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Tyrosol prevents apoptosis in irradiated keratinocytes

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ABSTRACT

Phenolic compounds, the biggest group of natural antioxidants, have attracted much attention due to their known and wide-ranging biological activities, as well as to their health effects. In particular, regardless their antioxidant activity, they play a key role in the control of several inflammation-associated processes as well as in improving antioxidant defense system. In an our previous work we have demonstrated the ability of Hydroxytyrosol, an ortho-diphenolic compound, essential component of oleuropein, in preventing apoptotic cell death induced by UVB radiation in HaCaT cell lines *in vitro*. In olive oil, besides Hydroxytyrosol, there are appreciable amounts of Tyrosol and its secoiridoid derivatives. It has been well established that Tyrosol has a significantly lower antioxidant activity than Hydroxytyrosol, but despite this, recent studies suggest that Tyrosol exerts a powerful protective effect against oxidative injuries in cell systems and that it is able to improve the intracellular antioxidant defenses. Here, Tyrosol effect has been evaluated in HaCaT cells exposed to UVB radiation by means of morphological and molecular analyses which revealed the polyphenol ability in reducing apoptotic markers and in protecting HaCaT cells from damage. These findings suggest an important role of Tyrosol in protecting cells from apoptotic cell death and encourage the use of this phytochemical as biological ingredient in topical preparations as possible tool to prevent skin damage.

1. Introduction

The skin has a complex defense system comprising both enzymatic and non-enzymatic components to counteract the adverse biological effects of reactive nitrogen/oxygen species [1]. As the first line of defense, reactive nitrogen/oxygen species are reduced by antioxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as by endogenous and exogenous small molecules, including glutathione and vitamins C and E [2,3]. When biomolecules are oxidized, they are repaired or replaced by biological protective systems. Nevertheless, biomolecules are gradually irreversibly oxidized, and their accumulation over time impairs biological functions, eventually leading to skin ageing and related diseases [4]. Sunscreen use is a widely accepted method for primary prevention against skin disorders, sunburn cells and photoageing. In fact, the antioxidant compounds used in these products seem to have a protective function against the ultraviolet (UV)-induced generation of reactive oxygen species (ROS) in the skin. Topical antioxidants, such as the flavonoid quercetin and ascorbic acid, have been reported to diminish UV radiation-mediated oxidative damage [5]. Recently, the attention focused on a wide range of phenolic compounds which have been identified in virgin olive oil, including phenolic alcohols, secoiridoid derivatives, phenolic acids, lignans, and flavonoids [6]. Phenolic compounds are responsible for the nutritional and organoleptic properties [7], and it has been suggested that high concentrations of them in olive oil may contribute to the healthy action of the Mediterranean diet [8] because they exhibit protective effects against neuro-degenerative and cardiovascular diseases. Moreover, they show antiproliferative effects [9], contributing, at the same time, to protect the organism against oxidative damage [10]. The major phenolic compounds identified and quantified in olive oil belong to three different classes: simple phenols (Hydroxytyrosol, HyT; Tyrosol, Tyr); secoiridoids (oleuropein, the aglycone of ligstroside, and their respective decarboxylated dialdehyde derivatives) and lignans. All classes have potent antioxidant properties. High consumption of extra-virgin

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olive oils, which are particularly rich in these phenolic antioxidants, should afford considerable protection against skin alterations, coronary heart disease, and ageing by inhibiting oxidative stress [9]. Among them, particular attention has been focused on HyT, an ortho-diphenolic compound, essential component of oleuropein, and present in virgin olive oil, mainly either as secoiridoid derivatives or as the acetate ester [11]. Its antioxidant and antiapoptotic effects have been demonstrated by several research groups and it has been the subject of various *in vitro* and *in vivo* studies [11]. Moreover, HyT has already been used as a bioactive ingredient in tomato juice [12] and fish products [13], showing good results.

Another natural olive oil phenol, Tyr seems to have a lower antioxidant capacity than HyT [14] due to the absence of the *ortho*-diphenolic group in its chemical structure. Nevertheless, Tyr exerts a protective effect against oxidative injury in cell models [15] and improves the intracellular antioxidant defence systems [16]. In particular, Tyr showed antigenotoxic activity against H_2O_2 induced damage and it was characterized as an efficient hydroxyl radical scavenger and also preventing Caco-2 cells from ox-LDL induced injury. It has been also reported that Tyr has moderate but stable antioxidant activity, exerting its effect only as a hydroxyl radical scavenger or, at most, as an α-tocopherol regenerator [17,18].

Recently, our research group have been demonstrated the HyT anti-apoptotic effect against UVB radiation in a human keratynocytic cell model confirming its beneficial properties and a possible application also in the sun skin protection [19]. To our knowledge, no applications has been studied for Tyr, which is present in the phenolic fraction of virgin olive oil, where it seems to exert a protective role. In this study Tyr activity has been analyzed in HaCaT cells exposed to UVB radiation, a known physical apoptotic trigger [19,20] by means of morpho-functional and molecular analyses.

2. Materials and methods

2.1. Cell culture

HaCaT cells (Ha = human adult, Ca = calcium, T = temperature) are a spontaneously immortalized human keratinocyte line, that has been widely used for skin biology and differentiation studies [21]. They grow in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% antibiotics and were maintained at 37 °C in humidified air with 5% CO_2. Cell behavior was progressively monitored by means of inverted microscope.

2.2. Apoptosis induction and Tyr administration

The irradiating source consisted of a lamp generating UVB light in the range of 290–320 nm with an emission peak at 312 nm (Transilluminator 2000, Bio-Rad Laboratories, Hercules, CA, USA).

For apoptosis induction cells were cultured to 80% confluence and washed with PBS, covered with a thin layer of PBS and exposed to UVB for 10 min [19]. The UVB dose is 250 mJ/cm^2 and has been calculated by means of UV light meter (UV-340 Lutron electronic). After UV irradiation, fresh media was added to each plate or flasks, and cells were maintained in regular culture conditions for a designated time until analysis. After the addition of fresh medium, cells were further incubated for 2 h.

HaCaT cells were pre-treated for 24 h with Tyr before apoptosis induction. The best polyphenol concentration has been chosen testing different Tyr doses and cell viability evaluated by means of trypan blue exclusion assay. Increasing the antioxidant concentration up 20 μM, a proliferation impairment could be observed. As a consequence 5 μM appeared the best treatment, which has nor harmful for HaCaT cells.

Cells were pre-treated with 5 μM Tyr before UVB exposure and then post-incubated as previously described. For each technique, control sample and 24 h Tyr alone treatment were also analyzed. Tyr was recovered with 95% purity from olive oil wastewaters, and further purified by column chromatography [11].

2.3. Tali image-based cytometer

The Tali® image-based cytometer (Life Technologies) is a 3-channel (bright field, green fluorescence, red fluorescence) benchtop assay platform that offers several advantages both coupling, in suspended cells, flow cytometry and fluorescence microscopy information. In particular, we applied the supravital propidium iodide technique (PI; Tali® Viability Kit; Life Technologies). A 100 μl aliquot of cells (1×10^6) from each sample, detached with trypsin from the substrate and re-suspended in DMEM, was treated directly in cell culture medium by means of 1 μg/ml PI for few minutes and then analyzed by means of Tali-image-based cytometer. Dot plots have been elaborated with the Attune Cytometric Software [22]. Differences in the percentages of viable/dead cells among groups were determined using *t*-Student test. Significance was set at $p < 0.05$. Data were collected from three independent experiments.

2.4. Acridine orange (AO) and PI nuclei staining

Cells, directly processed on coverslips in Petri dishes, were fixed with 4% paraformaldehyde in PBS pH 7.4 for 30 min, and washed twice using PBS. Cells were pre-treated with 10 μg/ml RNase A in PBS for 30 min and then exposed to an equal mixture of PI (1 μg/ml) and AO (1 μg/ml; Life Technologies) diluted in PBS at room temperature in the dark for 10 min [22].

Specimens were observed with a Leica TCS-SP5CLSM as described above (AO and PI excitation were at 488 nm and 500 nm respectively and their emission signals were detected at 617 and 525). CLSM Images are presented as maximum intensity projection or single-plane images. A count of living, apoptotic and necrotic cells has been performed.

2.5. Western Blot analysis

Protein assay was performed using the Bio-Rad protein assay according to the manufacturer's instructions. Cells were lysed at 107/ml in RIPA lysis buffer containing the Complete Protease Inhibitor Cocktail (Thermo Scientific, USA). Lysates were then briefly sonicated to shear DNA and reduce viscosity and boiled for 5 min with reducing sample buffer. Protein separated (25 μg) on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to nitrocellulose membranes using a semidry blotting apparatus. Membranes were saturated for 60 min at room temperature in blocking buffer (PBS supplemented with 5% non-fat milk), then incubated overnight at 4 °C with primary antibody for caspase-8, -9, -3 and PARP (Cell Signaling Technology, Danvers, MA, USA). After four washes in PBS containing 0.1% Tween 20, samples were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody diluted 1:2000 in PBS-Tween 20, and washed as above [23]. Bands were visualized by the ECL method and images were achieved through ChemiDoc-it2 Imager (UVP, Upland, CA, USA).

2.6. Scanning electron microscopy (SEM)

Control and treated cells were directly processed on coverslips in Petri dishes. After careful washing with 0.1 M phosphate buffer, monolayers were fixed with 2.5% glutaraldehyde in the same

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