymes comprising the antioxidant systems that regulate ROS levels in living cells include superoxide dismutase (SOD) and catalase (CAT), which are expressed in response to different stimuli [10]. In the skin, antioxidants confer protection against ROS-induced injury. In addition, antioxidants such as ascorbic acid, α-tocopherol and a mixture of other dietary antioxidants have been reported to inhibit UV-induced skin carcinogenesis [11–13]. Triphlorethol-A, an open-chain trimer of phloroglucinol, is a phlorotannin isolated from *Ecklonia cava,* a brown alga of the family Laminariaceae. Recently, we reported that triphlorethol-A protects cells from hydrogen peroxide (H_2O_2)-induced oxidative stress by a mechanism involving radical quenching and CAT activation [14]. Triphlorethol-A was also shown to protect cells against damage from ionizing radiation by the inhibition of apoptosis-related processes [15] and to induce the antioxidant enzyme heme oxygenase-1 by activating the transcription factor NF-E2-related factor 2 [16]. Furthermore, triphlorethol-A reduces matrix metalloproteinase-1 induction through the inhibition of extracellular-signal-regulated protein kinase and activator protein-1 [17]. Additional actions include the inhibition of damage resulting from formaldehyde-induced oxidative stress and the restoration of DNA integrity by the enhancement of DNA non-homologous end-joining and base-excision repair processes [18,19].

In this study, we determined the effect of triphlorethol-A on UVB-induced cell damage in HaCaT cells. By blocking the accumulation of intracellular ROS and by preventing UVB-induced apoptosis, triphlorethol-A was able to inhibit UVB-induced cell damage.

2. Materials and methods

2.1. Materials

Triphlorethol-A (Fig. 1), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) and Hoechst 33342 were purchased from

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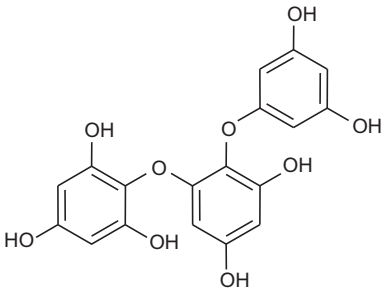

Fig. 1. Chemical structure of triphlorethol-A.

Sigma Chemical Company (St. Louis, MO, USA), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) was purchased from Invitrogen (Carlsbad, CA, USA). Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Molecular Probes (Eugene, OR, USA). Caspase 9, caspase 3, poly (ADP-ribose) polymerase (PARP), c-Jun NH_2-terminal kinase (JNK) and phospho JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture and UVB radiation

Human keratinocytes (HaCaT cells) were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 and were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml). Cells were exposed to UVB at 150 mJ/cm^2. The CL-1000M UV Crosslinker (UVP, Upland, CA, USA) was used as the UVB source and delivered a UVB energy spectrum of 280–320 nm.

2.3. Intracellular ROS measurement

Cells were treated with 10 μg/ml of triphlorethol-A and exposed to UVB radiation one hour later, followed by incubation for an additional 24 h at 37 °C. DCF-DA solution was added, incubated for 30 min, and 2′,7′-dichlorofluorescein fluorescence was detected using a Perkin Elmer LS-5B spectrofluorometer, a flow cytometer (Becton Dickinson, Mountain View, CA, USA) and a confocal microscope [20].

2.4. SOD activity

Cells were treated with 10 μg/ml of triphlorethol-A and exposed to UVB one hour later. Cells were incubated at 37 °C, washed with phosphate-buffered saline (PBS) and scraped. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 s. Triton X-100 (1%) was then added to the lysates and incubated for 10 min on ice. The lysates were centrifuged at 5000×*g* for 10 min at 4 °C and the protein content of the supernatant was measured. Fifty microgram of protein was added to 500 mM phosphate buffer (pH 10.2) and 1 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink-colored product, which was detected at 480 nm using a UV/VIS spectrophotometer in kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm and the amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity. Total SOD activity was expressed as unit per mg protein [21].

2.5. CAT activity

Fifty microgram of protein was added to 50 mM phosphate buffer (pH 7.0) and 100 mM H_2O_2, the mixture was incubated for 2 min at 37 °C and the absorbance at 240 nm was monitored for 5 min, as the change in absorbance is proportional to the breakdown of H_2O_2 [22]. CAT activity was expressed as unit per mg protein.

2.6. Lipid peroxidation assay

Lipid peroxidation was estimated using a fluorescent probe, DPPP [23]. Cells were incubated with 5 μM DPPP for 15 min in the dark and then exposed to UVB. DPPP fluorescence image was captured using a Zeiss Axiovert 200 inverted microscope at an excitation wavelength of 351 nm and an emission wavelength of 380 nm and quantified.

2.7. Single-cell gel electrophoresis (comet assay)

The degree of oxidative DNA damage was determined in a comet assay [24,25]. Cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA) at 39 °C and the mixture was spread on a fully frosted microscopic slide pre-coated with 200 μl of 1% normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 75 μl of 0.5% LMA and then immersed in a lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100 and 10% DMSO, pH 10) for 1 h at 4 °C. The slides were then placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow for DNA unwinding and the expression of the alkali-labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4 °C to draw the negatively charged DNA towards the anode. The slides were washed three times for 5 min at 4 °C in a neutralizing buffer (0.4 M Tris, pH 7.5), stained with 75 μl of propidium iodide (20 μg/ml) and observed using a fluorescence microscope and an image analyzer (Kinetic Imaging, Komet 5.5, UK). The percentage of total fluorescence in the DNA tails and the tail length of 50 cells per slide were recorded.

2.8. Cell viability

Cells were treated with 10 μg/ml of triphlorethol-A and with UVB. Forty-eight hours later, 50 μl of the MTT stock solution (2 mg/ml) was added to each well to obtain a total reaction volume of 200 μl. After incubation for 4 h, the plate was centrifuged at 800×*g* for 5 min followed by aspiration of the supernatants. The formazan crystals in each well were dissolved in 150 μl of dimethyl sulfoxide (DMSO) and the absorbance at 540 nm was measured on a scanning multi-well spectrophotometer [26].

2.9. Nuclear staining with Hoechst 33342

Cells were treated with 10 μg/ml of triphlorethol-A and exposed to UVB one hour later. After a further 48 h incubation at 37 °C, 1.5 μl of the DNA-specific fluorescent dye Hoechst 33342 (stock 10 mg/ml) was added to each well and the plates were then incubated for 10 min at 37 °C. The degree of nuclear condensation in the stained cells was determined by visualizing them using a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera.

2.10. DNA fragmentation

Cellular DNA fragmentation was assessed by analyzing the cytoplasmic histone-associated DNA fragmentation, using a kit from Roche Diagnostics (Portland, OR, USA) according to the manufacturer's instructions.