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Inhibition of melanogenesis by 5,7-dihydroxyflavone (chrysin) via blocking adenylyl cyclase activity

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ABSTRACT

Due to its multiple biological activities, 5,7-dihydroxyflavone (chrysin) in propolis has gained attention as potentially useful therapeutics for various diseases. However, the efficacy of chrysin for the use of dermatological health has not been fully explored. To clarify the action mechanism of the skin protecting property of chrysin, we firstly investigated the molecular docking property of chrysin on the mammalian adenylyl cyclase, which is the key enzyme of cAMP-induced melanogenesis. We also examined the involvement of chrysin in alpha-MSH and forskolin-induced cAMP signaling within a cell based assay. In addition, we inquired into the inhibitory effect of chrysin on melanogenesis and found that the pretreatment with chrysin inhibited the forskolin-induced melanin contents significantly without annihilating the cell viability. These results strongly suggest that chrysin directly inhibits the activity of adenylyl cyclase, downregulates forskolin-induced cAMP-production pathway, consequently inhibiting melanogenesis. Thus, chrysin may also be used as an effective inhibitor of hyperpigmentation.

1. Introduction

Adenylyl cyclase (AC) is a 12-transmembrane protein that catalyzes the conversion of ATP to cAMP upon the stimulation of various G-protein coupled receptors [1]. In Eukaryotes, two basic families of AC exist: the G protein-regulated transmembrane AC isoforms and the soluble AC. The soluble AC is regulated by bicarbonate and calcium and is insensitive to forskolin or activated $G\alpha_s$ [2]. The topology of transmembrane ACs consists of a variable intracellular N terminus and two large cytoplasmic domains separated by two membrane-spanning domains (six transmembranes each) [3]. The transmembrane class is generally considered the target of most hormone-sensitive cAMP control. So far nine mammalian AC isoforms have been identified. These isoforms show distinct tissue distribution and biological/pharmacological properties, while they share the same membrane topology and catalytic core formation that resembles transporters [1]. All nine membrane-bound AC isoforms are activated by GTP-bound $G\alpha_s$ and the plant diterpene forskolin, with the exception of AC9. Forskolin, a direct activator of AC, is a diterpene extract from *coleus forskolii*, which is a natural plant used in Indian traditional medicine for the treatment of heart failure, bronchial asthma, and alimentary diseases [4].

Drug discovery in skin pharmacotherapy is an enormous, continually expanding field. Researchers are developing novel and sensitive pharmaceutical products and drugs that target specific receptors to elicit concerted and appropriate responses [5]. Melanin is main target for skin pharmacotherapy, and is synthesized in special organelles called melanosomes that play an essential role in protecting skin from deleterious sunlight under normal conditions. However, enhanced generation and excessive accumulation of melanin can cause a number of skin problems such as freckles, age spots, and melasma [6]. Melanin synthesis is stimulated by a large number of effectors, including 1-oleyl-2-acetyl-glycerol [7], ultraviolet B radiations [8], cAMP-elevating agents (forskolin, IBMX, a-MSH) [9]. The major signaling pathway leading to melanin synthesis appears to be stimulation of adenylyl cyclase followed by an increase in the cAMP level and activation of cAMP-dependent protein kinases A (PKAs). cAMP increases the expression of the micropthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor crucial for melanocyte development and differentiation, through activation of the cAMP-dependent protein kinase A (PKA) and the cAMP-response element binding protein (CREB) transcription factor, which in turn stimulates tyrosinase gene expression to allow melanin synthesis [10]. Therefore, the inhibitors of the melanogenesis via blocking adenylyl cyclase activity and its signaling pathway, particularly from natural sources, can be a great interest to industries that produce skin medications and cosmetics.

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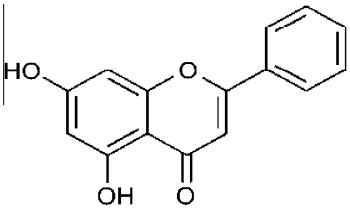

Fig. 1. Chemical structure of 5,7-dihydroxyflavone (chrysin).

In previous studies, propolis and honey showed wound healing and skin protective effects [11,12]. Chrysin (5,7-dihydroxyflavone, Fig. 1) is a natural flavonoid contained in many plant extracts, honey, and propolis [13,14]. Chrysin is also known for its multiple biological activities, such as anti-inflammation [15], anti-cancer [16], and anti-oxidation [17]. Therefore, using the well-characterized Autodock computation modeling system and B16 mouse melanoma cells, we attempted to determine the effects and mechanisms of chrysin on adenylyl cyclase and melanogenesis. In this report, we first demonstrated that chrysin down-regulates melanogenesis via blocking adenylyl cyclase activity and directly affects on cAMP production in B16 melanoma cells.

2. Materials and methods

2.1. Reagents

Chrysin, forskolin, α-MSH, MTT and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). [^{3}H]cAMP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

2.2. Molecular docking

Autodock4 [18] was used for the docking study of the chrysin on the mammalian adenylyl cyclase (mAC). Forskolin, a known ligand of mAC, was used as a controller for the test of the docking reliability. The atomic coordinates of the chain A and B corresponding to VC1 and IIC2, two catalytic subunits of mAC, respectively, were adopted from the PDB file 1UOH [19]. ADT [20] was used to prepare the ligands and the receptor molecules and to define a search box encompassing both binding sites of forskolin and 2′(3′)-*O*-(*N*-methylanthraniloyl) (MANT)-GTP (37.5 × 24.0 × 27.0 Å). The receptor molecule was assumed to be rigid or flexible, and thus four docking experiments for two ligands were performed in total. The residues of Asp A440, Leu A438, Lys B938, Asp B1018, Asn B1025, and Lys B1065 were defined as flexible. For each docking experiment, 100 runs of Lamarckian genetic search were done and the resulting docked coordinates of ligands were clustered based on their r.m.s.d. values. The result was analyzed and visualized by the software PyMol [21].

2.3. Cell culture

B16 cells were purchased from Korean Cell Line Bank (Seoul, Korea) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco, NY, USA.) and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO_2 in air at 37 °C. B16 cells were cultured in 24-well plates for each assay. All the experiments were performed in triplicate and repeated three times to ensure reproducibility.

2.4. Measurement of [^{3}H]cAMP

Intracellular cyclic AMP generation was determined by [^{3}H]cAMP competition assay in binding to cyclic AMP-binding protein as described previously by [22] with some modification. To determine the cyclic AMP production induced by forskolin, the B16 cells were stimulated with agonists for 20 min in the presence of the phosphodiesterase inhibitor Ro 20–1724 (5 μM) and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at 2500×*g* for 5 min at 4 °C. The cyclic AMP assay is based on the competition between [^{3}H]-labeled cyclic AMP and unlabeled cyclic AMP present in the sample for binding to a crude cyclic AMP-binding protein prepared from bovine adrenal cortex according to the method of [23]. Each sample was incubated with 5 μl of [^{3}H]-labeled cyclic AMP (5 mCi) and 100 μl of binding protein for 2 h at 4 °C. Separation of the protein-bound cyclic AMP from the unbound cyclic AMP was achieved by the adsorption of the free cyclic AMP onto charcoal (100 μl) followed by centrifugation at 12,000×*g* at 4 °C. The 200 μl of supernatant were then placed into an Eppendorf tube containing 1.2 ml of scintillation cocktail to measure the radioactivity. The cyclic AMP concentration in the sample was determined based on a standard curve and expressed as pmol per number of cells.

2.5. Estimation of melanogenesis in B16 cells

Melanin content was used as an index of melanogenesis. Determination of melanin content was performed using a modified method of previous study [24]. In brief, B16 cells (5×10^4) were plated on 24-well, multi-dishes and pretreated with or without chrysin at concentrations ranging from 5 to 100 μM. Then added and incubated with 100 nM of forskolin for 24 h. After washing twice with PBS, samples were dissolved in 100 ml of 1 N NaOH. The samples were incubated at 60 °C for 1 h and mixed to solubilize the melanin. Absorbance at 405 nm was compared with a standard curve of synthetic melanin.

2.6. Determination of chrysin for cell viability

The cell viability assay was performed as described in the previous study [25] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Shortly thereafter, 5×10^4 B16 cells/well were plated in a 24-well plate. After cells were exposed to chrysin at various concentrations for 24 h, MTT solutions were added and the insoluble derivative formed by cellular dehydrogenase was solubilized with EtOH–dimethyl sulfoxide (DMSO) (1:1 mixture solution); the absorbance of each well was read at 560 nm using a microplate spectrophotometer.

2.7. Statistical analysis

The values are expressed as mean ± standard error ($n = 3$) and the biological significance $p < 0.05$ was determined by the Student's *t*-test.

3. Results

3.1. Molecular docking study of chrysin on adenylyl cyclase

Autodock4 successfully identified the crystallographic binding mode of forskolin with a good separation from other binding modes (Fig. 2, panels A and B). The r.m.s.d. of the best docking solution of forskolin from the crystallographic structure was 1.1 Å. Out of 100 runs of Lamarckian genetic search, the best cluster had 64 runs. The best estimated free energy of binding given by Autodock4 was −12.33 kcal/mol and the estimated inhibition constant, Ki was 912.34 pM at 298.15 K for this cluster.

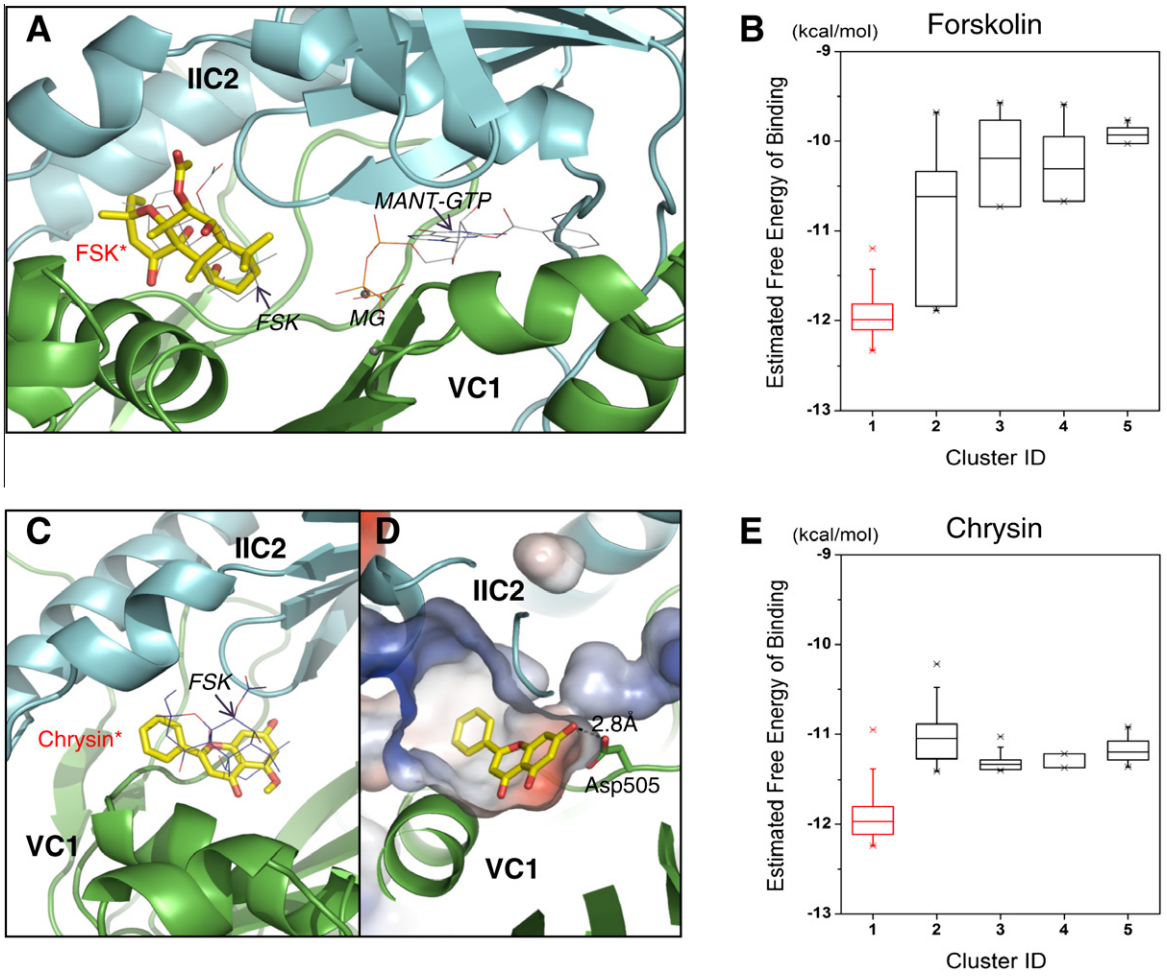

Fig. 2. The predicted docked conformation of forskolin (FSK) and chrysin. The best docking solution for forskolin and chrysin (yellow stick; denoted by *) was visualized on the mAC structure (ribbon). For comparison, the known binding mode of forskolin and MANT-GTP is shown as lines (A and C, respectively); the distribution of calculated binding free energy of top five docking clusters for forskolin and chrysin, respectively, were summarized as a box plot. The best cluster for each molecule is shown in red (B and E); the solvent accessible surface around the best docking conformation of chrysin was mapped by AMBER99 vacuum electrostatic potential calculated by PyMOL (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The best docking solution of chrysin was found at the forskolin binding site (Fig. 2, panels C, D and E). This well separated solution consisted of 17 runs, probably due to a more relatively complex energy landscape than that of forskolin. Autodock, however, suggests that the binding affinity of chrysin can be comparable to that of forskolin. The best estimated free energy of binding for chrysin was −12.24 kcal/mol and the estimated inhibition constant, Ki was 1.06 nM. For both ligands, consideration of flexible residues in the mAC protein did not affect the location of the best cluster.

The predicted binding mode of chrysin was mediated by hydrophobic interactions at the phenyl group and by one hydrogen bond at the 7-hydroxyl group with Asp505 from VCI. In addition, the narrow binding cleft formed between two catalytic subunits, VC1 and IIC2 (Fig. 2. panel D) served as a steric constraint, and thus the possible orientations of chrysin were limited. The existence of extra space in the cavity and two non-interacting polar groups, one ketone and one hydroxyl group in chrysin, suggests that further modification might be possible.

3.2. Inhibitory action of chrysin on adenylyl cyclase-induced cAMP production

To check whether chrysin really blocks adenylyl cyclase activity in living cellular system or not, we chose the B16 melanoma cell line and investigated the chrysin's inhibitory effect. Both α-MSH and forskolin are known to increase intracellular cAMP concentration via activating adenylyl cyclase signaling pathways in B16 cells [26]. We preliminarily checked the cAMP production induced by α-MSH and forskolin in B16 cells (data not shown), and EC_{50} for both were 100 nM. 5 min pretreatment of chrysin showed strong inhibitory effect on α-MSH – and forskolin-induced cAMP production in a concentration-dependent manner (Fig. 3) suggesting that chrysin inhibits cAMP production signaling pathways via blocking adenylyl cyclase.

3.3. Inhibitory action of chrysin on melanin content in B16 cells

In order to obtain evidence on the inhibitory effect of chrysin on forskolin-induced melanogenesis signaling pathway in B16 cells, melanin production in B16 cells was examined. As shown in Fig. 4A, the melanin content of the cells that were pretreated with chrysin decreased in dose-dependent manners, showing 51.6% at 10 μM, 40.90% at 100 μM compared to the forskolin alone treated control group.

3.4. The effect of chrysin on cell viability

The data from the cell viability assay using MTT for B16 cells are shown in Fig. 4B. At IC_{50} concentration of chrysin (10 μM), the B16 cells were treated for 24 h. Longterm treatment of chrysin did not

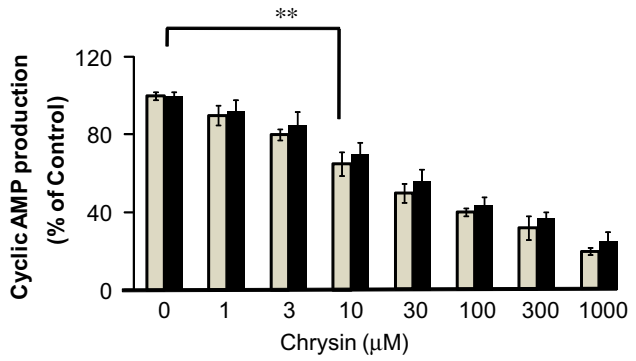

Fig. 3. The inhibition of α-MSH- and forskolin-induced cAMP production with the increase in the concentration of chrysin treatment. B16 cells were preincubated with various concentrations of chrysin for 5 min, then cells were stimulated with α-MSH (100 nM, gray bar) and forkolin(100 nM, closed bar), respectively. The data are the mean ± S.E.M. of assay triplicates with at least six independent experiments.

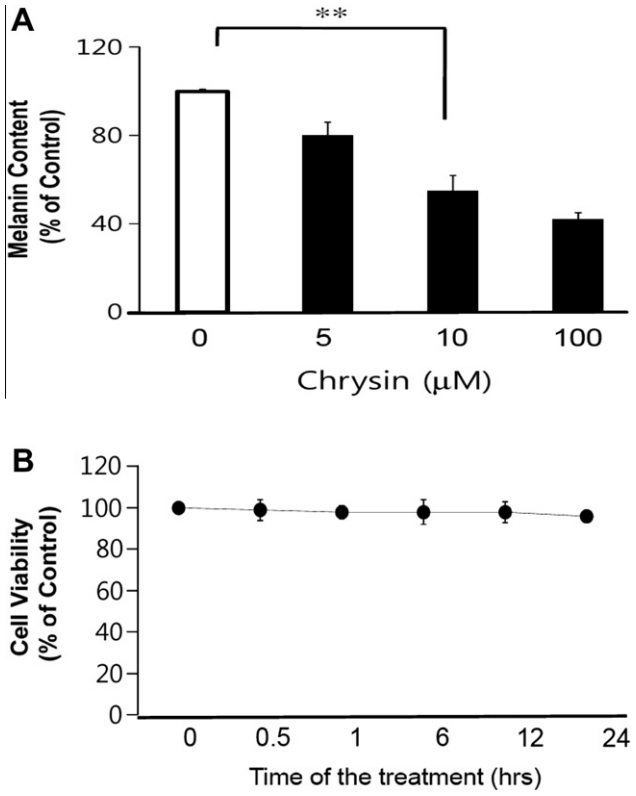

Fig. 4. (A) The melanin content reduction with the increase in the concentration chrysin treatment on the melanin biosynthesis. Open bar represents without chrysin, and closed bar with chrysin pretreatment. The results are expressed as percentages of control, and the data are the mean ± S.E.M. of at least three determinations. (B) The viability of B16 cells during the chrysin treatments. Cells were treated over the indicated time (from 0 to 24 h) with 10 μM of chrysin. The viability was evaluated by MTT assay and represented as percentages of control. The data are the mean ± S.E.M. of assay triplicates.

induce any cellular death. These data clearly indicate that the anti-melanogenesis effect of chrysin is not due to its cytotoxicity.

4. Discussion

The inhibition of melanogenesis can be useful not only for cosmetic as skin-whitening purposes, but also for the treatment of abnormal pigmentation. In the present study, we first provided the evidence that polyphenolic natural compound chrysin has a potent anti-melanogenic effects via blocking adenylyl cyclase activity.

The result of docking study implies the specific binding of chrysin to adenylyl cyclase. The best docking solution for chrysin found in the forskolin binding cleft is well separated from the next best solution. The difference in binding free energy between two solutions was statistically significant ($p < 0.05$, two samples unpaired t-test). The best cluster was the largest one. This indicates the success in finding the globally optimal docking conformation by search algorithm. In addition, the lowest free energy of binding for chrysin given by Autodock4 was as low as that for a known ligand, forskolin. The docking study predicted a preferential binding of chrysin to the forskolin binding site than to the MANT-GTP site. This may be partly due to two charged magnesium ions which create very strong polar environment in the MANT-GTP site, and thus repel large hydrophobic molecules. Or one may claim about the failure of docking at this site. In fact, still there is no guarantee of successive metal ion treatment in docking, despite a report of recent success [27]. It is also because of not yet efficient docking algorithm for the highly flexible region such as the MANT-GTP site. The test docking run with MANT-GTP as a ligand yielded non-separable clusters and failed in finding the crystallographic binding mode (data not shown). This, however, requires a careful interpretation. The search process for MANT-GTP with 11 torsions and metal ions is an extremely difficult case and can differ from the smaller and less flexible ligands such as forskolin (3 torsions) or chrysin (2 torsions). In our study, whether the receptor flexibility is considered or not, Autodock4 yielded consistent results for both forskolin and chrysin. In this regard, adenylyl cyclase may also have a great potential to be a new target drugs in regulating cyclic nucleotide signal in melanogenesis.

In many previous reports [28–32], polyphenolic hydroxyl compound may show skin protection and whitening activity because of its ability to donate an electron and/or chelate transition metals, such as copper or ferrous ions, thereby inhibit free radical reactions and diminish ROS generation. Hong et al. showed that chrysin has antioxidative property and inhibitory effect on matrix metalloproteinase activity in UVA-irradiated human dermal fibroblast [33]. One of the interesting findings of our study is that the potency of chrysin is likely related to its duel efficacy on anti-melanogenic and antioxidative activities. As shown in this study, chrysin inhibited melanogenesis induced by adenylyl cyclase activity and was able to decrease melanin content without showing any adverse effect on cell viability. These strong anti-melanogenic and skin protecting actions of chrysin are ascribed to its structural arrangement.

Overall, the elucidation of the binding site structure of for chrysin on adenylyl cyclase could provide a structural basis for the pharmacological modulation of ACs and clues for the treatment related incurable diseases. Chrysin was introduced as a potent therapeutic agent for skin hyperpigmentation such as melanosis.

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