Journal of Dermatological Science 88 (2017) 96–102

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Tranexamic acid inhibits melanogenesis by activating the autophagy system in cultured melanoma cells

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ARTICLE INFO

Article history:
Received 31 March 2017
Received in revised form 19 May 2017
Accepted 31 May 2017

Keywords:
Skin whitening
Tranexamic acid
Autophagy
Melanogenesis
p-ERK

ABSTRACT

Background: As interest in skin beauty increases, the development of new skin whitening agents has attracted substantial attention; however, the action mechanism of the agents developed so far remains largely unknown. Tranexamic acid (TXA) is commonly being used to reduce melanin synthesis in patients with melasma and also used as a raw material for functional whitening cosmetics, although its action mechanism is poorly understood. Autophagy has been well known to be essential for tissue homeostasis, adaptation to starvation, and removal of dysfunctional organelles or pathogens. Recent studies have shown that autophagy regulators might have prominent roles in the initial formation stage of the melanosome, a lysosome-related organelle synthesizing melanin pigments. However, there is still no direct evidence showing a relationship between the activation of the autophagy system and the melanogenesis.

Objective: To investigate whether TXA can inhibit melanogenesis through the activation of autophagy in a melanoma cell line.

Methods: B16-F1 melanoma cells were treated with TXA and the levels of autophagy- and melanogenesis-related proteins were determined by Western blottings. The direct effect of TXA-mediated autophagy activation on melanin production was further evaluated by transfecting the cells with 60 pmols of small interfering RNAs (siRNAs)-targeting the mechanistic target of rapamycin (mTOR) and the autophagy-related protein 5 (Atg5).

Results: The results of Western blottings showed that TXA enhanced the production of autophagy-related proteins such as mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK) 1/2, Beclin-1, Atg12, and light chain 3 (LC3) I–II, whereas it decreased the synthesis of the mTOR complex. Confocal microscopy clearly showed that TXA treatment resulted in the formation of autophagosomes in B16-F1 cells, as revealed by immunostaining with an anti-LC3 antibody. The production of melanogenesis-associated proteins, including microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related protein 1 and 2 (TRP1/2), were clearly downregulated by the treatments with TXA. These results suggest that TXA can mediate a decrease in melanin synthesis by alleviating the production of tyrosinase and TRP1/2, along with lowered MITF protein levels. Furthermore, after treatment with TXA, siRNAs- targeting to mTOR and Atg5 increased melanin synthesis by 20% and 40%, respectively, compared to that in non-transfected cells, in a dose-dependent manner. These results further confirmed that TXA can inhibit melanogenesis by activating the autophagy system.

Conclusion: Collectively, the results demonstrate that TXA can reduce melanin synthesis in melanoma B16-F1 cells by activating the ERK signaling pathway and the autophagy system.

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ERK, extracellular signal-regulated kinase; DHICA, 5,6-dihydroxyin-dole-2-carboxylic acid; LC3, light chain 3; L-DOPA, L-3,4-dihydroxyphenylalanine; mTOR, mechanistic target of rapamycin; MITF, microphthalmia-associated transcription factor; TGF-β, transforming growth factor-beta; TRP1/2, tyrosinase-related protein 1/2; TXA, tranexamic acid; uPA, urokinase-type plasminogen activator; UV, ultraviolet.

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http://dx.doi.org/10.1016/j.jdermsci.2017.05.019

1. Introduction

The colors of the skin, hair, and eye are derived from the biological pigmentation factor melanin, which is produced via melanogenesis within melanosomes [1]. The amount of melanin produced is influenced by various genetic and environmental factors, including exposure to ultraviolet (UV) light. One of the roles of melanin is to protect the skin, tissues, and genes from UV-induced skin injury [2]. In response to UV light, preexisting melanin pigments in melanocytes are immediately redistributed, which is followed by an enhanced melanogenesis in epidermal melanocytes and increased the transfer of melanin to epidermal keratinocytes [3]. However, the formation of excessive melanin in the skin results in hyperpigmentation, which can induce skin disorders, such as melasma, freckles, and geriatric pigment spots [4]. Therefore, studies regarding the regulation and mechanism of melanogenesis are important to identify targets for the prevention and treatment of hyperpigmentation disorders.

Melanin is initially produced in two different forms, eumelanin and pheomelanin, in which tyrosinase and tyrosinase-related protein 1/2 (TRP1/2) are involved as main factors. In the melanogenesis, tyrosinase is a rate-limiting enzyme that catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanin (L-DOPA) and subsequently oxidizes this molecule to form dopaquinone. Eumelanin is synthesized via TRP-2, which functions as a DOPA chrome tautomerase and catalyzes the rearrangement of DOPA chrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA); subsequently, TRP-1 oxidizes DHICA to its carboxylated form, indole-quinone [5]. Pheomelanin is synthesized through a reaction involving dopaquinone and cysteine. In addition, tyrosinase and TRP1/2 are transcriptionally regulated by microphthalmia-associated transcription factor (MITF) in melanocytes [6]. The phosphorylation of MITF by extracellular signal-regulated kinase (ERK) 2 results in the degradation of the former via the proteasome-mediated proteolytic pathway [1].

Cellular autophagy system is well known to play an important role in removing waste proteins (misfolded or aggregated proteins) and also in eliminating dysfunctional organelles, including the mitochondria, endoplasmic reticulum, and peroxisomes [5,7]. Recent studies show that the autophagy system might also be involved in the biogenesis of melanin and the degradation of the melanosome, a lysosome-related organelle synthesizing melanin pigments, suggesting that its activation can be closely related to skin color by reducing the production of melanin [8]. In fact, some studies have shown that autophagy regulators may have prominent roles in the initial stages of melanosome formation [9,10]. Beclin-1 [11], autophagic modulator WIPI1 (a mammalian homolog of ATG18) [12], and microtubule-associated protein light chain 3 (LC3) are potent regulators for the melanogenesis in melanocytic lesions [13]. In addition, a factor called mechanistic target of rapamycin (mTOR) acts as a critical inducer for autophagy system, as its positive regulation suppresses this process through Akt and mitogen-activated protein kinase (MAPK) signaling pathway; however, its negative regulation executed by AMPK and the p53 signaling pathway promotes autophagy [14,15]. The ERK1/2 pathway also plays a key role in regulating autophagy [1,16].

Tranexamic acid (TXA; *trans*-4-aminomethylcyclohexanecarboxylic acid) has been developed initially as a plasmin inhibitor that can be used for treating a heavy bleeding resulted from trauma, surgery, and menstruation [17]. The compound is a synthetic derivative of the amino acid lysine and exerts its effects by reversibly blocking lysine-binding sites on plasminogen molecules [18]. TXA has also emerged as a potential drug for treating melasma, since it can inhibit the melanin synthesis by inhibiting the plasminogen/plasmin pathway, thereby blocking the interaction between melanocytes and keratinocytes [19]. As plasminogen also exists in cultured human keratinocytes, which are known to produce plasminogen activators, it can be expected that TXA may have an effect on the function of keratinocytes [20]. However, the action mechanism of TXA related to its direct involvement in melanogenesis in view of signaling pathway(s) has not yet been studied in detail.

Based on the results from previous studies, we postulated that there would be a mechanistic link between autophagy activation and melanogenesis, which might help to understand the action mechanism of TXA related to its suppressive ability against melanin synthesis. We describe here a relationship between the melanogenesis and the activation of autophagy as well as how TXA involves in inhibiting the melanin synthesis by activating cellular autophagy system in cultured B16-F1 melanoma cell line. The results obtained by the present study demonstrate that TXA has a great potential of becoming a functional agent for the treatment of pigment disorders as well as a cosmetic product for skin whitening.

2. Materials and methods

2.1. Materials

The compound TXA was a kind gift from a Korean company Hugel (Chuncheon, Korea). Mushroom tyrosinase, arbutin, poly-L-lysine (0.01% solution), 1% penicillin-streptomycin, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) was from Lonza (Walkersville, MD, USA). Antibodies raised against MAP-LC3β, GAPDH, tyrosinase, and TRP1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Beclin-1, Atg12, phospho-Erk1/2, p44/42 MAP kinase, phospho-p38 MAP kinase, p38 MAPK, MITF, phospho-mTOR, and mTOR were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Murine melanoma B16-F1 cells were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea) and routinely used by this study. The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS; ATLAS Biologicals, Fort Collins, CO, USA) and 1% penicillin-streptomycin (Sigma-Aldrich; St. Louis, MO, USA) at 37 °C in 5% CO_2 incubator. The culture medium was changed every 2 days [2] and the cells were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Welegen; Daegu, Korea) when they reached to approximately 70% confluency.

2.3. Cell viability assay

Cell viability was evaluated using the CellTiter 96® Non-Radioactive cell proliferation assay (Promega; Madison, WI, USA). B16-F1 cells cultured at a density of 0.5×10^5 cells/well in 96-well plates for 24 h were treated with various concentrations of TXA (0.1–10 mg/ml), arbutin (0.1–10 mg/ml), and TGF-β (0.00002–0.2 ng/ml) for 24 h. At the end of the incubation, 15 μl of MTT dye solution was added to each well and further incubated at 37 °C in 5% CO_2 for 4 h. After adding 100 μl of the Solubilization Solution/Stop Mix per well, the reaction was continued for 1 h and the absorbance at 570 nm was measured using a 96-well plate reader.

2.4. Western blot analysis

B16-F1 cells were seeded at a density of 0.5×10^5 cells/well in 6-well culture plates. After incubating for 24 h, the cells were treated with TXA (1 mg/ml) or TGF-β (10 ng/ml) to induce autophagy for 15 min. In addition, to induce melanogenesis, the cells were treated