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Research Paper

IDH2 deficiency accelerates skin pigmentation in mice via enhancing melanogenesis

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ABSTRACT

Melanogenesis is a complex biosynthetic pathway regulated by multiple agents, which are involved in the production, transport, and release of melanin. Melanin has diverse roles, including determination of visible skin color and photoprotection. Studies indicate that melanin synthesis is tightly linked to the interaction between melanocytes and keratinocytes. α-melanocyte-stimulating hormone (α-MSH) is known as a trigger that enhances melanin biosynthesis in melanocytes through paracrine effects. Accumulated reactive oxygen species (ROS) in skin affects both keratinocytes and melanocytes by causing DNA damage, which eventually leads to the stimulation of α-MSH production. Mitochondria are one of the main sources of ROS in the skin and play a central role in modulating redox-dependent cellular processes such as metabolism and apoptosis. Therefore, mitochondrial dysfunction may serve as a key for the pathogenesis of skin melanogenesis. Mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDH2) is a key enzyme that regulates mitochondrial redox balance and reduces oxidative stress-induced cell injury through the generation of NADPH. Downregulation of *IDH2* expression resulted in an increase in oxidative DNA damage in mice skin through ROS-dependent ATM-mediated p53 signaling. IDH2 deficiency also promoted pigmentation on the dorsal skin of mice, as evident from the elevated levels of melanin synthesis markers. Furthermore, pretreatment with mitochondria-targeted antioxidant mito-TEMPO alleviated oxidative DNA damage and melanogenesis induced by IDH2 deficiency both *in vitro* and *in vivo*. Together, our findings highlight the role of IDH2 in skin melanogenesis in association with mitochondrial ROS and suggest unique therapeutic strategies for the prevention of skin pigmentation.

1. Introduction

Melanogenesis, the process of melanin pigment production, is a complex biosynthetic pathway regulated by multiple agents, which are involved in the production, transport, and release of melanin [1]. Melanin has diverse roles and functions in various organisms; for instance, the visible skin color is determined by the distribution of melanin in the skin epidermis [2]. Numerous studies have indicated melanin as an important photo-protective factor involved in the absorption and reflection of UV irradiation [3]. In addition, melanin functions as an antioxidant by scavenging oxidative free radicals [4].

Melanogenesis is tightly linked to the interaction between melanocytes and keratinocytes [5]. Melanogenesis in melanocytes is under the control of secreted factors from neighboring keratinocytes [6]. Keratinocytes produce and secrete factors such as α-melanocyte-stimulating factor (α-MSH), which regulates and stimulates the epidermal melanogenesis of melanocytes [7–10]. The stimulated melanosomes in melanocytes synthesize melanin and induce the transfer of melanin from melanocytes to keratinocytes [5]. The microphthalmia-transcription factor (MITF) is the most important factor that regulates the differentiation and development of melanocytes in melanogenesis [11]. MITF controls the expression of melanocyte-specific proteins such as tyrosinase and tyrosinase-related protein 1 and 2 (TRP1 and TRP2), which regulate the synthesis of melanin [12,13].

Reactive oxygen species (ROS) are shown to play a major role in skin melanogenesis [14]. Skin is continuously exposed to harmful environmental sources such as UV irradiation, resulting in the generation of ROS, which consequently cause DNA damage in both keratinocytes and melanocytes [15–17]. ROS may be transported from keratinocytes to melanocytes and affect several biosynthetic processes in melanocytes [18]. An increase in ROS level was observed during melanogenesis [15], and treatment with antioxidants was shown to inhibit skin pigmentation [19].

In human skin, mitochondria are one of the main sources of ROS

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[20] and are major organelles involved in the regulation of cellular redox status [21]. Accumulating evidence indicates that mitochondria are sensitive to oxidative stress, as these are the major sites of ROS generation and exhibit limited function to eliminate oxidants [22]. Excess ROS production induces mitochondrial dysfunction and impairs oxidative stress balance, which are believed to play a crucial role in the pathogenesis of metabolic diseases [23–25]. Numerous studies have indicated that mitochondria play a role in processes such as melanogenesis [26]. The complex V of mitochondria has been identified as the modulator of skin pigmentation [27]. In addition, mitochondria were shown to regulate the biogenesis of melanosomes [28].

Mitochondrial isocitrate dehydrogenase 2 (IDH2), a subtype of isocitrate dehydrogenases, is located in mitochondria and known to generate NADPH [29]. IDH2 plays the role of an antioxidant protein against ROS by converting $NADP^+$ to NADPH, which promotes the regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) [30]. The role of IDH2 is very important in the pathogenesis of some metabolic diseases, and the deficiency of IDH2 results in various ROS-mediated metabolic syndromes [31,32]. Therefore, IDH2 may play an important role in melanogenesis [33].

In the present study, we examined the role of IDH2 in skin melanogenesis by using both *in vitro* and *in vivo* models. We found that IDH2 deficiency results in the generation of ROS in the mitochondria and secretion of α-MSH in keratinocytes, eventually leading to an increase in melanin production in melanocytes. We believe that the knowledge of the involvement of mitochondria in skin melanogenesis may be useful to design potential therapeutic strategies for skin disorders.

2. Materials and methods

2.1. Materials

Gil No. 3 hematoxylin and eosin Y (H&E) solution, xylenol orange, L-DOPA, and mito-TEMPO were obtained from Sigma-Aldrich (St. Louis, MO). The Masson-Fontana staining kit was purchased from BioGnost (Zagreb, Croatia). JC-1 mitochondrial membrane potential probe was supplied by Thermo Fischer Scientific (Waltham, MA), and 2′,7′-dichloro-fluorescein diacetate (DCFH-DA) by Molecular Probes (Eugene, OR). MitoSOX Red was procured from Invitrogen (Eugene, OR), and the cAMP assay kit from Abcam (Cambridge, MA). The mouse α-MSH ELISA kit was obtained from Abbkine (Wuhan, China). The antibodies used in this study were acquired from Cell Signaling (Beverly, MA), Abcam (Cambridge, MA), Santa Cruz Biotechnology (Santa Cruz, CA), Virogen (Watertown, MA), Biorbyt (Cambridge, UK), Calbiochem (San Diego, CA), and AbFrontier (Seoul, Korea).

2.2. Cell culture

The murine keratinocyte cell line JB6 CI 41-5a was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Minimum essential medium (Eagle) containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 5% fetal calf serum (FBS), and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. B16F10 mouse melanoma cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured with growth medium as previously described [31]. For the conditioned medium, JB6 cells transfected with *IDH2* shRNA and non-target shRNA were cultured under conditions previously described for 48 h [34]. After 48 h, the supernatant was collected and used as the conditioned medium. B16F10 cells were cultured with the conditioned medium for 48 h. The conditioned medium was changed every day. For mito-TEMPO treatment, JB6 cells transfected with *IDH2* shRNA were cultured with the described condition for 24 h. After 24 h, cells were treated with 200 nM of mito-TEMPO for 24 h. Following incubation, the supernatant was collected and used as the conditioned medium. B16F10 cells were cultured in the presence of conditioned medium and mito-TEMPO-treated medium for 48 h. The conditioned medium was changed every day.

2.3. IDH2 shRNA knockdown

IDH2 short-hairpin RNA (shRNA) and non-target shRNA MISSION® lentiviral transduction particles were purchased from Sigma. JB6 cells were transduced with hexadimethrine bromide at a final concentration of 8 μg/mL, as per the manufacturer's protocol. Transduced cells were selected as single colonies in medium containing 5 μg/mL puromycin (Clontech, Mountain View, CA) and maintained in medium containing 1 μg/mL puromycin.

2.4. Animal protocols

All animal experiments were reviewed and approved by the Kyungpook National University Institutional Animal Care and Use Committee. We used 10–12 weeks old male C57BL/6 mice. The mice had the following genotypes: wild-type (WT, $IDH2^{+/+}$) or knockout (KO, $IDH2^{-/-}$). Mice were identified by polymerase chain reaction (PCR) genotyping, as previously described [31]. Animals were housed at a consistent 22 °C temperature and 12 h of light/dark cycle; the dorsal skin was shaved with a trimmer, followed by animal euthanization. For mito-TEMPO experiments, mice received a daily injection of mito-TEMPO (0.7 mg/kg, intraperitoneal) starting at week 6 for 30 days [35].

2.5. Immunoblot analysis

Total protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands were transferred onto nitrocellulose membranes and probed with appropriate primary antibodies. Proteins were visualized using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Protein expression was analyzed using Image J software.

2.6. Measurement of melanin content

B16F10 cells were incubated with the conditioned media for 2 days. After incubation, cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS), and centrifuged at 13,000 ×*g* for 15 min. Pellets were dissolved in 1 N sodium hydroxide (NaOH) containing 10% dimethyl sulfoxide (DMSO) for 30 min at 56 °C. Cell lysates were placed in a 96-well microplate and the absorbance measured at 405 nm wavelength. The mice dorsal skin samples were homogenized and centrifuged at 13,000 ×*g* for 15 min. The supernatants were collected. Skin tissue lysate was dissolved in 1 N NaOH and the procedure was performed, as previously described. Relative melanin production was calculated by normalizing the absorbance values with the protein concentrations (absorbance/μg protein).

2.7. Measurement of tyrosinase activity

B16F10 cells were incubated with conditioned media for 2 days. Cell were washed with ice-cold PBS and lysed with PBS containing 1% Triton X-100. After centrifugation at 13,000 ×*g* for 15 min, the supernatant was collected. The amount of cell lysate was adjusted with lysis buffer as protein concentration. A total of 50 μL cell supernatant and 10 μL of 2 mg/mL L-DOPA were added in a well of a 96-well plate. After incubation at 37 °C for 1 h, absorbance values were recorded at 475 nm wavelength. Skin tissue lysates were prepared, as previously described.