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Original article

Dual hypopigmentary effects of punicalagin via the ERK and Akt pathways

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ABSTRACT

Punicalagin is a phenolic compound with antioxidant properties. However, the effects of punicalagin on melanin synthesis have been poorly evaluated. Therefore, we investigated the effects of punicalagin on melanogenesis in Mel-Ab cells. Punicalagin significantly inhibited melanin synthesis in a dose-dependent manner. In accordance with the melanin content, punicalagin also dose-dependently decreased tyrosinase activity. Punicalagin did not directly inhibit tyrosinase in a cell-free system but did downregulate the expression of microphthalmia-associated transcription factor (MITF) and tyrosinase. Therefore, we examined the effects of punicalagin on melanogenesis-related signaling pathways. Punicalagin induced extracellular signal-regulated kinase (ERK) and Akt phosphorylation but had no effect on β-catenin level. We measured melanin content and MITF expression in the presence of the ERK pathway inhibitor PD98059 and/or the Akt pathway inhibitor LY294002. Cotreatment with PD98059 and LY294002 almost completely restored punicalagin-induced hypopigmentation. These data indicate that punicalagin inhibits melanin synthesis through ERK and Akt phosphorylation, with subsequent downregulation of MITF and tyrosinase.

1. Introduction

Melanin pigment is produced by melanocytes and is a major determinant of skin, hair, and eye color. It is protective against ultraviolet (UV) irradiation [1,2]. However, melanin accumulation can induce hyperpigmentary skin diseases, including melasma and freckles [3]. Therefore, many studies have focused on developing effective skin-whitening agents [4].

Tyrosinase catalyzes the first two reactions in mammalian melanogenesis [5]. Studies on skin-whitening agents have focused on direct inhibition of tyrosinase. For example, the phenolic derivatives of flavonoids are antioxidants that chelate the copper ion of tyrosinase [6]. These molecules can inhibit tyrosinase and decrease melanogenesis.

Many tyrosinase inhibitors have been identified and investigated. However, knowledge of their effects on melanogenesis is insufficient. Therefore, alternative approaches have also been investigated. Microphthalmia-associated transcription factor (MITF) stimulates tyrosinase expression and is a key transcription factor in melanin synthesis [7]. MITF and tyrosinase expression are regulated by several signal transduction pathways [8,9]. In particular, extracellular signal-regulated kinase (ERK) and Akt contribute to a major signaling pathway associated with melanogenesis [10,11]. One study found that ERK activation decreased melanin production through MITF regulation [12]. Activation of Akt also reduced melanin synthesis via downregulation of MITF [13]. In addition, GSK3β is involved in the melanogenic signaling cascade [11].

Punicalagin is a water-soluble ellagitannin and polyphenolic antioxidant molecule that is extracted from pomegranate fruit and *Terminalia catappa* leaves [14,15]. As a polyphenol, punicalagin has 16 dissociable –OH groups that are responsible for its high antioxidant activity (Fig. 1) [16]. Punicalagin has pharmacological effects, reduces oxidative stress and apoptosis [17], and shows hepatoprotective activity in rats [16]. It has chemopreventive

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase3β; MITF, microphthalmia-associated transcription factor.

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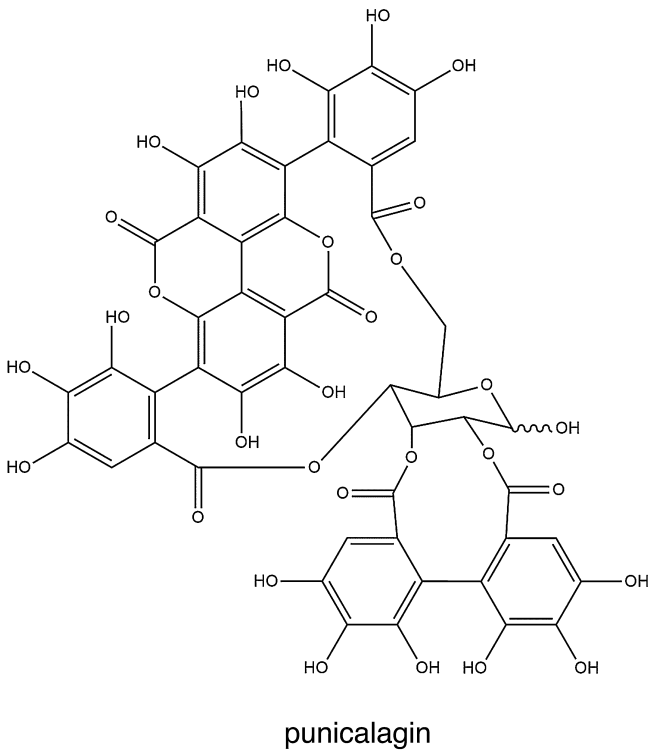

Fig. 1. Structure of punicalagin.

effects in NIH3T3 cells [18] and increases NO production in endothelial cells [19].

Although punicalagin is a bioactive molecule, its effects on melanogenesis remain unclear. A recent study found that punicalagin inhibits melanin production in melanocytes [20]. That study suggested that punicalagin directly inhibits melanin production. However, in our preliminary study, punicalagin did not have a direct inhibitory effect on tyrosinase, which is responsible for melanin production. Therefore, we sought to clarify the effects of punicalagin on melanin synthesis. We hypothesized that punicalagin regulates MITF and tyrosinase expression levels. This study investigated signaling pathways related to melanogenesis.

2. Materials and methods

2.1. Materials

Punicalagin (SLBC2256, Sigma), cholera toxin (CT), 12-O-tetradecanoylphorbol-13-acetate (TPA), and mushroom tyrosinase were from Sigma–Aldrich Co. (St. Louis, MO, USA). Antibodies specific to phospho-Akt (Thr308, 9271S), Akt (#9272), phospho-ERK1/2 (Thr202/Tyr204, #9101S), ERK1/2 (#9102), phospho-GSK3β (#9336S), GSK3β (#9315S), and β-catenin (#9581S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against actin (I-19) and tyrosinase (C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary specific anti-goat IgG (PI-9500), anti-rabbit IgG (PI-1000), and anti-mouse IgG (PI-2000) were purchased from Vector Laboratories (Burlingame, CA, USA).

2.2. Cell culture

The Mel-Ab cell line is a mouse-derived immortalized melanocyte cell line that synthesizes large quantities of melanin [21]. Mel-Ab cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 100 nM TPA, 10% fetal bovine serum (FBS), 1 nM CT, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37 °C in 5% CO_2.

2.3. Cell viability assay

Cell viability was measured using a crystal violet assay [22]. After incubation with punicalagin for 24 h, culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and washed 4 times with distilled water. Crystal violet remaining in adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

2.4. Measurement of melanin content and microscopy

Melanin content was quantified as described previously [23]. Briefly, the cells were treated with punicalagin in DMEM containing 10% FBS for 3 days, dissolved in 550 μL 1 N NaOH at 100 °C for 30 min, and centrifuged at 15,000 rpm for 5 min. The optical density of supernatants containing the same amount of protein was measured at 400 nm with an ELISA reader. Before measuring melanin content, the cells were observed under a phase contrast microscope (Olympus IX50, Tokyo, Japan) and photographed using a DCM300 digital microscope camera (Scopetek, Inc., Hangzhou, China), supported by ScopePhoto software (Scopetek, Inc.).

2.5. Tyrosinase activity

Tyrosinase activity was analyzed as described previously [24], with slight modifications. Mel-Ab cells were seeded in 6-well plates and incubated with punicalagin for 3 days. Cells were washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. Cells were disrupted by freezing and thawing, and lysates were clarified by centrifugation at 15,000 rpm for 10 min. After quantifying protein levels of lysates and adjusting concentrations with lysis buffer, 90 μL lysate (containing the same amount of protein) was placed in wells of 96-well plates, and 10 μL 10 mM L-DOPA was added. Control wells contained 90 μL lysis buffer and 10 μL 10 mM L-DOPA. Following incubation at 37 °C, absorbance at 475 nm was measured every 10 min for at least 1 h using an ELISA reader. A cell-free assay system was used to examine the direct effects of punicalagin on tyrosinase activity. For this assay, 60 μL phosphate buffer containing punicalagin was mixed with 20 μL 53.7 units/mL mushroom tyrosinase, and 20 μL

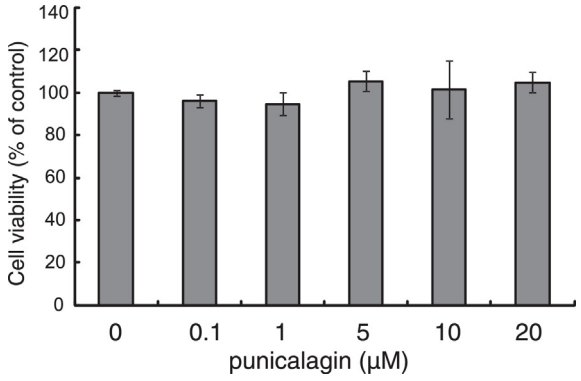

Fig. 2. Effect of punicalagin on cell viability. Mel-Ab cells (4×10^4 cells/well) were seeded into 24-well plates and serum-starved for 24 h. Cells were treated with 0–20 μM punicalagin for 24 h. Cell viability was measured by crystal violet assay.