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Original article

Autophagy participates in isoliquiritigenin–induced melanin degradation in human epidermal keratinocytes through PI3K/AKT/mTOR signaling

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ABSTRACT

Melanin is the pigment responsible for the color of human skin and hair. Melanin serves as a double-edge sword which can exert both protective and spot-causing effects on skin. Although melanin has an important role in protecting the skin against UV damage, an excessive or uneven melanin production can lead to the formation of freckles and age spots. Isoliquiritigenin (ISL) has been reported to inhibit melanin synthesis; however, its role in melanin degradation remains unclear. In the present study, we evaluated the detailed function of ISL in melanin degradation in human epidermal keratinocytes. Since autophagy has been reported to be related to melanin degradation, we also examined the activation of autophagy by ISL treatment in keratinocytes by measurement of autophagy-related proteins, ATG7, LC3 and p62. Moreover, si-ATG7-induced ATG7 knockdown and autophagy inhibitor 3-MA decreased LC3 II protein levels and increased PMEL17, p62 and melanin levels in HaCaT cells, which could be partially reversed by ISL treatment, indicating that autophagy participated in melanin degradation. The decreased p-AKT and p-mTOR proteins upon ISL treatment indicated the involvement of PI3K/AKT/mTOR signaling in ISL-induced melanin degradation. Taken together, we demonstrated that autophagy participates in ISL-induced melanin degradation in human epidermal keratinocytes through PI3K/AKT/mTOR signaling.

1. Introduction

Melanin is the pigment responsible for the color of human skin and hair. It is secreted in the basal layer of the dermis by melanocyte cells [1–3]. Its roles are to protect the skin from ultraviolet (UV) damage and remove reactive oxygen species (ROS) [4,5]. Although melanin has an important role in protecting the skin against UV damage, an excessive or uneven melanin production can lead to the formation of melasma, freckles, age spots and sites of actinic damage. Given the essential role of melanin in determinant of the color of mammalian skin, the inhibition of melanin formation may result in a reduction in skin darkness [6]. The inadequacy of current therapies to treat the indicated problems as well as high cytotoxicity and mutagenicity, poor skin penetration, and low stability of formulations led us to seek new whitening agents to meet the medical requirements for depigmenting.

Isoliquiritigenin (ISL) is an abundant dietary flavonoid with a chalcone structure, which is an important constituent in Glycyrrhizae Radix (GR). ISL has been reported to inhibit the activity of tyrosinase, which is known to be a key enzyme in melanin biosynthesis [7]. The effect of ISL on tyrosinase activity was correlated to its ability to inhibit melanin formation in melanocytes [7]. However, the function of ISL in melanin degradation remains to be uncovered. In the present study, we investigated the role of ISL in melanin degradation in human epidermal keratinocytes, and further explored the possible mechanism.

Autophagy is an intracellular process whereby the cytosol and organelles are sequestered within double-membrane-bound structures, called autophagosomes, which subsequently deliver their contents to lysosomes/vacuoles for degradation [8–11]. Recently, autophagy has been reported to be associated with melanin degradation in human epidermal keratinocytes. Murase et al. revealed that variations in autophagy are associated with ethnic alteration in skin color, via the regulation of melanosome degradation in epidermal keratinocytes [12].

Herein, we evaluated the function of ISL in regulation of melanin degradation in human epidermal keratinocytes; further, the effect of ISL on autophagy-related protein expression was also monitored; by knocking down ATG7, we evaluated the role of autophagy in melanin degradation in human epidermal keratinocytes; finally, the involvement of PI3K/AKT/mTOR signaling in ISL regulation of melanin content in human epidermal keratinocytes was confirmed. Taken together, we demonstrated that autophagy participates in isoliquiritigenin (ISL)-induced melanin degradation in human epidermal keratinocytes through suppressing PI3K/AKT/mTOR signaling.

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2. Materials and methods

2.1. Cell culture and cell transfection

The human melanoma cell line A375 and human epidermal keratinocytes HaCaT cells were obtained from Shanghai Biological Institute (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 mg/ml), and streptomycin (100 mg/ml) and incubated at 37 °C in a humidified chamber using 5% CO_2.

Melanosomes were isolated from A375 cells followed by the method reported previously [13]. Melanosomes released from A375 were collected from cultured medium, according to the method reported [14]. The cultured medium was centrifuged at 800*g* for 5 min at 4 °C. The supernatant was centrifuged at $2 \times 10^4 g$ for 1 h at 4 °C to pellet melanin. Melanin concentration of melanosomes was determined from 490-nm absorbance values using standard solutions made from synthesized melanin. Melanosomes containing 4 μg melanin were added to each well of 6-well plates in which human epidermal keratinocytes (HaCaT cells) had been seeded 1 day earlier. 48 h after treatment, non-incorporated melanosomes were removed by washing three times with phosphate buffered saline (PBS).

For inhibition of autophagy, autophagy inhibitor 3-MA (Sigma, USA) or ATG7 knockdown was utilized,. For 3-MA treatment, after incubating with melanin, HaCaT cells were treated with 5 mM 3-MA for 2 h, then the solution was removed and treated with 8 μg/ml ISL for 24 h. Cells were harvested for further experiments. For ATG7 knockdown, specific small interference RNA (si-ATG7) and negative small RNA control (si-NC) were purchased from Genepharm (Shanghai, China) and transfected into HaCaT cells with si-Mate transfection reagent (Genepharm, China). Cells were harvested after 48 h for further experiments.

2.2. MTT assay

A modified MTT assay was used to evaluate cell viability. 24 h after seeding into 96-well plates (5×10^3 cells per well), cells were incubated with different concentrations of ISL (purity ≥ 98%, purchased from Jiangxi Herb Tiangong Technology Co., Ltd., Jiangxi, China) (0, 1, 2, 4, 8, 16 and 32 μg/ml) for 0, 24, 48 and 72 h; 20 μl MTT (at a concentration of 5 mg/ml; Sigma–Aldrich) was added, and the cells were incubated for an additional 4 h in a humidified incubator. 200 μl DMSO was added after the supernatant discarded to dissolve the formazan. OD_{490nm} value was measured. The viability of the non-treated cells (control) was defined as 100%, and the viability of cells from all other groups was calculated separately from that of the control group.

2.3. Measurement of melanin in keratinocytes

Melanin contents in HaCaT cells were measured according to the method described previously [15]. After incubation with melanosomes for 48 h, HaCaT cells were treated with different concentrations of ISL (0, 1, 2, 4 and 8 μg/ml) for 24 h, HaCaT cells were lysed with 200 μl 1 N NaOH and pipetted repeatedly to homogenize them. After homogenization, the cell extract was transferred into 96-well plates. Relative melanin content was determined by absorbance at 405 nm in an ELISA plate reader.

2.4. Western blot

For PMEL17 protein level determination, HaCaT cells were incubated with melanosomes (isolated from A375 melanocytes) for 48 h, and then cultured in mediums containing different concentrations of ISL (0, 1, 2, 4 and 8 μg/ml) and cultured for 24 h.

For ATG7, LC3 and p62 protein level determination in Fig. 2, HaCaT cells were incubated with melanosomes (isolated from A375 melanocytes) for 48 h, and then cultured in mediums containing 8 μg/ml ISL for 24 h.

For ATG7, LC3, p62 and PMEL17 protein level determination in Fig. 3, HaCaT cells were transfected with si-ATG7, incubated with melanosomes (isolated from A375 melanocytes) for 48 h, and then cultured in mediums containing 8 μg/ml ISL for 24 h.

For AKT, p-AKT, mTOR, p-mTOR and PMEL17 protein level determination in Fig. 4, HaCaT cells were co-incubated with melanosomes for 48 h, and then co-treated with AKT-mTOR inhibitor, LY294002 (10 μM, Beyotime, China) and 8 μg/ml ISL for 24 h.

After indicated treatment, HaCaT cells were rinsed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl p H8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentration was determined using the BCA protein assay kit (Pierce). Protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 3% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then probed with anti-PMEL17 (clone HMB-45, DAKO, USA), anti-ATG7 (A2856, Sigma, USA), anti-LC3 (L8918, Sigma, USA), anti-p62 (ab56416, Abcam, USA), anti-AKT (Cat# Y89, Abcam, USA), anti-p-AKT (Cat# EP2109Y, phospho S473, Abcam, USA), anti-mTOR (ab2732, Abcam, USA) and anti-p-mTOR (Cat# EPR426(2), Abcam, USA). Subsequently the membranes were reacted with secondary IgG antibodies coupled with horseradish peroxidase, and signals were detected by enhanced chemiluminescence (ECL) and visualized with ChemiDOC XRS system (Bio-Rad).

2.5. Statistical analysis

Data were exhibited as mean ± SD of three independent experiments and processed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). The difference among the groups was estimated by Student's *t*-test (two groups) or one-way ANOVA (more than two groups). *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. The effect of ISL on the melanin content in HaCaT cells

To evaluate the function of ISL on melanin content in HaCaT cells, we first tested the safe dose of ISL. HaCaT cells co-incubated with melanosomes were treated with a series of doses of ISL (0, 1, 2, 4, 8, 16 and 32 μg/ml) for 0, 24, 48 and 72 h; then the cell viability was determined using MTT assays. As shown in Fig. 1B, the cell viability was significantly suppressed by 32 μg/ml ISL on 24 h after treatment; 48 h later, the cell viability was significantly suppressed by 16 and 32 μg/ml ISL; moreover, the cell viability under 1, 2, 4 and 8 μg/ml ISL treatments exhibited no significant changes. Based on this, 1, 2, 4 and 8 μg/ml ISL treatments for 24 h were regarded as safe doses of ISL treatment for further experiments.

Further, the effect of ISL treatment on melanin content and melanin protein PMEL 17 was evaluated. HaCaT cells co-incubated with melanosomes were treated with 0, 1, 2, 4 and 8 μg/ml ISL for 24 h; the melanin content and protein level of melanin protein PMEL 17 were then determined. As shown in Fig. 1C, the melanin content in HaCaT cells was significantly reduced by 4 and 8 μg/ml ISL treatment; results from Western blot assays showed that PMEL 17 protein levels were significantly reduced by 4 and 8 μg/ml ISL treatment, more strongly reduced by 8 μg/ml ISL treatment (Fig. 1D). Based on this, 8 μg/ml ISL treatment for 24 h was chosen for further experiments.

3.2. The effect of ISL on autophagy-related proteins

It has been reported that melanosomes intracellularly transferred to