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Glycyrrhizic acid (GA), a triterpenoid saponin glycoside alleviates ultraviolet-B irradiation-induced photoaging in human dermal fibroblasts

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ABSTRACT

Glycyrrhizic acid (GA), a triterpenoid saponin glycoside from the roots and rhizomes of licorice is used in traditional and modern medicine for the treatment of numerous medical conditions including skin diseases and beauty care product. In the present study, we investigated the effect of GA against ultraviolet B (UVB) irradiation-induced photoaging in human dermal fibroblasts (HDFs) and its possible mechanism of action. HDFs were subjected to photoaging by sub-toxic dose of UVB (10 mj/cm^2) irradiation. Cell viability, matrix metalloproteinase 1 (MMP1), pro-collagen 1, cellular and nuclear morphology, cell cycle, intracellular reactive oxygen species (ROS), caspase 3 and hyaluronidase inhibition assays were performed. Western blotting was used to evaluate the expression of NF-kappa B (NF-κB) and cytochrome-C proteins. GA treatment significantly inhibited photoaging. It achieved this by reducing ROS, NF-κB, cytochrome c, caspase 3 levels and inhibiting hyaluronidase enzyme. The main mechanism seems to be, most likely by blocking MMP1 activation by modulating NF-κB signaling. These findings may be useful for development of natural and safe photoprotective agents against UVB irradiation.

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Introduction

Skin is the largest human organ and is directly exposed to the harmful irradiation, toxic materials and microbial invasion and raises a physical, biochemical and immunological barrier against environmental insults (Rittié and Fisher 2002; Ding and Wang 2003). Ultraviolet (UV) irradiation has deleterious effects on human skin, including tanning, sunburn, immune suppression, cancer, and connective tissue degradation (photoaging) (Jones et al. 1999; Offord et al. 2002). Out of all subtypes of UV irradiation, only UV-B is capable of producing physiological response and is considered most hazardous environmental carcinogen (Katiyar et al. 2001; Fisher et al. 2002). Higher doses of UVB irradiation induces severe oxidative stress resulting in cell death (Morliere et al. 1995). While as sub-lethal UVB irradiation (physiological UV) induces oxidative stress and activates various intracellular signal transduction pathways leading to “mammalian UV response” (Vicentini et al. 2011). This response results in activation of nuclear factor kappa B (NF-kappa B). Upon activation, NF-kappa B-induces various genes including interleukin-1 (IL-1), tumor necrosis factor alpha (TNF alpha), and matrix metalloproteinase-1 (MMP-1). MMP-1 is the cause for extracellular matrix (ECM) degradation resulting in skin photoaging and eventually leading to cancer (Fisher et al. 1998; Jenkins 2002; Varani et al. 2002; Oh et al. 2004; Wenk et al. 2004).

Botanical antioxidants have been shown to be associated with reduced incidence of photocarcinogenesis and photoaging (F'guyer et al. 2003). Consistent with this understanding, botanical antioxidants have attracted considerable attention because of their skin photoprotective effects (Afaq et al. 2005).

Glycyrrhizic acid (GA), a triterpenoid saponin glycoside, is a major active constituent of licorice root, has been attributed with numerous pharmacologic effects, including anti-inflammatory, anti-viral, anti-tumor, hepatoprotective, and antioxidant activities (Asl and Hosseinzadeh 2008; Ni et al. 2011). Traditionally licorice has been used in various skin disorders, in cosmetics and personal care products. Licorice-derived ingredients are used in the formulation of makeup products, hair care products and skin care products.

Recently it has been suggested that GA down-regulates UVB induced signal transduction cascades in carcinogenesis and confers photo-protective effect in SKH-1 murine skin via down regulation of cell proliferation involving thymine dimer, proliferating cell nuclear antigen (PCNA), apoptosis and transcription factor NF-kappa B and inflammatory processes involving cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and nitric oxide (NO) (Lee et al.

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2005; Cherng et al. 2011). Here we report the inhibitory effect of GA against UVB-induced photoaging in human dermal fibroblasts (HDFs) and the mechanisms involved.

Materials and methods

Materials

The normal human newborn foreskin fibroblast cell line, HS68 cell (ATCC CRL 1635), was obtained from American Type Culture Collection (Rockville, MD, USA). Glycyrrhizic acid 75% ($C_{42}H_{62}O_{16}\cdot NH_3$, FW = 840.0), Dimethyl sulphoxide (DMSO), Dulbecco's modified eagle's media (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin–EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA), propidium iodide, ribonuclease A (RNase A), hyaluronic acid (HA), hyaluronidase (ENZ) were purchased from Sigma Aldrich Chemicals Private Limited (St. Louis, MO). Pro-collagen type 1 C-peptide protein and matrix metaloproteinase-1 ELISA kits were procured from Takara, Japan; Cat.#MK101 and GE Healthcare; Code: RPN2610, respectively. NF-kappa B (p50), cytochrome C, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) All other biochemicals used were of high purity biochemistry grade.

Cell culture

HS68 cells used for the experiments were between 10 and 25 passages of their growth period. HS68 cells were plated in 175 cm^2 culture flasks and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma, St. Louis, Mo, USA). The cells were pretreated with GA for 24 h, washed with phosphate buffer saline (PBS; Sigma, St. Louis, Mo, USA) and irradiated under UVB radiation. After UVB irradiation, cells were rinsed twice with PBS and incubated in fresh culture media without serum, in presence of GA for further 24 h. All UVB irradiations were performed under a thin layer of PBS.

Ultraviolet irradiation

The source of UVB radiation was a band of four UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH, USA) equipped with digital controller to regulate UV dosage at a fixed distance of 24 cm from the lamps to the surface of the cell culture plates. The majority of the resulting wavelengths (>90%) were in the UVB range (280–320 nm) and UVA was less than 10%. The peak emission was recorded at 314 nm.

Cytotoxicity/cytoprotection

Cell viability was determined as described by (Moon et al. 2008), by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For cytotoxicity experiments, cells were treated with GA at mentioned concentrations and incubated for 24 h. For cyto protection experiments, cells were pretreated with GA for 24 h, washed twice with PBS and subjected to UVB-irradiation. After irradiation, cells were again washed twice with PBS and cultured in fresh medium in presence of GA for further 24 h. After corresponding treatments the medium was removed and cell viability was evaluated by assaying the ability of functional mitochondria to catalyze the reduction of MTT (0.5 mg/ml, at 37 °C for 3 h) to form formazan salt by mitochondrial dehydrogenases, as determined by ELISA reader at 565 nm (Multiskan Spectrum; Thermo Electron Corporation, USA).

Cellular and nuclear morphology

The cellular and nuclear morphology was observed under the light microscope (Nikon Eclipse TE2000U), at 20X magnification, or under fluorescent microscopy, using Hoechst 33258 staining, as described earlier (Tasduq et al. 2008).

Pro-collagen-1 and matrix metalloproteinase-1 immunoassay

The levels of type 1 procollagen (Takara, Japan) and matrix metalloproteinase-1 proteins (GE Healthcare) in cell-free supernatants were determined by ELISA. The cell free supernatants of cultured fibroblasts after indicated treatments were collected and stored at −80 °C until used. The assays were performed according to manufacturer's instructions.

Hyaluronidase inhibition assay

Hyaluronidase (HA) was assayed as described by (Sumantran et al. 2007), based on precipitation of HA with cetyl pyridinium chloride. Enzyme (800 U/ml) and HA substrate (0.40 mg/ml) were incubated at 37 °C for 1 h. Enzyme activity was measured by monitoring the percentage of undigested HA substrate in the cetyl pyridinium chloride precipitate at absorbance 415 nm (A 415 nm) after the enzyme reaction.

Cell cycle analysis

Cell cycle was analyzed as described by Yang et al. (2007) with some modifications. Briefly, non-treated and treated skin fibroblast cells were harvested by trypsinization, centrifuged at 1500 × g for 5 min, washed with PBS, and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed twice with PBS and incubated in PBS containing 1.5 mg/l RNase A for 1 h at 37 °C, followed by staining with 5 μl PI (1 mmol/l stock) for 20 min on ice. The cells were analyzed for DNA content using BD FACS Calibur cytometer using blue (488 nm) excitation from argon laser. Data were collected in list mode on 10,000 events for FL2-A vs. FL2-W.

Intracellular reactive oxygen species (ROS)

The intracellular production of ROS levels were determined as described by Heo et al. (2009) using 2′,7′-dichlorofluorescein diacetate (DCFDA). Cells grown in 96-blackwell (Nunc, Denmark), after pre-treatment of GA for 24 h, were washed twice with PBS and irradiated with UVB (10 mj/cm^2) under a thin layer of PBS. Immediately after UVB irradiation, cells were again washed twice with PBS and DCFDA was added (5 μM) and incubated for 30 min at 37 °C in a CO_2 incubator. DCFDA is oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence was read at ex/em of 488/525 nm with Perkin Elmer, LS 55, USA.

Caspase 3 assay

Caspase activation was measured using a caspase 3 fluorometric assay kit (BD Apoalert caspase 3 fluorescent assay kit). HDFs, treated or untreated were harvested, and centrifuged (approximately 1 mg protein) at 400 × g for 5 min. The cell pellets were re-suspended in 50 μl of chilled cell lysis buffer and incubated on ice for 10 min, and the lysates were centrifuged at 15,000 × g for 10 min at 4 °C to precipitate cellular debris. A total of 50 μl of cell lysates was incubated with 50 μl of reaction buffer/DTT mix. DEVD-CHO was used as an inhibitor of caspase 3 in an induced sample. Five μl of

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