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Research article

Anti-melanogenic property of ginsenoside Rf from *Panax ginseng* via inhibition of CREB/MITF pathway in melanocytes and *ex vivo* human skin

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ABSTRACT

Background: Ginsenosides of *Panax ginseng* are used to enhance skin health and beauty. The present study aimed to investigate the potential use of ginsenoside Rf (Rf) from *Panax ginseng* as a new anti-pigmentation agent.
Methods: The anti-melanogenic effects of Rf were explored. The transcriptional activity of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and the expression levels of tyrosinase, microphthalmia-associated transcription factor (MITF), and tyrosinase-related proteins (Tyrps) were evaluated in melanocytes and UV-irradiated *ex vivo* human skin.
Results: Rf significantly inhibited Forskolin (FSK) or UV-stimulated melanogenesis. Consistently, cellular tyrosinase activity and levels of MITF, tyrosinase, and Tyrps were downregulated. Furthermore, Rf suppressed MITF promoter activity, which was stimulated by FSK or CREB-regulated transcription coactivator 3 (CRTC3) overexpression. Increased CREB phosphorylation and protein kinase A (PKA) activity induced by FSK were also mitigated in the presence of Rf.
Conclusion: Rf can be used as a reliable anti-pigmentation agent, which has a scientifically confirmed and reproducible action mechanism, via inhibition of CREB/MITF pathway.

1. Introduction

Melanin synthesized in melanosomes of melanocytes is the determinant for skin and hair color. Although melanin protects the skin against UV radiation (UVR), overproduced melanin in the skin causes pigmentary diseases such as post-inflammatory hyperpigmentataion (PIH), lentigines, freckles, and melasma [1,2]. Thus, attenuating melanin biosynthesis as well as reducing subclinical inflammation have been the aims of cosmeceuticals in skin beauty and health [1,2]. In addition to a key enzyme—tyrosinase, enzymatic proteins involved in melanin production such as tyrosinase-related protein (Tyrp)1 and Tyrp2, also called as dopachrome tautomerase (DCT) regulate melanogenesis [1–4]. Microphthalmia-associated transcriptional factors (MITF) play crucial roles in the modulation of melanin production and the tyrosinase transcription [3,4]. UV is the most significant physiological stimulus for melanogenesis and the cAMP response element binding protein (CREB) axis is an established pathway for its regulation. UVR sequentially activates cyclic adenosine monophosphate (cAMP) production, cAMP-dependent protein kinase (PKA), and the transcription factor CREB, which successively promotes the expression of MITF and downstream genes involved in melanogenesis. Along with PKA induced phosphorylation of CREB, previous studies have shown that the recruitment of CREB-regulated transcription coactivator 3 (CRTC3) to the CREB transcription complex is also needed for the activation of cAMP/MITF pathway for melanin synthesis [5,6]. Thus,

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we used CRTC3 overexpression to stimulate melanin synthesis and explore the relevant anti-pigmentation mechanisms.

Ginseng characterized by the existence of ginsenosides and gintonin is the root of plants in the genus *Panax*, such as Korean ginseng (*P. ginseng*) [7], South China ginseng (*P. notoginseng*), and American ginseng (*P. quinquefolius*) [8]. Ginsenosides are the main bioactive compounds and are categorized into several groups according to their particular chemical structures, including protopanaxadiols, protopanaxatriols, propanaxatriols, and oleanolic acids [8–10]. *Panax ginseng* originated from Korea 5,000 years ago and is a well-accepted tonic and remedy used in oriental medicine [7].

Several investigations have demonstrated that ginsenosides have antioxidant, anti-neoplastic, anti-inflammatory, immune modulating, and melanogenesis modulating action; however, the mechanisms of these actions have not been robustly established to date [10–17]. Ginsenoside Rf (Rf) is a ginseng saponin present in *P. ginseng* and modulates lipid metabolism, neuroprotection, and anti-inflammatory processes [18–23]. Rf content has been found to be much higher in *P. ginseng* from Korea and New Zealand than in *P. ginseng* from China [24,25]. Ginsenoside Re attenuated melanin content as well as tyrosinase activity in mouse melanoma B16BL6 cells stimulated by alpha-melanocyte stimulating hormone (α-MSH) [14]. Ginsenoside Rd reduced melanogenesis in Melan-A mouse melanocytes [16]. The ocotillol-type saponins extracted from *P. vietnamensis* Ha *et* Grushv. have been reported as possible depigmentation agents [26]. The ginsenoside Rg3, a tetracyclic triterpenoid saponin monomer, has antioxidant and pigment-modulating properties [8,9,13,15,17]. The ginsenoside Rh23 and 20-O-β-d-glucopyranosyl-3β,6α,12β,20β,25-pentahydroxydammar-23-ene extracted from *P. ginseng* have shown whitening effects in Melan-A cells [27]. Melanin biosynthesis and MITF were inhibited by ginsenoside Rb2 extracted from *P. ginseng* berries in Melan-A cells [28]. Compound-Y, a minor ginsenoside and a ginsenoside Rb2 metabolite from *P. quinquefolius*, was shown to have anti-pigmentation effects by attenuating tyrosinase activity in Melan-A cells [29]. Ginsenoside F1 has been demonstrated to have anti-melanogenic effects in humans, but the mechanism is not clear [30,31]. Ginsenoside Ia, synthesized from ginsenoside F1 showed melanogenesis inhibition in BL6B16 cells [32]. In another study, dammarane-type triterpenoid saponins and 20(S)-ginsenoside-Rf-1a showed a weaker anti-melanogenic effect than 20Z-ginsenoside-Rs4 or 23-O-methylginsenoside-Rg11, showing less attenuation of tyrosinase activity in B16 melanoma cells stimulated by α-MSH [33]. Hydrolyzed ginseng extract has shown anti-melanogenic activity in B16F10 melanoma cells possibly by blocking the c-Jun N-terminal kinase signaling pathway [34]. Nevertheless, the anti-melanogenic mechanisms of these ginsenosides are uncertain and have only been shown in mouse or melanoma cells, which may not correspond to results in human cells. Besides, usually only tyrosinase activity was tested in the previous reports. As melanogenesis of melanocytes is tightly regulated by keratinocytes and other neighboring cells, *ex vivo* human skin is a more reliable *in vitro* setting for the exploration of an whitening agent. Here, we investigate the anti-pigmentation effects of Rf included in protopanaxadiols from *P. ginseng* in melanocytes and *ex vivo* human skin in order to establish its potential as a natural whitening product.

2. Materials and methods

2.1. Chemicals

Korean Red Ginseng (*P. ginseng*) which contains Ginsenoside Rg3s, 19.25 mg/g; Rb1, 18.92 mg/g; Rc, 9.37 mg/g; Rh1, 8.38 mg/g; Rb2, 7.84 mg/g; Rg2s, 7.82 mg/g; Rg3r, 5.96 mg/g; Rf, 5.95 mg/g; Rd, 4.12 mg/g; Re, 2.79 mg/g; Rg1, 2.27 mg/g was supplied by the Korea Ginseng Corporation (Daejeon, Korea). The ginsenoside content analyzed by ultra-performance liquid chromatography was provided by the Korea Ginseng Corporation R&D center (Daejeon, Korea).

Ginsenoside Rf protopanaxatriol (3β,12β,20-trihydroxydammar-24-en-6α-yl 2-O-β-D-glucopyranosyl-β-D-glucopyranoside) with 98.3% purity by high-performance liquid chromatography, ginsenosides Rb1, Rb2, Rc, Rd, Re, F2, and Rh2 were provided by BTGin Co., Ltd. (Daejeon, Korea).

The preparations were resuspended in dimethyl sulfoxide (DMSO) and applied as indicated in the figures. Unless specified, Rf was applied at 80 μM. Forskolin (FSK, [3R-(3<, 4aβ, 5β, 6β, 6a<, 10<, 10aβ, 10b<)]-5-(Acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1H-naphtho[2,1-b]pyran-1-one) was obtained from Tocris Bioscience (Bristol, UK) and used at 10 μM.

2.2. Cell cultures

Mel-Ab cells which are well-known and non-tumorigenic mouse melanocytes [5,35,36] were kindly provided by Professor Park and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Corning Life Sciences, Corning, NY, USA), 1% penicillin/streptomycin (P/S), 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO, USA), and 1 nM cholera toxin (Cayman Chemicals, Ann Arbor, MI, USA). To examine the effects of saponin fractions and Rf on melanin production, melanocyte culture media was replaced with DMEM supplemented 10% FBS and 1% P/S and either saponin fractions, Rf, and/or forskolin (FSK). HEK-293T cells (Marc Montminy Lab, Salk Institute for Biological Studies, La Jolla, CA, USA) were cultured in DMEM supplemented 10% FBS and 1% P/S. Cells were maintained in a humid environment at 5% CO_2 and 95% O_2.

2.3. Cell viability assay

Mel-Ab cells were treated with saponin fractions or Rf with culture medium containing vehicle or drugs, and replaced every 24 h. At 72 h, MTT solution was added to culture media to a final concentration of 1 mg/ml MTT and incubated another 1 h in the culture incubator. After washing with phosphate-buffered saline (PBS), MTT incorporated into cells was solubilized in DMSO, and the optical density was measured at 562 nm absorbance using microplate reader (Biotek, Winooski, VT, USA). Cell viability is displayed as percent change relative to that of vehicle-treated controls.

2.4. Melanin content and tyrosinase activity

Mel-Ab cells were treated with saponin fractions, Rf, and FSK as indicated in the figures. For cotreatment experiments, pretreatment with Rf was performed 30 min prior to FSK treatment. The culture medium was replaced every 24 h. At 72 h after initial treatment, cells were solubilized with 1 N NaOH and boiled for 30 min with intermittent vortexing and centrifugation. Melanin content was measured using the supernatant and optical density measurements were taken at 405 nm with microplate reader (Biotek). Melanin content was normalized to the protein amount in the lysate and displayed as percent change relative to that of vehicle-treated controls. For tyrosinase activity assay, cells were lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. After quantifying the protein levels of the lysate and adjusting the protein concentrations with lysis buffer, the lysate was treated with

5 mM L-DOPA. After incubation at 37 °C, tyrosinase activity was measured using a microplate reader at 475 nm. Cell-free tyrosinase activity was assessed using a mushroom tyrosinase (Sigma-Aldrich, St. Louis, MO, USA) and 5 mM L-DOPA.

2.5. Immunoblots and antibody

Protein samples were prepared by washing Mel-Ab cells on culture plates with ice cold PBS followed by applying lysis buffer (10 mM Tris (pH 7.4) with 5 mM ethylenediaminetetraacetic acid and 1% sodium dodecyl sulfate (SDS)). *Ex vivo* human skin derived protein samples were made by first grinding the tissue in liquid nitrogen prior to lysis in buffer with 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor cocktail (Tech & Innovation, ChunCheon, Korea), and phosphatase inhibitors (5 mM Na-pyrophosphate, 20 mM β-glycerophosphate, and 50 mM NaF). Protein samples were boiled for 10 min in lysis buffer followed by separation via 6%–10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (ATTO Technology, Amherst, NY, USA). Immunoblotting was conducted using antibodies against tyrosinase, Tyrp1, DCT (Santa Cruz Biotechnology, Dallas, TX, USA), MITF (Neomarkers, Fremont, CA, USA), PKA substrate, phospho-protein kinase C (PKC) substrate, phospho-ACC, ACC, phospho-raptor, raptor, phospho-AMPKα, and CRTC3 (Cell Signaling Technology, Danvers, MA, USA). CREB and phospho-CREB antibodies were gift from Dr. Montminy (Salk Institute for Biological Studies, La Jolla, CA, USA) and HSP90 (Santa Cruz) was used as an internal loading control. The protein expression level was quantified by densitometry after normalized to the optical density of HSP90 using Image J (National Institute of Health, Bethesda, MA, USA).

2.6. mRNA analysis

Total RNA was isolated from Mel-Ab cells using the FavorPrep Blood/Cultured Cell total RNA purification kit (Farvorgen Biotech, Changzhi Township, Taiwan). 700 ng of total RNA was subjected to first strand cDNA synthesis with a random hexamer using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Relative expression levels of mRNAs were analyzed by quantitative real-time reverse transcriptase PCR (qRT-PCR) using a THUNDERBIRD SYBR qPCR mix (Toyobo) and a Lightcycler 480 (Roche Applied Science, Indianapolis, IN, USA). L32 expression was used as an internal reference; specific primer sets used for the amplification of the genes in this study were performed as described previously [36].

2.7. MITF, Tyr promoter and CREB activity

A MITF promoter (494 bp) or tyrosinase promoter (390 bp) was cloned into pGL3 plasmids and co-transfected with RSV promoter regulated β-galactosidase plasmids into HEK-293T cells using polyethylenimine (PEI) reagents as described previously [5]. 24 h after transfection, cells were treated with the saponin fractions, Rf, and/or FSK for 6 h as indicated in the figures. Following, luciferase and β-galactosidase activity was measured. MITF or tyrosinase promoter are displayed as normalized luciferase activity relative to β-galactosidase activity. CREB activity was measured by EVX1 promoter activity as described previously [36]. Briefly, reporter plasmids (pGAS-hEVX1Pr-Luc) and pRSV-β-gal plasmids were transfected into HEK-293T cells. Luciferase activity was measured and normalized to β-galactosidase activity.

2.8. Ex vivo human skin cultures

In line with the Declaration of Helsinki, skin tissue was obtained from a 39-year-old female patient (IRB number 2014-0837) with informed consent. Skin tissues were washed for a moment with 100% and 70% EtOH consecutively, cut into 1-cm^2 sections, and positioned on metal grids in 6-well plates in contact with DMEM containing 10% FBS and 10% penicillin/streptomycin, plus either vehicle or Rf under a humidified environment of 5% CO_2. Culture medium with vehicle or Rf was replaced daily. For UVR-induced melanin synthesis, skin tissue was exposed to 70 mJ/cm^2 UVB for 50 s using TL20W/12RS UV lamp (Philips, Eindhoven, Netherlands). After 96 h, skin tissues were harvested and split into two pieces. One piece was embedded in paraffin for histological evaluation with immunohistochemistry and the other was ground in liquid nitrogen for extraction of protein samples as described above. Fontana–Masson stain was used to visualize melanin pigment and multiple randomly selected areas were photographed by a phase-contrast microscope (BX53, Olympus, Tokyo, Japan). The melanin index was calculated by measuring the stained area normalized to total epidermal area using Image J (National Institute of Health, Bethesda, MA, USA) and expressed as percent change relative to vehicle-treated controls.

2.9. Statistics

Data are presented as mean and ± standard error of the mean (SEM). Statistical significance was determined by unpaired student's t-test using the GraphPad Prism program. In this study $P < 0.05$, $P < 0.01$, $P < 0.001$ represent *, **, and ***, respectively, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Rf reduced melanogenesis in melanocytes with or without cAMP stimulation while it did not affect cell viability

To identify potential melanogenesis modulating ginsenosides, we first investigated the effects of various ginsenosides such as Rb1, Rb2, Rc, Rd, Re, Rf, F2, and Rh2 on CREB activity as measured by EVX1 promoter activity. In this screen, Rb1, Rb2, Rd, and Rf significantly attenuated FSK-stimulated CREB activity (Fig. 1A). As Rf appeared to attenuate CREB activity most effectively among them, we decided to perform further experiments with Rf.

To test whether saponin fractions and/or Rf exhibit anti-melanogenic activity, we first conducted cell viability assays and determined the doses required to see their specific effects. The viability of Mel-Ab cells was not affected by 72-hour (h) treatment of 12.5–100 μg/ml saponin fractions or 20–100 μM of Rf (Fig. 1B and C). Based on these results, we treated Mel-Ab cells with up to 100 μg/ml saponin fractions or 40–80 μM of Rf and examined their effects on melanin accumulation. Whereas no significant changes of melanin content was observed in Mel-Ab cells treated with 12.5–100 μg/ml saponin fractions (Fig. 1D), compared with vehicle treatment, Rf at a concentration of 40 μM decreases melanin accumulation in Mel-Ab cells by 12% and 80 μM Rf reduces it by 15% (Fig. 1E and F). Because 80 μM of Rf has a larger anti-melanogenic response without cytotoxicity, subsequent experiments were conducted with the fixed dose.

Physiologically, cAMP signaling is a key player linking UVR stimulation to melanogenesis [36]. Thus, we asked whether Rf attenuates UVR/cAMP-stimulated melanogenesis using FSK which is an adenylate cyclase agonist known to elevate intracellular cAMP level. As expected, in comparison with vehicle treatment, treatment with 10 μM of FSK increased the melanin content in Mel-Ab

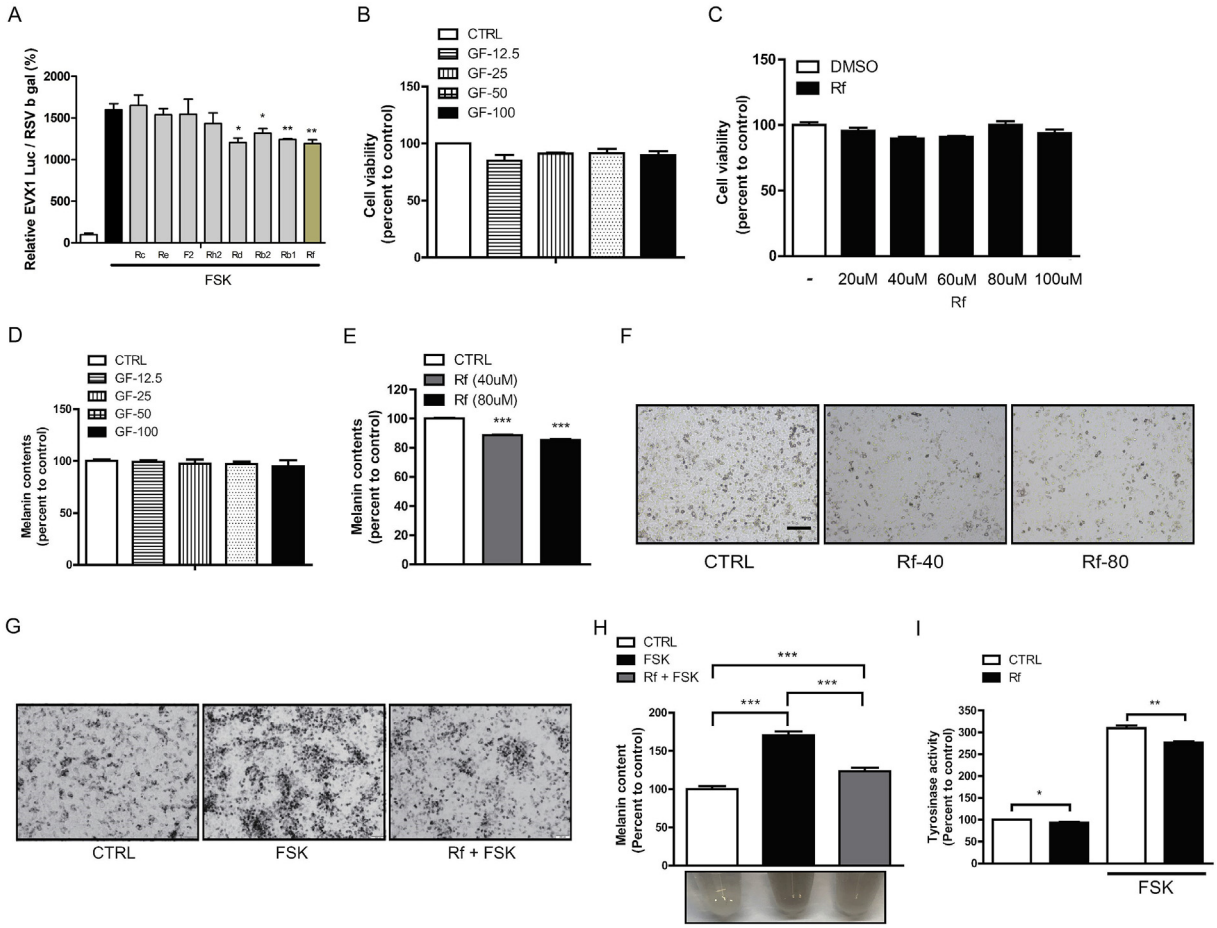

Fig. 1. Rf reduced melanogenesis in Mel-Ab mouse melanocytes with or without cAMP stimulation while it did not influence cell viability. (A) The effects of 60 μM of individual ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, F2, and Rh2) on forskolin (FSK)-stimulated CREB activity. Mel-Ab cells were treated with (B) 12.5–100 μg/ml of saponin ginsenosides fractions (GF) (C) 20–100 μM of Rf for 72 h; cell viability was examined by MTT assays. Both preparations were not cytotoxic at concentrations less than 80 μM. The melanin content of Mel-Ab cells treated with (D) 12.5–100 μg/ml of saponin ginsenosides fractions (GF) or (E) 40 and 80 μM of Rf were measured at 72 h. Melanin content is displayed as percent change of vehicle-treated controls. (F) Microscopic images of Mel-Ab cells treated with vehicle, 40 μM, or 80 μM of Rf for 72 h. bar = 1000 μm
Mel-Ab cells were treated with vehicle (CTRL), FSK, or ginsenoside Rf (Rf, 80 μM) + FSK. 72 h after treatment (G) microscopic images of Mel-Ab cells were obtained using phase-contrast microscopy, (H) melanin content, and (I) tyrosinase activity was measured and displayed as percent change relative to vehicle-treated controls. (A–E, H, I) The experiments were repeated at least three times and quantified and recorded as mean ± SEM.

cells by 70%; however, this FSK-induced increase in melanin production was inhibited in the presence of Rf (Fig. 1G and H). The reduced melanin content by Rf correlated with cellular tyrosinase activity. Mel-Ab cells treated for 72 h with FSK exhibited enhanced tyrosinase activity and it was downregulated by pretreatment with Rf (Fig. 1I).

3.2. Rf reduced melanogenesis in UVR-stimulated ex vivo human skin culture

We next explored the anti-melanogenic properties of Rf in *ex vivo* human skin culture. Physiologically, melanogenesis process is not exclusive to melanocytes but is rather intricately regulated via interaction with surrounding keratinocytes [37]. Thus, melanogenesis system can be assessed via an *ex vivo* system precisely. Rf showed anti-melanogenic effects in the *ex vivo* human skin culture, given that it reversed the UVR-induced melanin accumulation (Fig. 2A and B). In addition, Rf treatment suppressed the UVR-induced upregulation of MITF, tyrosinase, Tyrp1, and DCT expression (Fig. 2C and D).

3.3. The anti-melanogenic effects of Rf via reduction of melanogenesis-related genes expression

It is well known that PKC regulates melanogenesis via directly inducing phosphorylation-dependent modulation of tyrosinase activity [38,39]. To investigate whether reduced melanin accumulation in the presence of Rf is due to a direct effect on PKC and/or tyrosinase activity, we performed cell-free tyrosinase activity assay. Treatment with 20 to 160 μM of Rf did not inhibit mushroom tyrosinase activity (Fig. 3A). Additionally, we treated Mel-Ab cells with Rf for a short period of time (hours) and examined its effect on PKC and/or tyrosinase activity. Contrary to a decrease of cellular tyrosinase activity in Mel-Ab cells 72 h after Rf treatment (Figs. 1I), 3 h treatment with Rf did not alter cellular tyrosinase activity or PKC activity (Fig. 3B and C). Having confirmed that Rf has no direct inhibitory effects on tyrosinase activity, we hypothesized that reduced tyrosinase activity and melanin production by Rf might be associated with the expression levels of tyrosinase and other proteins related to melanogenesis. To evaluate whether Rf alters the expression of tyrosinase or other proteins related to melanogenesis, western blot analysis and qRT-PCR were performed. Rf suppressed

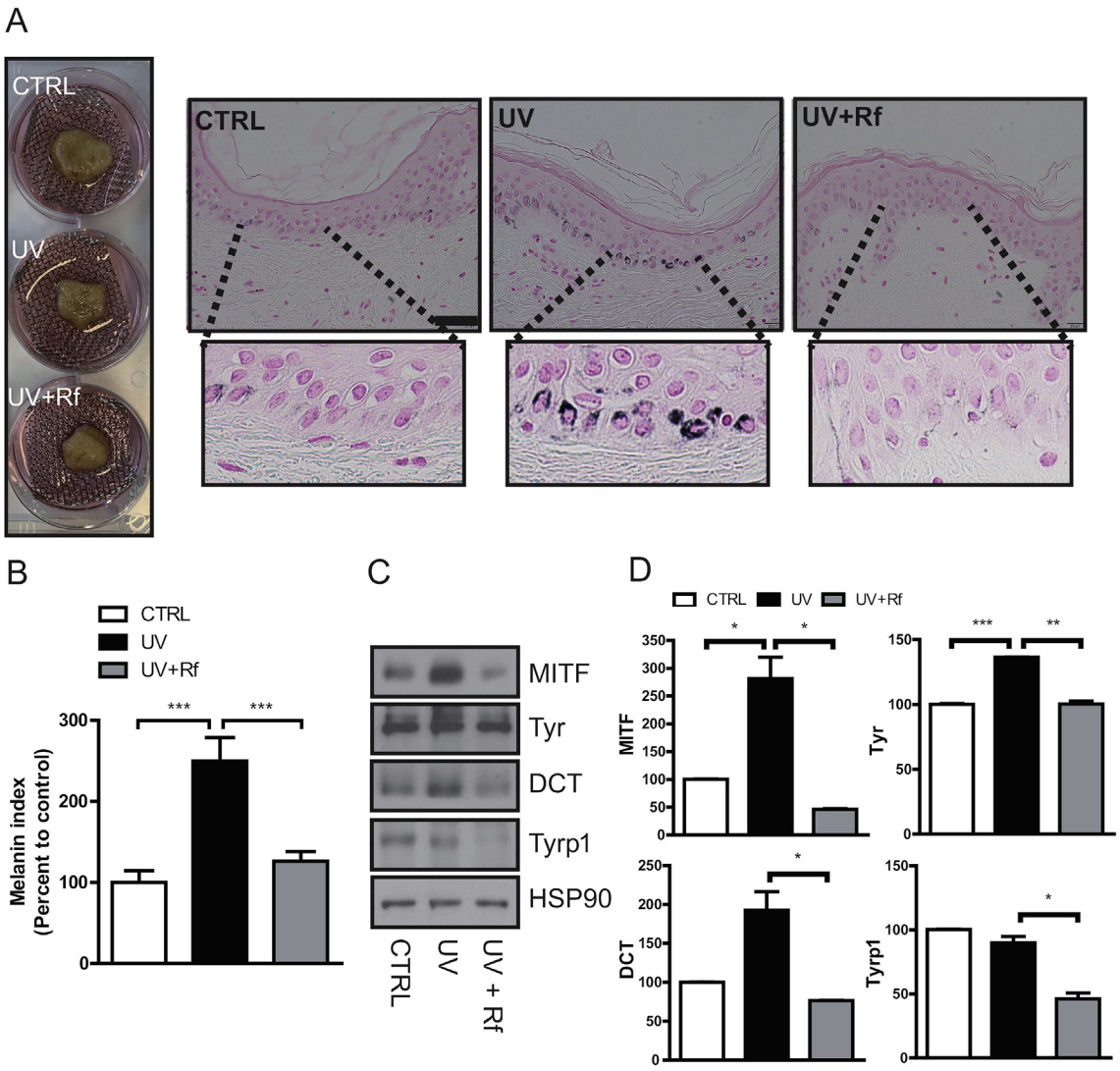

Fig. 2. Rf reduced melanogenesis in UV irradiation-stimulated *ex vivo* human skin culture. (A) Fontana-Masson-stained microscopic images of *ex vivo* human skin with vehicle, UV irradiation, and UV irradiation plus 400 μM of Rf for 96 h are shown (original magnification ×400). (B) Melanin index of *ex vivo* human skin culture was calculated by Image J analysis and displayed as percent change of vehicle-treated controls. The melanin index was significantly increased by UV irradiation, whereas 400 μM of Rf for 96 h reversed it. (C) Protein levels of MITF, tyrosinase (Tyr), Tyrp1, and DCT were examined by western blotting and HSP90 was used as a loading control. (D) Densitometry graphs of the western blot analyses. (B, D) The experiments were repeated at least three times and quantified and recorded as mean ± SEM.

the expression of MITF and tyrosinase compared with that observed in the vehicle-treated controls in both basal and FSK-stimulated conditions at 72 h (Fig. 3D–F, Supplementary Fig. 1, 2).

3.4. Rf downregulated tyrosinase expression via suppressing MITF expression

Based on our observations that both tyrosinase and MITF expression was downregulated by Rf, we examined whether Rf regulates MITF transcriptionally. The assay for the burst-attenuation kinetics of MITF expression during cAMP stimulation demonstrated that MITF mRNA expression peaks within 1 h of FSK treatment and decreases thereafter. FSK-stimulated MITF expression was strongly suppressed by Rf treatment (Fig. 4A). The mRNA expression levels of tyrosinase and Tyrp1 were lower at 1 h in Rf + FSK treated Mel-ab cells compared with FSK treated cells. DCT mRNA expression levels were not altered during the 6 h period of Rf treatment. In promoter activity assay in HEK-293T cells, tyrosinase promoter activity was not altered, while Rf suppressed MITF promoter activity stimulated by FSK or overexpression of CRTC3, a CREB transcription cofactor that enhances CREB activity (Fig. 4B and C). These results suggest the specificity of Rf on the suppression of MITF transcription and downregulation of tyrosinase expression by Rf is secondary to the decreased MITF expression.

3.5. Effects of Rf on melanogenesis intracellular signaling and CREB transcriptional activity

Based on the above observations, we suspected that Rf attenuates MITF expression via inhibition of CREB transcriptional activity. No change in melanin accumulation occurs using saponin fractions; even 80 μg/ml of saponin fractions did not alter CREB activity (Fig. 5A). Rf dose dependently inhibited FSK-stimulated CREB activity as well as CREB activity stimulated by CRTC3 overexpression (Fig. 5B and C). The phosphorylation status of Ser133 of CREB by protein kinase A reflects the transcriptional activity of CREB and melanogenesis [40]. To decipher the molecular mechanisms of CREB inhibition by Rf, we investigated the effects of Rf on CREB

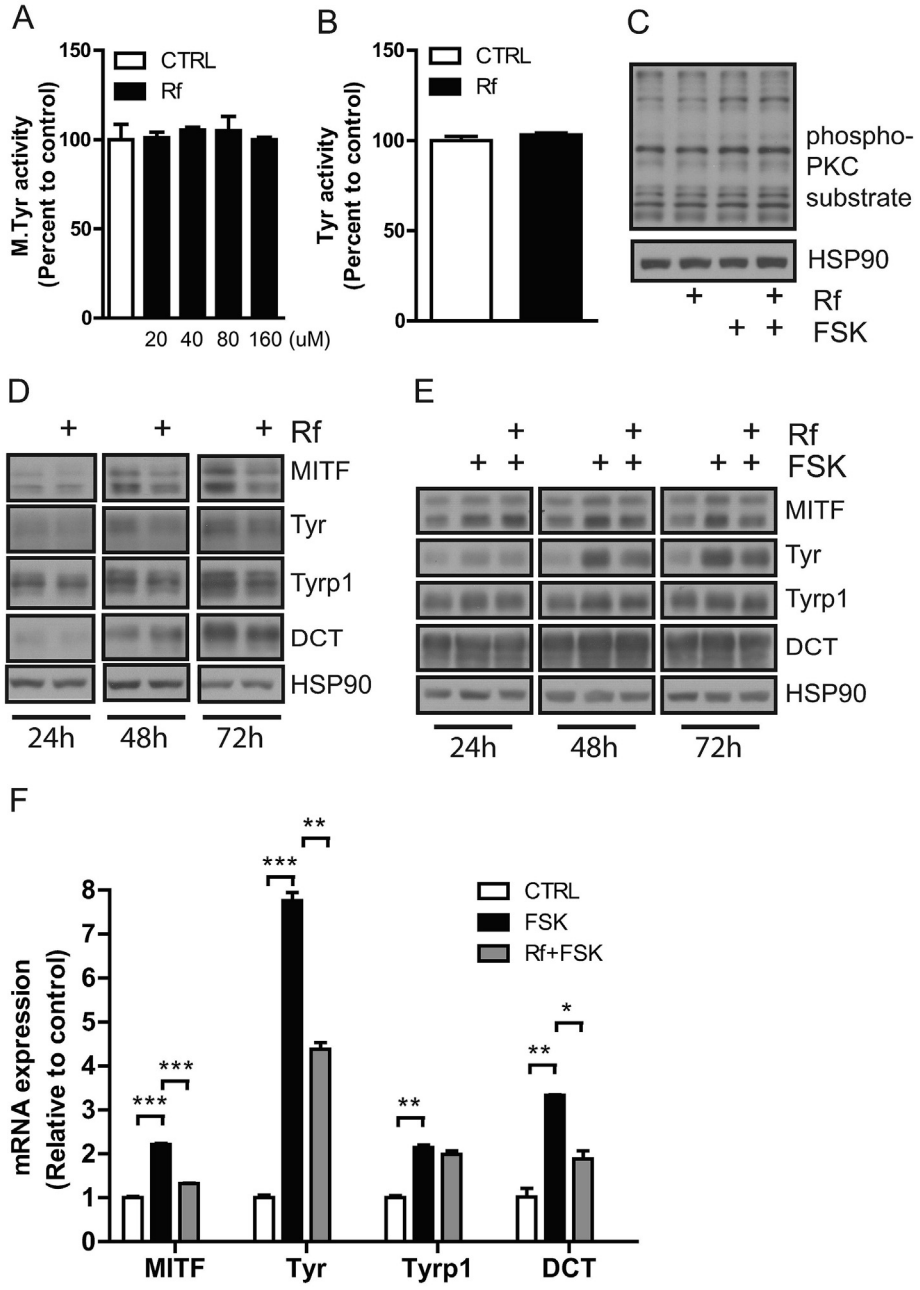

Fig. 3. Rf suppressed expression of melanogenesis-related genes with or without cAMP stimulation. Cell-free mushroom tyrosinase (M. Tyr) activity was examined with 20–160 μM of Rf (A). Rf treatment for 3 h (B) tyrosinase activity and (C) PKC activity was examined in Mel-Ab cells. (D) The expression levels of melanogenesis-related genes including MITF, tyrosinase (Tyr), Tyrp1, and DCT when treated with vehicle or 80 μM of Rf were determined by immunoblotting. Mel-Ab cells were treated with vehicle, FSK, or 80 μM of Rf + FSK and (E) protein (at 24, 48 and 72 h after treatment) and (F) mRNA (at 72 h) levels of MITF, Tyr, Tyrp1, and DCT were compared by immunoblotting and qRT-PCR, respectively. HSP90 and L32 was used as a loading control. (A, B, F) The experiments were repeated at least three times and quantified and recorded as mean +/-SEM.

phosphorylation and relevant signaling pathways. CREB phosphorylation was markedly increased at 1 h after FSK stimulation, which was reversed by Rf treatment (Fig. 5D). Similarly, PKA activity was increased at 1 h after FSK stimulation, which was mitigated in the presence of Rf (Fig. 5D). AMPK activity, which involves the downregulation of CREB activity [41] as assessed by AMPK and its downstream target Raptor and ACC phosphorylation, was not altered (Fig. 5D). These results imply that the anti-melanogenesis mechanism of Rf is associated with the inactivation of the PKA/CREB signaling axis; thus, diminished CREB transcriptional activity likely leads to the inhibition of tyrosinase expression via suppressing the expression of MITF, a key transcription factor of the tyrosinase gene.

4. Discussion

Some ginsenosides of *P. ginseng* have been demonstrated to modulate skin pigmentation. In a previous study, ginsenoside Rh2 appeared to induce the differentiation of B16 melanoma cells, thereby stimulating melanogenesis [15]. Ginsenoside Rg1 has been reported to increase melanin accumulation in human melanocytes by activating PKA/CREB/MITF signaling [42]. Compound K, a

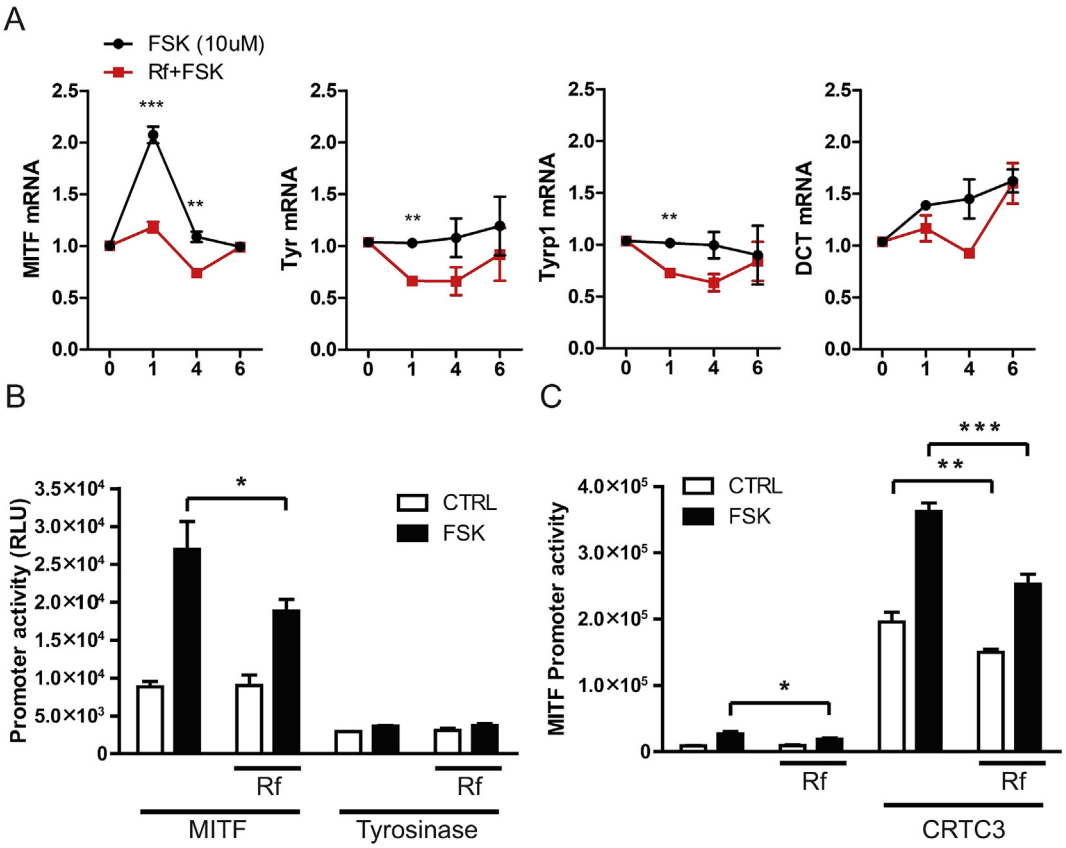

Fig. 4. Rf directly suppressed cAMP-stimulated MITF expression and transcription levels. (A) Mel-Ab cells were treated with FSK or 80 μM of Rf + FSK for 1–6 h and the mRNA levels for MITF, Tyr, Tyrp1, and DCT were compared. (B) The effect of Rf (80 μM) on MITF and tyrosinase promoter activity was examined. (C) The effect of Rf (80 μM) on the cAMP CRTC3-stimulated MITF promoter was examined in HEK-293T cells. (A–C) The experiments were repeated at least three times and quantified and recorded as mean +/-SEM.

metabolite of ginsenoside Rb1, has been reported to elevate pigment accumulation in B16F10 cells via an unknown mechanism [43]. Increasing melanogenesis is a good strategy for amelioration of vitiligo or hypopigmented skin conditions. Conversely, ginsenoside Rb2 inhibits melanin biosynthesis by decreasing tyrosinase and MITF expression in Melan-A cells [28], which can be considered as a skin whitening agent.

However, most of these previous studies modulating pigmentation by ginsenosides were conducted in mouse melanoma cells or melanocytes alone and thus obtained contradictory results depending on the cell culture conditions [42,44]. For human trials, confirmative experiments using human skin are necessary. Moreover, refined individual ginsenoside should be further developed. It is known that steam temperature is an essential factor that determines the components and ratio of ginsenosides from saponin extracts. For example, ginseng processed at low temperatures enriches Rf, in addition to Re, Rg, and Rb1 compared with that with extraction at high temperatures [45].

In our experiment, when whole saponin fractions were treated, they neither reduced melanin in melanocytes nor decreased CREB transcriptional activity. By CREB activity screening of various individual ginsenosides, we found that Rf effectively reduced melanin content by profoundly attenuating CREB transcriptional activity. In agreement with our findings, a previous study reported that Rf injection decreased phospho-CREB levels in ectopic endometrial tissues in rats [18]. The anti-inflammatory properties of ginseng extracts were revealed with purified ginsenosides, such as ginsenosides Rb1, Rg1, Rg3, Rh2, and compound K [46]. The down-regulation of inflammatory cytokine and enzyme expressions, including TNF-α, IL-1β, IL-6, iNOS, and COX-2, were identified as the anti-inflammatory mechanisms of ginsenosides in M1-polarized macrophages [46,47]. Rf regulated lipoprotein metabolism in a peroxisome proliferator-activated receptor (PPAR) alpha dependent manner and also has anti-inflammatory, neuroprotective, and analgesic effects by reducing interleukin (IL)-1β, IL-6, and iNOS [18–22]. Rf is an component of *P. ginseng,* which has been reported to actively inhibit hypoxia-induced COX-2 via PPAR γ [23]. Considering that the most common hyperpigmentation skin disorders include PIH, Rf's anti-inflammatory property could provide strong advantages for the purpose of reducing pigmentation in human skin.

Meanwhile, most skin whitening agents, whether naturally or chemically derived, may cause skin toxicity or irritation; which can predicted to a certain extent using *in vitro* cellular viability assays with melanocytes. It is well known that hydroquinone creams can result in undesired hypopigmentation and skin toxicity [48,49]. Furthermore, some whitening cosmetics have disastrous consequences by inducing vitiligo through degradation of tyrosinase proteins [50]. Indeed, safe and healthy skin whitening agents are continuously under exploration; In order to attain the goal of newer, reliable, and reproducible mechanisms of anti-melanogenesis should be pursued in parallel.

For whitening agents, MITF transcription is the main target as MITF is central to most biological aspects of melanocytes including cell survival, differentiation, proliferation, migration, senescence, metabolism, DNA damage repair, UV or stress responses, and so on [3,5,35,36]. Post-translational degradation of MITF by serine phosphorylation is a strong whitening agent mechanism, but

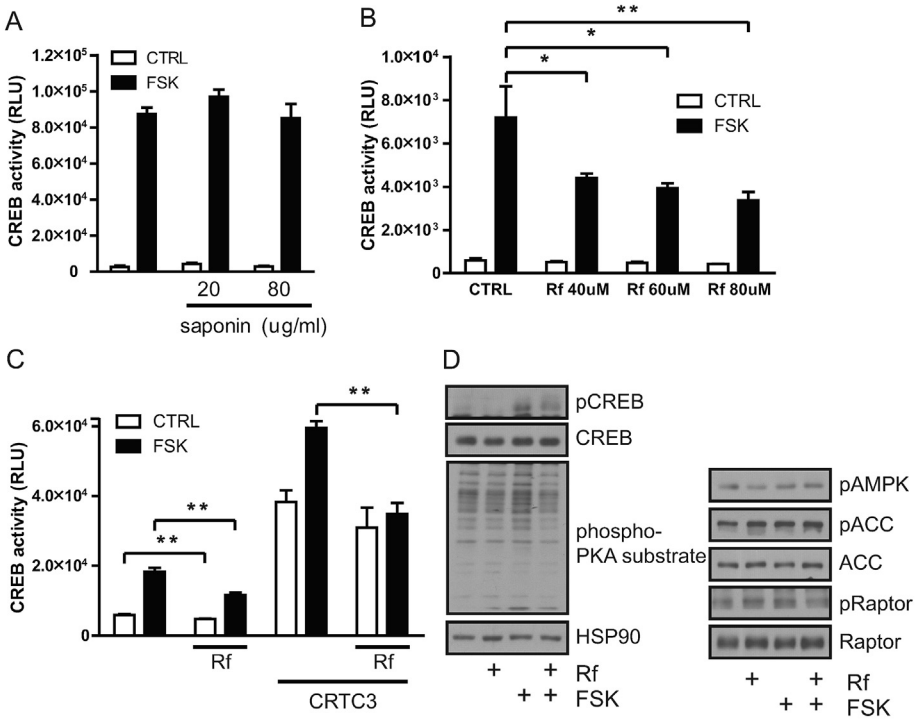

Fig. 5. Rf inhibited CREB activity via suppression of PKA activity. The effect of (A) saponin fractions and (B) Rf on cAMP-stimulated CREB activity was analyzed using a CREB-reporter assay. (C) The effect of Rf (80 μM) on cAMP CRTC3-stimulated CREB activity was analyzed using a CREB-reporter assay. (D) Mel-Ab cells were treated with vehicle, Rf (80 μM for 1.5 h), FSK (1 h), or Rf (1.5 h) + FSK (1 h) and the expression levels of total and phospho-CREB, PKA activity (phospho-PKA substrate), and genes in AMPK pathways (AMPK, Raptor, and ACC) were examined by immunoblotting. HSP90 was used as a loading control. (A–C) The experiments were repeated at least three times and quantified and recorded as mean +/- SEM.

considering that MITF is essential for melanocyte survival and proper biological function, deficits in MITF proteins alter melanocytic viability, resulting in possible hypopigmentation [50]. We previously reported that the ginsenoside Rg3 strongly inhibits melanin by degrading MITF proteins via delayed ERK activation [17]. In this context, only lower doses of Rg3 may be suitable for anti-melanogenesis when considering the viability of melanocytes. As whitening agents can be used at higher doses to maximize skin whitening effects depending on the users and as cosmetics are applied on the entire face and not only on hyperpigmented spots, a wide safe drug tolerance margin is mandatory. To that point, Rf has distinct advantages over previously discovered whitening ingredients as Rf has lower risk of hypopigmentation as it attenuates cellular tyrosinase activity over time, not abruptly. It is well known that CREB delivers UV-induced cAMP signals to MITF; thus CREB phosphorylation by PKA is a main axis for regulation of MITF transcription in melanocytes [5,36]. Considering that MITF mRNA gene regulation is intricately controlled and rescued by other intracellular signaling molecules and coactivators, transcriptional level regulation of MITF is a promising strategy for exploring healthy skin whitening ingredients as the survival function of MITF is preserved and rescued. Indeed, when we investigated FSK-induced MITF transcription, for up to 6 h, MITF mRNA has its own rescue response curve for cellular survival and biological functions [6].

Rf could be utilized for functional foods or topical agents. For topical treatment of skin hyperpigmentary disorders, enhancing penetration is the most important for proper bioavailability [51]. To this point, Rf is a small lipophilic molecule, enabling efficient topical delivery.

Most other ginsenosides and naturally sourced chemicals act by inhibition of direct or cellular tyrosinase activity. In contrast, we found that Rf may be utilized as a safe and healthy skin lightning agent via the inhibition of CREB transcriptional activity. Furthermore, UV radiation or cAMP aggravated skin hyperpigmentation and PIH were suggested to be a better and real world target of Rf. Notably, in accordance with our study, aglycone of Rh4 was reported to inhibit FSK-stimulated melanin synthesis by inhibiting PKA/CREB, although this study was performed with B16 melanoma cells [52].

Further screening for *P. ginseng* ginsenosides, and other natural agents that regulate the transcriptional activity of CREB, may provide a beneficial treatment effect for skin diseases with hyperpigmentation and lightning skin tone. Furthermore, our study showed a possible role for CRTC3 regulating the transcriptional activity of CREB beyond previously established pathways of CREB phosphorylation by PKA [5,36].

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.11.003.

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