Research article

Melanogenesis inhibition activity of floralginsenoside A from Panax ginseng berry

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ABSTRACT

Background: Panax ginseng is a traditional herb used for medicinal purposes in eastern Asia. P. ginseng contains various ginsenosides with pharmacological effects. In this study, floralginsenoside A (FGA), ginsenoside Rd (GRD), and ginsenoside Re (GRE) were purified from P. ginseng berry.

Methods: Chemical structures of FGA, GRD, and GRE were determined based on spectroscopic methods, including fast atom bombardment mass spectroscopy, 1D-nuclear magnetic resonance, and infrared spectroscopy. Inhibitory activities of these compounds on melanogenesis were studied by measuring the expression of protein and melanin content in the melan-a cell line. This inhibitory activity was confirmed by observing pigmentation and tyrosinase activities of zebrafish.

Results: GRD, GRE, and FGA were not cytotoxic at concentrations less than 20μM, 80μM, and 160μM in melan-a cells, respectively. GRD, GRE, and FGA inhibited melanin biosynthesis in melan-a cells by 15.2%, 22.9%, and 23.9% at 20μM, 80μM, and 160μM, respectively. FGA was observed to display the most potent inhibitory effect.

Conclusion: FGA showed the most potent inhibition of melanogenesis in both in vitro and in vivo studies. This study suggests that FGA purified from P. ginseng may be an effective melanogenesis inhibitor.

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1. Introduction

Melanin pigment has several functions, including protection of the skin from ultraviolet light and removal of reactive oxygen species [1,2]. Nevertheless, excessive production of melanin pigment and its accumulation in the skin can cause pigmentation disorders, such as solar lentigo, melasma, freckles, and post-inflammatory hyperpigmentation [3,4]. Several known depigmenting agents such as kojic acid and arbutin have already been the focus of studies and are currently being utilized as cosmetic additives [5]. However, it is necessary to find more effective and safer depigmenting agents because of the potential carcinogenic side effects of kojic acid [6]. Commercially available skin depigment cosmetics have displayed many safety concerns and low efficacy. Therefore, research into the separation of novel compounds from a natural source to prevent pigmentation is increasing.

Ginsenosides normally have the steroidal structures connected with sugar moieties. They are found in about 30 different forms of Panax ginseng [7]. Ginsenosides are reported to have various cellular activities, including immunomodulating, antitumor, anti-oxidative, and anti-inflammatory effects, and the ability to enhance cerebral blood flow [8–10]. Several studies have reported the discovery of inhibitors of melanin synthesis in P. ginseng [11–13]. However, these studies were performed with phenolic compounds.
and cinnamic acid [11–13]. We isolated floral ginsenoside A (FGA), ginsenoside Rd (GRD), and ginsenoside Re (GRE) from P. ginseng berry. In other studies of GRD and GRE, various physiological activities have been reported, such as antioxidant, anti-inflammatory, antidiabetic, anticancer, and immunomodulating activities [14–17]. However, there has been no research on the whitening activity of FGA, GRD, and GRE purified from P. ginseng berry, and estimated its potential as a new depigmentation substance.

2. Materials and methods

2.1. General

Kieselgel 60 and LiChroprep RP-18 resins were used for column chromatography (Merck, Darmstadt, Germany). Kieselgel 60 F254 (Merck) and RP-18 F254S (Merck, Darmstadt, Germany) were used as solid phases for the TLC experiment. Fast atom bombardment–mass spectrometry (FAB–MS) spectrum was recorded on a JMS–700 (JEOL, Tokyo, Japan). The IR spectra were obtained from a Spectrum (One FT-IR spectrometer (Perkin Elmer, Buckinghamshire, England)). The NMR spectra were recorded on a Varian Inova AS 400 spectrometer (400 MHz; Varian, Palo Alto, CA, USA). The compounds 12-O-tetradecanoylphorbol-13-acetate, 1-phenyl-2-thiourea (PTU), L-3,4-dihydroxyphenylalanine (L-DOPA), and L-tyrosine were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), antibiotics (penicillin, streptomycin) and cell culture media were obtained from Gibco BRL Life Technology (Rockville, MD, USA). Antiextracellular signal-regulated kinase (anti-ERK)1/2, anti-phosphorylated ERK1/2, anti-Akt, anti-phosphorylated Akt, and antismouse and antirabbit immunoglobulin G antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against microphthalmia-associated transcription factor (MITF) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Tyrosinase was gifted by Dr V.J. Hearing, National Institutes of Health, Bethesda, MD, USA. The cell culture media and additives were purchased from Gibco (Grand Island, NY, USA).

2.2. Plant materials

Six-yr-old P. ginseng berry was preserved and cultivated at the experimental field of the National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration, Chungbuk Province, Korea, in 2014. Voucher specimen (NIHHS140101) was deposited at the herbarium of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong, Korea.

2.3. Extraction and isolation

Fresh ginseng (5 kg) was extracted twice with 90% methanol at room temperature. Subsequently, the methanol extract was partitioned with water, ethylacetate, and n-butanol (n-BuOH) which were purchased from solvent (DAEJUNG Chemicals, Korea). Repeated silica gel and octadecyl silica gel resins used for column chromatography, and prep-liquid chromatography of BuOH fractions yielded three ginsenosides. A rare compound, FGA, along with two well-known ginsenosides, GRE and GRD, was isolated from the BuOH fraction. Identities of these compounds were confirmed by comparing their measured spectroscopic data with those reported in the literature [18–20].

2.4. Physicochemical characteristics of FGA, GRD, and GRE

FGA: white amorphous powder; Mp: 167–168°C; FAB/MS m/z: 831 [M–H]–; IR (KBr, v): 3,469 cm–1, 1,655 cm–1, 1,462 cm–1, 1,076 cm–1; 1H- and 13C-NMR data are same as the literature value [18]. GRE: white powder; Mp 186–187°C; FAB/MS m/z: 945 [M]+; IR (KBr, v): 3,359 cm–1, 2,929 cm–1, 1,642 cm–1, 1,072 cm–1, 1,045 cm–1; 1H- and 13C-NMR data are same as the literature value [19]. GRD: white powder; Mp 182–183°C; FAB/MS m/z: 969 [M]+; IR (KBr, v): 3,366 cm–1, 2,943 cm–1, 1,647 cm–1, 1,077 cm–1, 1,034 cm–1; 1H- and 13C-NMR data are same as the literature value [20].

2.5. Maintenance of cells

Melan-a cells are an immortalized normal murine melanocyte cell line derived from C57BL/6 mice, with high melanin pigmentation. The melan-a cell line was donated by Dr Dorothy Bennett (St. George’s Hospital, London, UK). These cells were maintained in RPMI-1640 culture medium supplemented with 10% (v/v) FBS (fetal bovine serum), 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 µM 12-o-tetradecanoylphorbol-13-acetate in a humidified atmosphere of 5% CO2 at 37°C. Cell viability was measured using CCK-8 cell counting kit-8 (Dojindo, Japan).

2.6. Measurement of melanin content

Melan-a cells were treated with three ginsenosides (FGA, GRD, and GRE). The cells were then lysed in 1N NaOH (Ducsan, Korea) at 60°C for 30 min. The melanin content was then determined in the lysates at 450 nm. The data were normalized by measuring the protein content of the cell lysates with a BCA (Bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

2.7. Western blot analysis

Melan-a cells were lysed in lysis buffer (Radioimmuno-precipitation assay buffer with Noniodet P-40, protease inhibitor, and phosphatase inhibitor). Cell debris was removed, and the resulting supernatant was used for Western blot analysis. Protein concentration of the cell lysate was determined using the BCA protein assay kit. Proteins in cultured cell homogenates were separated on 8% SDS (sodium dodecyl sulfate)-polyacrylamide gel, transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), and subsequently subjected to Western blot analysis using particular antibodies. The immunoreactive antigen was then visualized using the HRP (Horseradish peroxiase)-coupled secondary antibodies and the enhanced chemiluminescence detection kit from Pierce Biotechnology (Rockford, IL, USA).

2.8. Origin and maintenance of zebrafish embryo

Zebrafish embryos were obtained from a zebrafish organogenesis mutant bank in Gyeongbuk National University. Zebrafishes were kept in a 3.5-L acrylic tank. The acrylic tank was maintained at a constant temperature (28 ± 1°C) with 14/10-h cycle of light and dark. Zebrafishes were fed live brine shrimps (Artemia salina). The embryos were obtained from natural spawning that was induced in the morning by turning on the light. Embryo collection was completed in 30 min.

2.9. Phenotype-based assessment

Synchronized embryos were collected and arrayed using a pipette (10 embryos per well in 24-well plates containing 1 mL of
egg water). FGA was dissolved in dimethyl sulfoxide (Sigma Chemical Co., St Louis, MO, USA) and then added to the egg water 9–72 h postfertilization. Effects on the pigment spots of zebrafish were observed under a stereomicroscope. In all experiments, 0.1 mM PTU (Sigma Chemical Co., St Louis, MO, USA) was used to generate transparent zebrafish without interfering in the developmental process [21] and considered as a positive control. Phenotype-based assessment of pigmentation was carried out by dechorionation using forceps, anesthetization in tricaine methane sulfonate solution (Sigma Chemical Co., St Louis, MO, USA), mounting in glycerol on a 35-mm dish, and observation under a stereoscopic microscope to take pictures [22].

2.10. Tyrosinase activity and melanin content

Tyrosinase activity was spectrometrically determined. About 100 zebrafish embryos were treated with FGA 9–48 h postfertilization and sonicated in Pro-Prep protein extraction solution (Intron, Daejeon, Korea). The lysate was clarified by centrifuging at 10,000g for 5 min. After quantification, 250 µg of total protein was added, followed by 100 µL of 5 mM L-DOPA. After incubation for 60 min at 37°C, absorbance was measured at 475 nm using a spectrophotometer. For the determination of melanin content, about 100 zebrafish embryos were treated with FGA 9–48 h postfertilization and sonicated in protein extraction solution. After the centrifugation, the pellet was lysed in 1N NaOH at 100°C for 10 min. Optical density of the supernatant was measured at 490 nm [22].

2.11. Statistical analysis

Statistical analysis was performed using SPSS 18 software (IBM, USA). All experiments described were performed three or more times. The data are expressed as the mean ± standard deviation. Significant differences between results were determined by Student t test. The statistical differences were considered significant at p < 0.05.

3. Results and discussion

3.1. Inhibitory activity of three ginsenosides on melanogenesis in melan-a cells

Melanin biosynthesis activity was studied by isolating FGA, GRD, and GRE from P. ginseng berry (Fig. 1). Cell viability was determined using the CCK-8 cell-counting kit after treating melan-a cells with the three ginsenosides (0–160 µM) for 3 d. FGA did not show cytotoxicity toward melan-a cells in all tested concentrations. However, GRE and GRD were cytotoxic at concentrations 40 µM and 160 µM, respectively (Table 1).

To examine the activity of three ginsenosides on melanogenesis, we measured the melanin contents of melan-a cells up to the previously measured cytotoxic concentrations. As shown in Fig. 2,
Ginseng ginsenoside is the main biologically active compound that is widely studied. In recent years, it not only is used for its effect on the skin, but is also clearly a scientific approach for the treatment of various diseases [23,24]. It has been reported that several ginsenosides and cinnamic acid are inhibitory agents of melanogenesis, and are found mainly in P. ginseng [11,25,26]. Therefore, we studied the expression of a protein found in melanin synthesis by nontoxic FGA (it has not been studied with respect to the physiological activities, as mentioned in the introduction) in melan-a cells.

3.2. Effect of FGA on the melanogenesis-related signaling pathway

Tyrosinase is involved in the biosynthesis of melanin by catalyzing the first two steps of melanin as a rate-limiting enzyme [27]. Tyrosinase hydroxylates tyrosine to DOPA and then oxidizes DOPA into dopaquinone [28]. Gene expression of tyrosinase is reported to be regulated by the MITF, which binds to the M-box (GTCATGTGCT) within the tyrosinase promoter [29]. Downregulation of MITF expression through tyrosinase is the most significant mechanism underlying the antimelanogenesis of inhibitory agents [30]. In addition, intracellular signaling kinase family ERK and Akt have been reported to have an influential function in melanin biosynthesis [31]. The action of ERK causes phosphorylation of MITF at serine-73, which leads to ubiquitination and subsequent MITF degradation, finally decreasing tyrosinase synthesis and melanogenesis [32]. Further, melanin production is also reduced by the phosphorylation of MITF at serine-29, which activates the signaling pathways of Akt [33].

Accordingly, it was hypothesized that FGA might inhibit melanogenesis-related signaling pathways, including the inhibition of MITF–tyrosinase signaling and/or activation of ERK–Akt signaling. Therefore, we studied the effect of FGA on the expression of tyrosinase and MITF, as well as ERK and Akt, in an attempt to further elucidate the molecular mechanisms. As shown in Fig. 3, tyrosinase and MITF expression were reduced in a dose-dependent manner. FGA indicated a significantly high melanin inhibitory activity (23.9%) at 160μM concentration without inducing any cytotoxicity in the cells.
manner. Furthermore, phospho-ERK and Akt signaling pathways were significantly enhanced in a dose-dependent manner by FGA treatment until 160µM concentration.

Our findings support that Akt and ERK pathway activation may be responsible for the discovered inhibition of melanin biosynthesis by FGA (Fig. 3). In order to confirm that these signaling pathways are responsible for FGA-induced inhibitory effects on melanogenesis, we activated both Akt and ERK signaling in the presence of FGA in melan-a cells.

3.3. Effect of FGA on melanogenesis in zebrafish

The zebrafish has become a favored model for biochemical studies because it is an efficient and robust replacement for animal experiments [34]. Observation of the pigment on the surface of zebrafish is a simple method to study the pigmentation process without complex laboratory procedures [22]. PTU (sulfur-containing tyrosinase inhibitor) is a widely used positive control in zebrafish investigations, which was included in this study [35]. The number of melanin pigments on the zebrafish embryo treated with FGA gradually decreased in a dose-dependent manner (Fig. 4A). Moreover, the total content of melanin and tyrosinase activity was measured using the whole zebrafish extract. FGA showed a potential reduction in the total melanin content and tyrosinase activity after treatment (Fig. 4B). More than 95% of the zebrafish survived treatments with PTU and treatment concentration of FGA (data not shown).

A recent study reported that the melanogenesis inhibitory effect of ginsenoside Rb1 from the P. ginseng root on the B16 melanoma cells is stimulated by α-melanocyte-stimulating hormone in a dose-dependent manner [36]. Lee et al. [25] reported that minor ginsenosides purified from the leaves showed inhibitory activity on pigmentation in zebrafish. There has been no study on the biological activity of FGA after isolation by Yoshikawa et al. [18]. Florphalsenosides Ka, Kc, and J were the only ginsenosides reported to inhibit melanogenesis in B16F0 cells by regulating the CREB/MITF/tyrosinase pathway [37,38].

In conclusion, we have found inhibitory activities of FGA, GRD, and GRE isolated from P. ginseng and Panax notoginseng. Anti-diabetic effects of minor ginsenosides isolated from the leaves of P. ginseng and radix trichosanthis on the melanogenesis. Biol Pharm Bull 2015;38:1663–7.

Conflicts of interest

The authors have no conflicts of interest to declare.

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