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Original article

Anti-melanogenesis effect of dehydroglyasperin C through the downregulation of MITF *via* the reduction of intracellular cAMP and acceleration of ERK activation in B16F1 melanoma cells

Ji Won Lim, Ji Hoon Ha, Yoon Ju Jeong, Soo Nam Park*

Department of Fine Chemistry, Cosmetic R&D Center, Cosmetic Industry Coupled Collaboration Center, Seoul National University of Science and Technology, Nowon-gu, Seoul, Republic of Korea

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ABSTRACT

Background: In mammals, UV radiation induces melanin synthesis in melanocyte for protecting their skin through the stimulation of α-melanocyte stimulating hormone (α-MSH) from keratinocytes. In this study, the inhibitory effects of dehydroglyasperin C (DGC), an useful component of *Glycyrrhiza uralensis* (*G. uralensis*), was investigated on melanogenesis induced by α-melanocyte stimulating hormone (α-MSH) and its mechanisms.

Methods: Melanogenesis suppression effect of DGC on α-MSH induced B16F1 melanoma cells. The cell viability was measured by MTT assay. Expression and phosphorylation of melanogeic protein were conducted using western blot. cAMP acceleration was measured by cAMP immunoassay kit. To investigate whitening mechanism, we used ERK inhibitor (PD98059).

Results: DGC decreased intra cellular tyrosinase (TYR) activity and expression of melanin synthesis related proteins (TYR and TRP-1) in a dose-dependent manner on α-MSH induced melanogenesis. In addition, DGC induced the downregulation of MITF (melanocyte-specific transcription factor) through suppression of cAMP-CREB pathway. Also, phosphorylation of extracellular signal regulated kinase (ERK) decreased MITF by DGC treatment.

Conclusion: Therefore, DGC could be used as a whitening ingredient in skin and clinical usage against hyperpigmentation.

Introduction

Melanin is synthesized for skin protection from UV exposure. But, immoderate melanin formation and accumulation induces hyperpigmentation in skin such as melasma, age spots and freckles [1]. Melanin is produced to melanosomes in melanocytes and are delivered to keratinocytes from the dendritic tips of melanocytes; this process is called melanogenesis [2,3]. In mammals, UV radiation stimulates the production of α-melanocyte stimulating hormone (α-MSH). It stimulates adenylyl cyclase to accelerate the level of cAMP, which binds to melanocortin receptor 1 (MC1R) within the cell membrane and increase the activity of cAMP-dependent protein kinase A (PKA). PKA subsequently activates the phosphorylation of cAMP response element-binding protein 1 (CREB1) as a regulator, which expresses microphthalmia-associated transcription factor (MITF) gene [4,5]. MITF, a major transcription factor for melanin synthesis related proteins, controls the production of tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and TRP-2 (dopachrome tautomerase) [6]. Tyrosinase hydroxylates L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and oxidizes DOPA into dopaquinone. TRP-2 oxidizes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), whereas TRP-1 catalyzes the conversion of DHICA to carboxylated indole-quinone *via* oxidation reaction [7–9].

Glycyrrhiza uralensis (*G. uralensis*) Fischer has been widely used as a medicinal plant with advantageous effects such as anti-inflammatory, antimicrobial, anti-melanogenic, and antioxidant activities. [15–17]. Dehydroglyasperin C (DGC) is one of prenyl-flavonoids isolated from licorice (Fig. 1) and has anticancer, anti-inflammatory, and antioxidant effects by inducing the production of phase II naturalizing and antioxidant enzymes [18–21]. However, DGC have not been reported on melanin synthesis.

In the present study, we estimated the inhibitory effects of DGC on melanogenesis and elucidated its mechanism in B16F1

* Corresponding author.
E-mail address: snpark@seoultech.ac.kr (S.N. Park).

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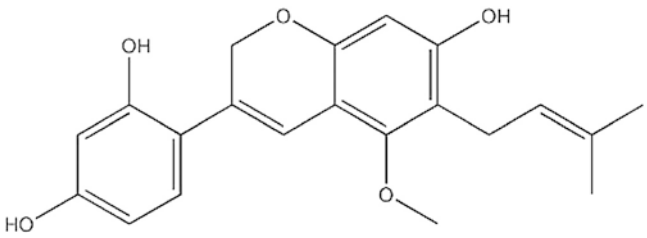

Fig. 1. Chemical structure of dehydroglyasperin C.

melanoma cells. This was the first attempt to identify the potential whitening effect of DGC to be used as a whitening ingredient in skin and clinical usage against hyperpigmentation.

Materials and methods

Materials

DGC, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), L-DOPA, and α-MSH were purchased from Sigma Chemical Co. (St. Louis, USA). Primary antibodies against tryosinase, TRP1, and TRP-2, MITF were purchased from Santa Cruz (CA, USA).

Antibodies against ERK, p-ERK, CREB and p-CREB, were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and mouse anti-GAPDH antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Cell culture

B16F1 cells were gained from Korean Cell Line Bank (Seoul, Korea) [22] and were cultured in Dulbecco's modified Eagle's medium (DMEM; Capricorn, Germany) supplemented with 10% fetal bovine serum (FBS; Capricorn, Germany) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell viability

The cell viability effect of DGC on B16F1 cells was estimated by measuring the reduction of MTT to formazan. B16F1 cells (1×10^4 cells) were cultured in a 96-well plates and incubated for 24 h at 37 °C. And then, the cells were treated with indicated concentrations of DGC (1, 2, 4, 6, and 8 μM) and incubated for 72 h at 37 °C. MTT solution (200 μL) was added to each well and incubated for 1 h. The resulting crystals were solubilized in DMSO, and the absorbance was measured using an ELISA reader (TECAN, Salzburg, Austria) at 570 nm.

Measurement of melanin content

Melanin content was estimated according to the methods of Hosoi et al. [23]. In brief, B16F1 cells were cultured at 1×10^5 cells with 200 nM α-MSH in 6-well plates and pre-treated with indicated concentrations of DGC (1, 2, 4, and 6 μM) for 24 h, and treated with 1 μM of α-MSH for additional 48 h. The extra-cellular melanin contents was measured in the culture medium at 405 nm using an ELISA reader. To analysis of intracellular melanin contents, treated cells were washed twice with PBS to eliminate the medium. The supernatant was collected and assayed for protein concentration using the BCA kit (Pierce, USA). The cell pellet was dissolved in 1 N NaOH containing 10% DMSO at 80 °C for 2 h. The intracellular melanin contents were estimated at 405 nm using the microplate reader.

Intracellular tyrosinase inhibitory activity

B16F10 cells (1×10^5 cells/well) were seeded in each 6-well plates and pre-treated with indicated concentrations of DGC (1, 2, 4, and 6 μM) for 24 h, and stimulated with 1 μM α-MSH for additional 48 h. To measure cellular TYR activity, cells were washed twice with PBS and lysed with RIPA buffer. The cell lysates were clarified by centrifugation at 13,000 rpm for 30 min. The supernatant was harvested and estimated to protein concentration using the BCA protein assay kit (Pierce, USA). Each lysate (90 μL) containing the same amount of protein was placed in each well of a 96-well plate and 10 μL of 10 mM L-DOPA was then added to each well. Following incubation at 37 °C, absorbance was measured after 1 h at 475 nm using microplate reader.

cAMP immunoassay

cAMP level was measured with cell lysate using cAMP assay kit (BioVision, USA). B16F1 cells were cultured on 24-well plates (5×10^4 cells/well) and were treated for 24 h with 4 μM DGC. cAMP levels measured at 20 min after 200 nM α-MSH stimulation. Cells were lysed using 0.1 M HCl, and cell lysate incubated for 20 min at 25 °C and centrifuged at 4000 rpm at 4 °C. Harvested supernatant added to the specific cAMP polyclonal antibody-coated plates. They were incubated for 1 h at room temperature. Then, standard and cAMP-HRP conjugate were added to the wells and incubated for 1 h on a shaker at room temperature and then add 0.1 M HCl for stopping of reaction. cAMP level was determined at 450 nm using an microplate reader.

Western blot analysis

Incubated cells were lysed with RIPA buffer containing protease inhibitors, and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was harvested and estimated to protein concentrations using the BCA protein assay kit (Pierce, Rockford, IL, USA). Total protein (60–80 μg) was loaded on 10% SDS-polyacrylamide gels and separated by electrophoresis, and then transferred to PVDF membranes. Blocking was performed in Tris-buffer saline containing 5% skim milk powder (or 5% BSA (Sigma–Aldrich, USA)) and 0.1% Tween-20 (Sigma–Aldrich, USA). Samples were incubated the appropriate primary antibodies and then incubated with HRP-conjugated secondary antibodies for 1 h. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

Statistical analysis

All experiments were performed in triplicate. Values are presented as mean ± SD. Statistical significance of the differences between groups was assessed using analysis of variance (ANOVA).

Results

Effects of DGC on cell viability

We investigated cell viability of DGC on B16F1 melanoma cells. The cytotoxicity of DGC was estimated by the MTT assay. The cell viabilities treated with DGC (1–6 μM) were over 90% and had no significant effect compared to non-treated cell. However, the cell viability was significantly reduced at 8 μM (Fig. 2). Based on the data, we decided to select the safe concentrations for following study.

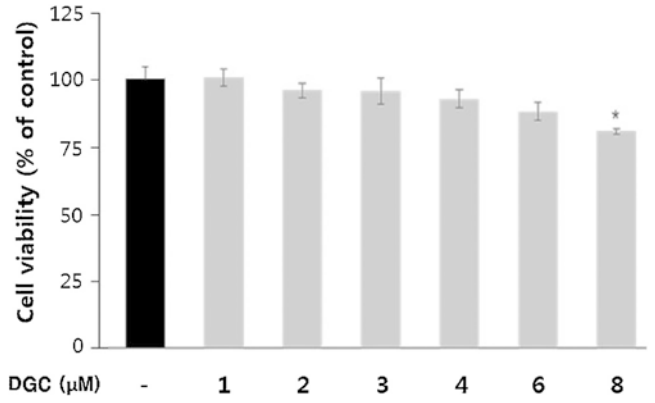

Fig. 2. B16F1 cell viability with various concentrations of DGC. B16F1 cells were incubated with various concentrations of DGC for 72 h, and then the cell viability was determined by MTT assay. Values are expressed as the mean ± SD of triplicate determinations. *$p < 0.05$ compared with untreated control.

DGC inhibits melanogenesis and tyrosinase activity

To investigate the effect of DGC on melanin synthesis, we measured the melanin contents and TYR activity in α-MSH-induced B16F1 melanoma cells. As shown in Fig. 3A and B, extra/intracellular melanin contents indicated the decrease by DGC treatment (extracellular melanin contents: control, 100 ± 3.2%; α-MSH, 202 ± 7.3%, 1 μM, 187 ± 3.6%; 2 μM, 182 ± 3.4%; 4 μM, 136 ± 5.3%; 6 μM, 95 ± 3.4%. Intracellular melanin contents: control, 100 ± 3.8%; α-MSH, 181 ± 20.4%, 1 μM, 174 ± 8.7%; 2 μM, 169 ± 4.6%; 4 μM, 131 ± 13.2%; 6 μM, 91 ± 4.9%). Tyrosinase, a key regulator in melanogenesis, acitivates melanogenesis. We evaluated the TYR inhibiton effects of DGC to determine whether DGC directly affects TYR activity or not. Our results showed DGC treatment decreased TYR activity in a dose-dependent manner (Fig. 3C). The inhibition effect of TYR is contributed by structural specificity of DGC containing hydroxyl group at the para position, which is similar to the structure of tyrosine as TYR substrates. Also, DGC possesses the catechol moiety (2,4-dihydroxyl groups) that is an significant pharmacophore to inhibit TYR activity. But these effects is not enough for describing at all. To find out additional mechanism in cell as well as the structural specificity, we investigated the level of cellular proteins involved melanogenesis.

DGC inhibits melanogenesis-related proteins expression in α-MSH-induced B16F1Cells

We measured the activity of DGC on the expression level of melagenic proteins (TYR, TRP-1 and TRP-2). DGC reduced the

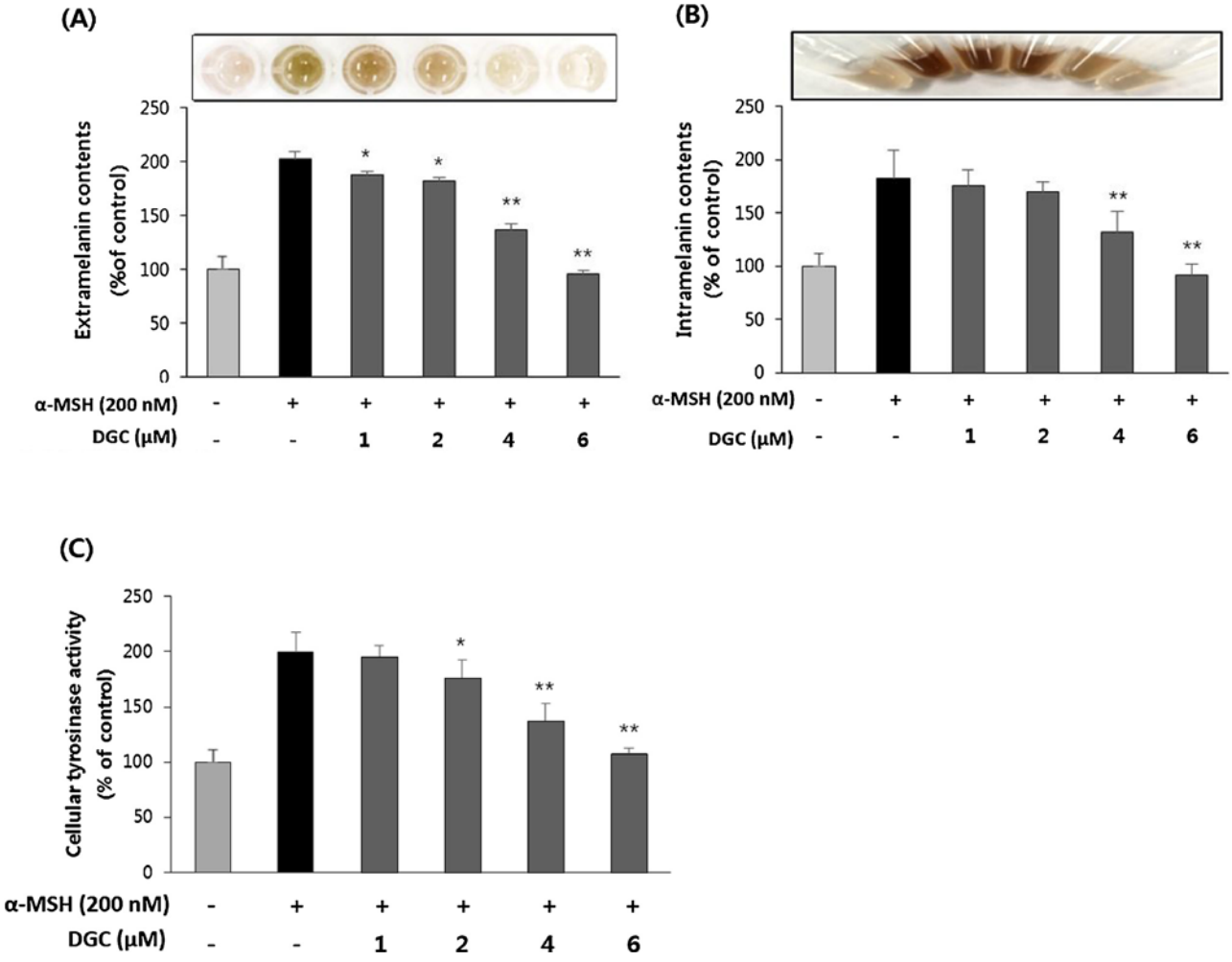

Fig. 3. Inhibition Effects of DGC on melanogenesis in B16F1 cells. Extramelanin (A) and intramelanin (B) inhibition and cellular tyrosinase activity (C) on melanocytes in the presence of various concentration of DGC and a-MSH used as a melanin stimulator at 200 nM. Values are expressed as the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with control treated by a-MSH only.

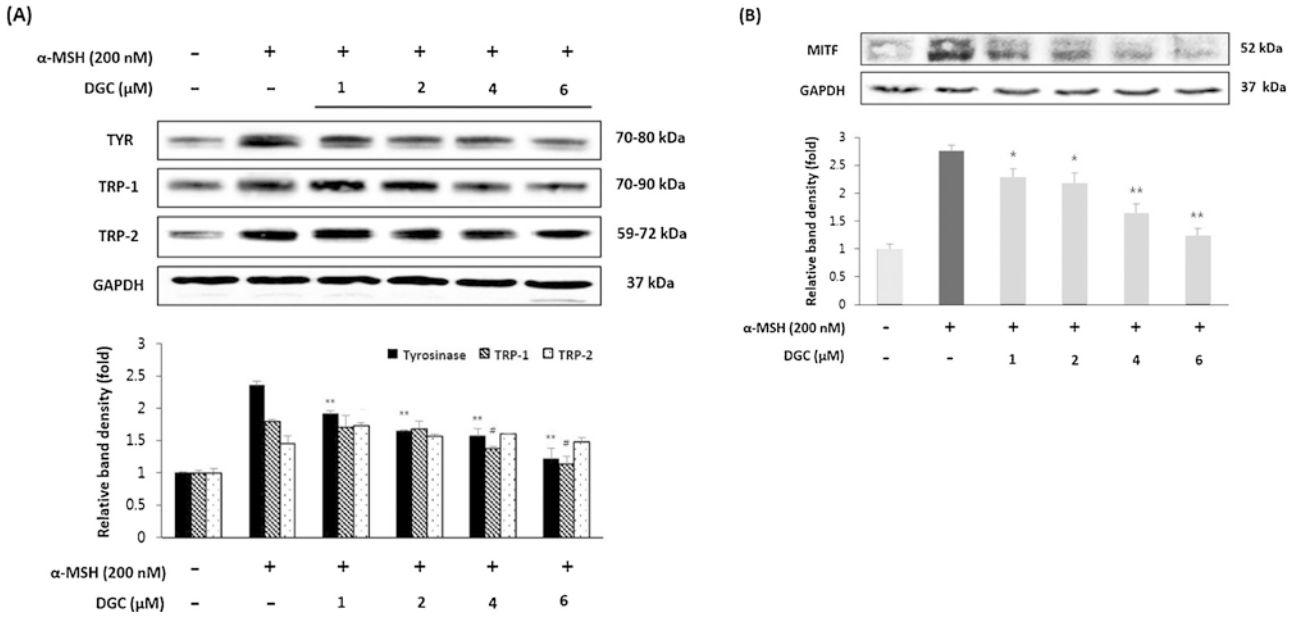

Fig. 4. Inhibitory effect of DGC on protein expression related to melanin biosynthesis. Tyrosinase, TRP-1, TRP-2 (A) and MITF (B) were measured in B16F1 cells treated with various concentrations of DGC (1, 2, 4 and 6 μM) and stimulated by 200 nM α-MSH for 48 h and 3 h respectively. The protein expression of tyrosinase, TRP-1, TRP-2 and MITF was measured by western blot analysis and quantified by Image J. Values are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with tyrosinase or MITF, $^{\#}p < 0.01$ compared with TRP-1 on control treated by α-MSH only.

expressions of TYR and TRP-1, but not TRP-2 (Fig. 4A). Especially, DGC contribute to reduce TYR expression from low concentration (1 μM). MITF, a key transcription factor, controls the expression of TYR, TRP-1 and TRP-2 [24]. Expression of MITF is affected by protein kinase signaling pathways and ERK patheway. Also, DGC decreased MITF expression in a concentration-dependent manner (Fig. 4B). Thus, we expected that anti-melanogenesis effect of DGC was contributed by a mechanism related to MITF expression.

DGC downregulated cAMP-CREB pathway in α-MSH-induced B16F1Cells

cAMP accelerated by activated adenylyl cyclase acts as the second messenger. It activates PKA, which phosphorylates cAMP-responsive element-binding protein (CREB). The cAMP-CREB pathway is the main stream induced by α-MSH. To investigate whether the decreased/inhibited MITF was due to DGC, the

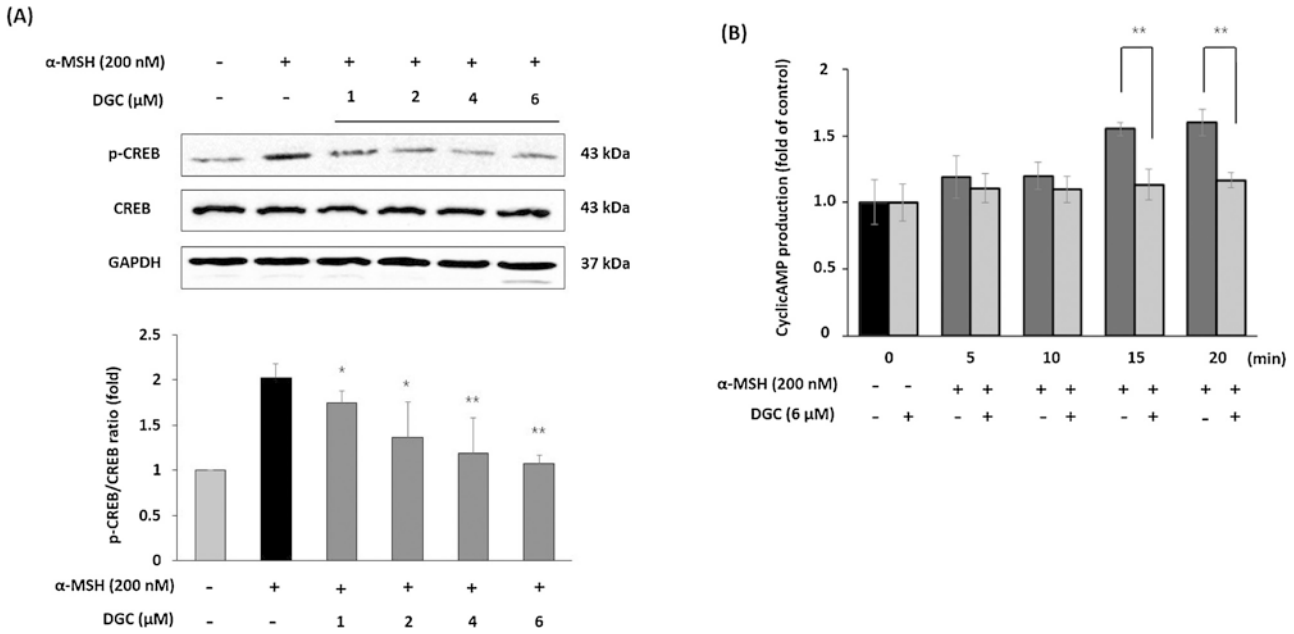

Fig. 5. Effect of DGC on the cAMP and CREB signaling pathway in B16F1 cells. (A) Phosphorylation of CREB treated with various concentration of DGC in the presence of 200 nM α-MSH for 45 min. Protein expression levels of phospho-CREB and CREB were determined by western blot analysis and quantified by Image J. Equal protein loading was confirmed using an anti-GAPDH antibody. (B) Cellular cAMP level was determined after 20 min. Data shown represent the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control treated by α-MSH only.

upstream CREB phosphorylation was analyzed (Fig. 5A). The CREB phosphorylation was accelerated by α-MSH. DGC significantly decreased phosphorylated CREB in a concentration-dependent manner. This result suggest that DGC could inhibit CREB phosphorylation leading to the subsequent decrease of MITF transcription. Based on these results, it was possible to suppose that DGC could exert cAMP associated signaling to α-MSH-induced melanogenesis. The level of cAMP increased on α-MSH induced B16F1 cells, and DGC reduced the increased cAMP in a concentration-dependent manner (Fig. 5B). Therefore, our results suggest that DGC significantly inhibited the expression of melanogenic proteins through the cAMP-CREB pathway.

Effects of DGC on ERK activation

The phosphorylation of ERK regulates melanin synthesis through the stimulation of MITF. The phosphorylation of ERK phosphorylation induces reduction of MITF leading to subsequent ubiquitin-dependent proteasomal degradation [25–27]. Thus, we analyzed the effect of DGC on ERK activation, ERK phosphorylation by western blot analysis. DGC induced the phosphorylation of ERK at 6 μM only in the presence of α-MSH and did not affect total ERK level (Fig. 6A and B). Especially, ERK phosphorylation effect of DGC presented after 30 min. To estimate the inhibition effects of DGC on MITF expression through ERK phosphorylation, melanocytes were treated with the ERK inhibitor, α-MSH and DGC. As shown in Fig. 6C–E, α-MSH increased MITF expression and melanin synthesis not increase the ERK phosphorylation significantly. DGC upregulated ERK phosphorylation, and suppressed MITF expression and melanin synthesis, while PD98059, ERK inhibitor, decreased the activities of DGC on α-MSH-induced B16F1Cells. These results suggest that DGC inhibits melanin synthesis through degradation of MITF from acceleration of ERK.

Discussion

In this study, we investigated the inhibitory effect of DGC, the active component of *G. uralensis* on melanogenesis whether DGC can be used as a whitening cosmetic agent. We found that DGC inhibited melanin production on B16 cells stimulated α-MSH in a dose-dependent manner.

G. uralensis is a kind of licorice, traditionally herbal, and widely used in Europe and Asia as a medicinal plant with beneficial effects such as anti-inflammatory, antimicrobial, anti-melanogenic activities. Licorice has various medical components containing glycyrrhizin, glabridin, isoliquiritigenin, DGC and etc. Especially, glabridin is well known for a whitening agent and they were included in oil soluble fraction of licorice [28]. DGC, a kind of prenylflavonoid, has anticancer, anti-inflammatory, and antioxidant effects [20,21]. However, its whitening effect hasn't been known yet. We have identified a new whitening agent from licorice in this research.

Our results showed DGC decreased *in vitro* activities of both mushroom and cellular TYR. DGC has hydroxyl group at the para position, which is similar to the binding group of TYR substrate. Additionally, DGC also has 2,4-dihydroxyl groups, the catechol moiety, which is a functional group for chelating on TYR. The skin cells respond to a wide range of environmental stimulus (UV, ROS and etc.) through means like paracrine and/or autocrine factors, and can secret many factors as α-MSH, increase cellular cAMP, stimulates adenylyl cyclase in melanocytes. Then, cellular melanin contents were significantly increased in melanocyte [29]. DGC subsequently suppressed melanogenesis by decreasing related protein expression such as TYR, TRP-1 and MITF. DGC reduced the phosphorylation of CREB and accelerated cAMP in B16F1 melanoma cells which stimulated α-MSH. These results support the depigmenting mechanism of DGC on the cAMP-CREB signaling pathway [4]. Additionally, MITF degradation mechanism of DGC

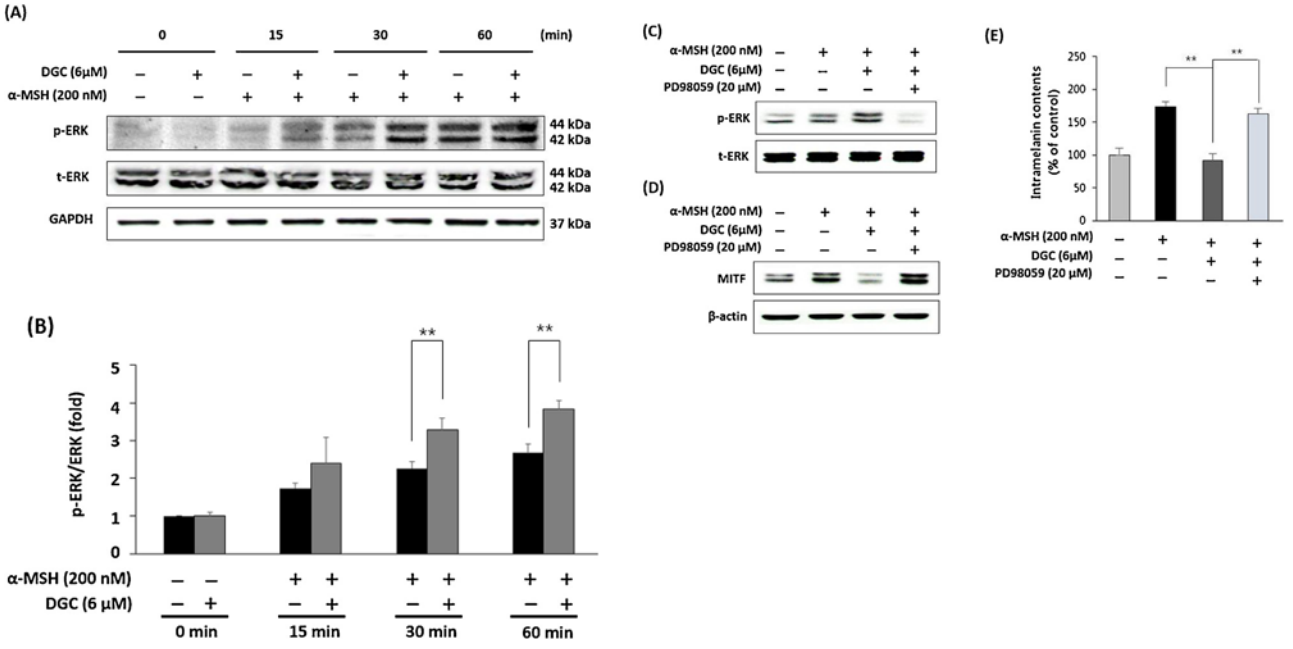

Fig. 6. Effects of DGC on the ERK signaling pathway. B16F1 cells were serum-starved for 24 h and treated with 6 μM DGC for the indicated time period (0–60 min). Protein expression levels of phospho-ERK and ERK were determined by western blot analysis followed by quantitation with Image J (A). Phosphorylation of ERK (B), MITF expression (C) and melanin contents (D) were determined in presence or absent of α-MSH, 6 uM DGC and PD98059 at 30 min or 3 h respectively. Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ compared with untreated control.

was contributed by increase of the ERK phosphorylation, which induced the downregulation of melanogenic proteins as TYR and TRP-1. But, inhibitory effect of DGC slightly indicated on melanogenesis through ERK pathway. These results suggest that main pathways of DGC is cAMP-CREB pathway and TYR inhibitory activity on melanogenesis. Interestingly, DGC can induce G1 arrest and apoptosis in mammary and prostate cancer cells in previous articles [30,31]. These cancer therapeutic effects suggest that DGC can regulate on the proliferation and activity of melanoma cells. The additional study require for underlying depigmenting effects of DGC.

Safety of therapeutic compounds is very important. Recently, many nature herbal extracts have gained attention as hypopigmenting agents [5,8,23]. DGC observed no toxicity after injection (5 mg/kg) for 24 h in rats [32]. This evidence suggests the DGC has potential as a safe whitening agent. Taken together, DGC may be useful in skin-depigmenting agent for a therapeutic treatment of hyperpigmentation.

Conflict of interest statement

All authors have declared that there is no conflict of interest.

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