Antimelanogenic effect of ginsenoside Rg3 through extracellular signal-regulated kinase-mediated inhibition of microphthalmia-associated transcription factor

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ARTICLE INFO
Article history:
Received 5 October 2014
Received in Revised form 26 December 2014
Accepted 8 January 2015
Available online 22 January 2015

Keywords:
- antimelanogenic effect
- ginsenoside Rg3
- Panax ginseng

ABSTRACT

Background: Panax ginseng has been used to prolong longevity and is believed to be useful for improving skin complexion. Ginsenosides are the active components isolated from ginseng, and ginsenoside Rg3 (G-Rg3) in particular has been demonstrated to possess antioxidative, antitumorigenic, and anti-inflammatory properties. The aim of this study was to examine the ability of G-Rg3 to inhibit melanogenesis.

Methods: The effects of G-Rg3 on melanin contents and the protein levels of tyrosinase, microphthalmia-associated transcription factor (MITF), and tyrosinase-related protein 1 (TRP1) were evaluated. Melanogenesis-regulating signaling molecules such as Akt and extracellular signal-regulated kinase (ERK) were also examined to explore G-Rg3-induced antimelanogenic mechanisms.

Results: G-Rg3 was found to significantly inhibit the synthesis of melanin in normal human epidermal melanocytes and B16F10 cells in a dose-dependent manner. The activity of cellular tyrosinase and the expression of MITF, tyrosinase, and TRP1 were all reduced, whereas ERK was strongly activated. PD98059 (a specific inhibitor of ERK) attenuated the G-Rg3-induced inhibition of melanin synthesis and tyrosinase activity.

Conclusion: Taken together, these results showed that G-Rg3 induces the activation of ERK, which accounts for its antimelanogenic effects. G-Rg3 may be a promising safe skin-whitening agent, adding to the long list of uses of P. ginseng for the enhancement of skin beauty.

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1. Introduction

Melanin is the pigment responsible for skin and hair color that is synthesized in melanosomes by melanocytes. Although melanin plays an important protective role against UV light, melanin overproduction and accumulation can cause serious cutaneous pigmentation disorders such as freckles, melasma, and age spots [1,2]. Thus, the inhibition of melanogenesis has been the focus of medicinal and cosmetic treatments for skin pigmentation diseases [1,2]. Although most of previous antimelanogenesis experiments were carried out in B16 melanoma cells, reduction of melanin activity in human normal epidermal melanocytes should be demonstrated to confirm the effect of possible whitening agents in human skin [3–5]. Melanogenesis is regulated by the key enzyme, tyrosinase, and additional enzymatic proteins such as tyrosinase-related proteins TRP1 and TRP2 [1–4]. Microphthalmia-associated transcriptional factor (MITF) plays a critical role in the regulation of melanin synthesis and transcription of the key enzyme tyrosinase [3–5].

Panax ginseng has been commonly used as an herbal medicine in Asia for more than 2,000 years and currently occupies an important place among the tonic remedies used in Oriental medicine. In North America, ginseng species such as Panax quinquefolius represent an important industry for both the domestic and export markets [6,7]. Currently, over 40 ginsenosides have been explored and classified into several types in accordance with their specific chemical
structures, such as protopanaxadiols, protopanaxatriols, and ole-anic acids [6–8]. Recently, many investigational studies have shown that ginsenosides are biologically active components in antioxidant, antineoplastic, anti-inflammatory, and biomodulatory processes [7,9–13]. Ginsenoside Rg3 (G-Rg3), a tetracyclic terpenoid saponin monomer, is the primary bioactive component of ginseng extract and has been reported to have various biological effects, including antioxidant effects that may influence melanogenesis [7,8,14,15]. However, the inhibitory effect of G-Rg3 on melanogenesis has not been reported to date. In this study, we have evaluated the inhibitory effect of G-Rg3 on melanin biosynthesis in B16F10 cells and normal human melanocytes. In addition, the molecular mechanisms underlying the antimelanogenic action of G-Rg3 were further evaluated.

2. Materials and methods

2.1. Chemicals and antibodies

Arbutin, alpha-melanocyte-stimulating hormone (α-MSH), 3,4-dihydroxyphenylalanine (l-DOPA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and PD98059 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies recognizing phospho-extracellular signal-regulated kinase (p-ERK, 49/42), MSH receptor 1 (α-MSH), TRP1 (H-90), MITF (H-50), and actin (H-300) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Melanocytes at between three and seven passages were used for the experiments. Antibodies recognizing phospho-extracellular signal-regulated kinase (p-ERK, No. 9101) and phospho-AKT (p-AKT, No. 9271) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antityrosinase (H-109), TRP1 (H-90), MITF (H-50), and actin (H-300) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and cell viability assay

B16F10 mouse melanoma cells [CRL 6475] were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Normal human melanocytes (neonatal/moderately pigmented) were cultured in medium 254 supplemented with human melanocyte growth supplement (Cascade Biologics, Invitrogen, Carlsbad, CA, USA). Melanocytes at between three and seven passages were used for analysis. The melanocyte culture was fed two times weekly and incubated in a humidified atmosphere at 37°C and 5% CO2.

After incubating the cells with 20, 40, 60, 80, or 100 μM of G-Rg3 for 48 h at 37°C in an atmosphere containing 5% CO2, MITT was added to each well at one tenth of the volume of media. The cells were incubated at 37°C for 3 h, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a spectrophotometer.

2.3. Measurement of melanin content

Normal human melanocytes and B16F10 cells were pretreated with various concentrations (20, 40, and 60μM) of G-Rg3 or with 50 μg/mL arbutin control for 72 h. Cell pellets were then dissolved in a 200 μL aliquot of 1N NaOH in 10% DMSO at 100°C for 30 min and centrifuged at 13,000 rpm for 10 min. The relative melanin content was measured using a microplate reader at 415 nm. The value of each measurement is expressed as a percentage change from the control.

2.4. Tyrosinase activity assay

Tyrosinase activity was estimated by measuring the rate of dopachrome formation from l-DOPA. Cells grown in six-well plates were treated with 200μM α-MSH in the presence of 20, 40, or 60μM G-Rg3 or 50 μg/mL arbutin (control) in DMEM for 72 h. The cells were then washed in ice-cold phosphate-buffered saline and lysed in 150 μL of sodium phosphate buffer (0.1M, pH 6.8) containing 0.5% Triton X-100 and 0.1mM phenylmethylsulfonyl fluoride. The cellular extract was centrifuged at 13,000 rpm for 20 min at 4°C. The tyrosinase substrate, 10 μL of 10mM l-DOPA, was added to 90 μL of the supernatant sample. Dopachrome formation was assayed by measuring the absorbance at 492 nm.

2.5. Western blot analysis

B16F10 cells were cultured in 60-mm diameter dishes with or without 200nM α-MSH and 20, 40, or 60μM G-Rg3. Cell pellets were harvested and lysed using radioimmunoprecipitation assay lysis buffer (EMD Millipore, Billerica, MA, USA). The samples were resolved by 4–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and then exposed to the appropriate primary antibodies such as MITF, tyrosinase, TRP1, p-ERK, p-AKT, and β-actin. The proteins were visualized by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) using horseradish peroxidase-conjugated antirabbit secondary antibodies.

2.6. Statistical analysis

The data in this report are representative of three or more experiments under same conditions and are expressed as the mean ± standard error of the mean. Differences between means were tested for significance by one-way analysis of variance test using a statistical software package (SPSS version 18.0; SPSS Inc., Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of G-Rg3 on cell viability

To exclude the possibility that the inhibitory effects of G-Rg3 on melanogenesis are caused by suppression of cell growth, a cell viability test was performed. Treatment with G-Rg3 at concentrations ranging from 20 to 60μM had no cytotoxic effect in B16F10 cells. Based on these results, G-Rg3 concentrations in the range of 20–60μM were used for the remainder of the experiments. Normal human melanocyte, no significant changes in cell viability were found at concentrations as high as 40μM G-Rg3, and more than 80% of cells were alive at 60μM G-Rg3 concentration (Fig. 1).

3.2. Inhibition of melanin biosynthesis and tyrosinase activity

To investigate the antimelanogenic mechanism of G-Rg3, cells were exposed to tyrosinase inhibitors α-MSH (200nM) or arbutin (50 μg/mL) in the presence of G-Rg3 (20–60μM), and subsequently, the melanin content and cellular tyrosinase activity of cells were measured. The melanin content was found to be remarkably reduced by the G-Rg3 treatment in a dose-dependent manner in both B16F10 cells (Fig. 2A) and normal human melanocytes (Fig. 2B). At concentration ≥ 40μM, G-Rg3 caused a significant reduction in melanin content in normal human melanocytes (Fig. 2B). Tyrosinase activity also showed a consistent and dose-dependent decrease in response to the G-Rg3 treatment (Fig. 2A). The melanin content and tyrosinase activity in the 60μM G-Rg3-treated and arbutin-treated cells were significantly lower than those in α-MSH-treated cells (p < 0.05). Moreover, the melanin content and tyrosinase activity in 60μM G-Rg3-treated cells were
remarkably lower than the levels seen in arbutin-treated cells. These results indicate that G-Rg3 inhibits tyrosinase activity and melanin production without affecting the cellular viability. Moreover, the inhibitory activity of G-Rg3 on melanogenesis is due to inhibition of the melanogenesis pathway involving the key melanogenesis enzyme, tyrosinase.

3.3. Effects of G-Rg3 on melanogenic protein expression and intracellular signaling involved in melanogenesis

To determine whether the inhibitory activity of G-Rg3 is related to the melanogenesis pathways involving MITF, tyrosinase, and TRP1, B16F10 cells were treated with G-Rg3, followed by stimulation with α-MSH for 48 h. The cells were then analyzed by Western blot analysis. The protein levels of MITF, tyrosinase, and TRP1 in α-MSH-stimulated cells decreased following treatment with G-Rg3 (Fig. 3A). The decreased expression levels of MITF, tyrosinase, and TRP1 were most prominent following treatment with 60 μM G-Rg3, consistent with the melanin content and tyrosinase activity measurements. These results indicate that G-Rg3 suppresses the expression of tyrosinase protein by downregulating the expression of MITF, a master transcription factor of the tyrosinase gene.

As for the intracellular signaling, ERK and/or Akt activation is well-known to be involved in the regulation of melanogenesis. We then investigated the effects of G-Rg3 on the ERK and AKT signaling pathways. Akt signaling was activated inconsistently. The activation of p-ERK was induced by the G-Rg3 treatment, most notably at concentrations of 40 and 60 μM (Fig. 3A). Moreover, serial time-dependent blotting of ERK activation revealed that p-ERK levels were markedly increased 48 h after the α-MSH treatment (Fig. 3B). These results suggest that the suppressive mechanism of G-Rg3 in melanogenesis is related to the activation of ERK signaling.

3.4. Role of the ERK pathway in the inhibition of melanin synthesis

We cultured B16F10 cells with or without PD98059 (a specific inhibitor of the ERK pathway) for 72 h to determine whether G-Rg3
inhibits melanogenesis through the ERK pathway. Treatment with PD98059 inhibited the G-Rg3-induced antimelanogenic effect in α-MSH-stimulated cells and led to the recovery of tyrosinase activity and melanin content. The melanin content and tyrosinase activities of cells co-treated with G-Rg3 and α-MSH were significantly increased following treatment with PD98059 ($p < 0.05$, Fig. 4). These results suggest that G-Rg3-mediated ERK activation contributes to reduced melanin synthesis through MITF and its downstream expression of tyrosinase and TRP1.

4. Discussion

The abnormal production and accumulation of melanin on the exposed area such as the face and neck are characteristic of several common hyperpigmentation disorders such as melasma, post-inflammatory hyperpigmentation, freckles, uneven skin tone, and solar lentigo. These skin disorders can pose a serious esthetic problem for many people, especially for those in Asian countries and exhibits a wide range of medicinal effects [10–14,23]. As a result, many studies have been conducted in attempts to develop new and safe skin-whitening agents.

Today, natural extracts are a valuable resource for the development of new drugs. Ginseng, which refers to the root of _Panax ginseng_ and its related species, has been used for thousands of years in Asian countries and exhibits a wide range of medicinal effects [10–14,23]. The pharmacological effects of ginseng have been demonstrated in the central nervous system as well as in the cardiovascular, endocrine, and immune systems [15,24–28]. In addition, ginseng and its constituents have been ascribed to have antineoplastic, antistress, and antioxidant activities [27–30]. The herb ginseng has many active components, and numerous studies have suggested that ginseng has a variety of beneficial effects. The active constituents found in most ginseng species are ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids. However, most of the pharmacological actions of ginseng are attributed to ginsenosides [7,15,24,27–30]. Approximately 40 ginsenoside compounds have been identified to date, each having different pharmacological effects and underlying mechanisms due to their different chemical structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiols and protopanaxatriol groups. The sugar moieties in the protopanaxadiol group attach to position 3 of dammarane-type triterpene structures. Based on their chemical structures, the ginsenosides are generally classified into two groups, namely, the protopanaxadiol group attaches to position 3 of the dammarane-type triterpene structure.
moieties in the propanoxatril group attach to position 6 of dammarane-type triterpene such as in Re, Rf, Rg1, Rg2, and Rh1. Among these compounds, the most commonly studied ginsenosides are Rb1, Rg1, Rg3, Re, Rd, and Rf [25–27,30]. Rg3, a tetracyclic triterpenoid saponin monomer, is one of the most active ingredients found in ginseng extracts and is reported to possess various biological effects such as anti-inflammatory, anti-melanogenic, antioxidant, and anti-inflammatory activities [7,8,14,15,24]. Rg3 exerts protective effects attributed to its antioxidant ability by increasing cellular antioxidant enzyme levels and acting as a free-radical scavenger. Moreover, G-Rg3 has been shown to have the strongest antioxidant activity among all ginsenosides [25,26,30]. Oxidative stress caused by excess reactive oxygen species and ultraviolet light is causally linked to skin disorders. Inhibition of reactive species and scavenging of reactive species are thought to reduce hyperpigmentation [28,29]. Antioxidants, such as ascorbic acid derivatives and α-tocopherol, may prevent or delay pigmentation [29]. Because of these potential roles, we hypothesized that G-Rg3 may possess an antimelanogenic effect. To date, there have been no reported scientific studies addressing this possibility. Therefore, we investigated the inhibitory effects of G-Rg3 on α-MSH-induced melanogenesis and the possible underlying molecular mechanisms. Melanogenesis is regulated by various intracellular signaling mechanisms by the action of protein kinases such as cyclic adenosine monophosphate-dependent protein kinase A, protein kinase C-α, protein kinase C-β, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K). Among these, ERK was shown to regulate the expression and function of melanogenic enzymes and MITF [5,31]. Moreover, inhibition of the ERK pathway in B16F10 melanoma cells leads to hyperpigmentation by the upregulation of tyrosinase activity and cellular differentiation. In addition, the PI3K/Akt signaling pathway downregulates the expression of MITF and causes antimelanogenic effect [1,18–21].

Previous studies have also shown that tyrosinase, TRP1, and TRP2 are regulated by MITF, which is downregulated by both ERK and Akt activation [1,19–21]. Our present results show that G-Rg3 downregulates the expression of MITF, tyrosinase, and TRP1, leading to a reduction in the activity of cellular tyrosinase and melanin content. Furthermore, G-Rg3 induces the phosphorylation of ERK; however, the addition of PD98059, a specific inhibitor of the ERK pathway, attenuates the G-Rg3-induced inhibition of melanin synthesis and tyrosinase activity. Taken together, G-Rg3 inhibits melanogenesis by activating the ERK pathway-mediated suppression of MITF and downstream signaling molecules such as tyrosinase and TRP1. To our knowledge, this is the first report on the inhibitory effects of G-Rg3 on melanin production. These results indicate that G-Rg3 is a potential new and safe skin-whitening agent for enhancing skin beauty and for the treatment of hyperpigmentation disorders.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by a 2010 Samsung Biomedical Research Institute grant (Grant No. C-B0-313-1).

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