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Stimulation of melanogenesis by glycyrrhizin in B16 melanoma cells

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Abbreviations: DHICA, 5,6-dihydroxyindole-2-carboxylic acid; GA, glycyrrhetinic acid; GR, glycyrrhizin; IBMX, isobutylmethylxanthine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; TCA, trichloroacetic acid; TRP, tyrosinase-related protein

Abstract

Glycyrrhizin (GR), triterpenoid saponin composed of one glycyrrhetinic acid (GA) and two glucuronic acids, is a main constituent of the hydrophilic fraction of licorice (*Glycyrrhiza glabra*) extracts and is known to have a wide range of pharmacological actions. In this study, we investigated the mechanism of GR effect on melanogenesis in B16 murine melanoma cells. The cellular levels of tyrosinase mRNA, protein, enzyme activities and melanin contents were increased by GR in a dose dependent manner. Expression of tyrosinase-related protein-2 (TRP-2) mRNA was also increased by GR, however, no significant change was observed on TRP-1. No cytotoxicity was observed at the effective concentration range of GR. GA showed no effect on melanogenesis at the equivalent nontoxic concentrations, indicating that glycoside structure is important in the stimulatory effect of GR on melanogenesis. These results indicate that GR-induced stimulation of melanogenesis is likely to occur through the transcriptional activation.

Keywords: melanogenesis, glycyrrhizin, tyrosinase

Introduction

The crude extract of licorice (*Glycyrrhiza glabla L.*) has been widely used for gastric ulcers and other diseases. Glycyrrhizin (GR) and its aglycone, glycyrrhetinic acid (GA) are the main constituents of the hydrophilic fraction of licorice extracts and are known to have a wide range of pharmacological actions including anti-inflammatory (Inoue *et al.*, 1986), anti-allergic (Takeda *et al.*, 1991), anti-viral (Pompei *et al.*, 1979), anti-carcinogenic (Nishino *et al.*, 1984), thrombin inhibitor (Francischetti *et al.*, 1997), and anti-immune-mediated cytotoxicity (Yoshikawa *et al.*, 1997).

Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein I (TRP-1), and tyrosinase-related protein 2 (TRP-2) (Hearing, 1999). Tyrosinase is a bifunctional enzyme which plays a pivotal role in the modulation of melanin production, first by catalyzing the hydroxylation of tyrosine to DOPA and secondly by catalyzing the oxidation of DOPA to DOPA-quinone (Hearing and Jimenez, 1987). TRP-2, which functions as DOPAchrome tautomerase, catalyzes the rearrangement of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Yokoyama *et al.*, 1994), and TRP-1 oxidizes DHICA to a carboxylated indole-quinone (Kobayashi *et al.*, 1994). Both of them work at the downstream points in the melanin biosynthetic pathway.

Recently, glabridin, a main constituent of the hydrophobic fraction of licorice extracts, has been reported to have inhibitory effects on melanogenesis (Yokota *et al.*, 1998). It is a kind of flavonoids acting as an inhibitor of tyrosinase activity. In an earlier study (Abe *et al.*, 1987) GA inhibited the growth of B16 melanoma cells and stimulated melanogenesis. However, GR induced the similar changes requiring 20 times more concentration. Our studies showed that GR was a far more effective inducer of melanogenesis than GA at nontoxic concentration. In this study, the mechanism of GR and GA effects on melanogenesis was investigated at a cellular level using B16 murine melanoma cells.

Materials and Methods

Cell Culture

B16/F10 murine melanoma cells (kindly supplied by Professor D.G. Kim, Department of Internal Medicine, Chonbuk National University Medical School, Chonju, Korea) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells were seeded on 6-well plates and drug treatment began 24 h

after seeding. GR was obtained from Sigma Chemical Co. (St. Louis, U.S.A.) and GA from Aldrich Chemical Co. (Milwaukee, U.S.A.). The cells were harvested three days later and the melanin contents and tyrosinase activities were determined in triplicate for each treatment (Ando *et al.*, 1998).

Melanin content measurement

Melanin contents of cultured B16/F10 cells were measured according to the method of Kim *et al.* (1998) with a slight modification. Cells were washed twice with phosphate-buffered saline (PBS) and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). Cell lysate was precipitated with same amount of 20% trichloroacetic acid (TCA). After washing twice with 10% TCA, pellets were treated with ethyl alcohol : diethyl ether (3 : 1) and diethyl ether successively. Samples were air-dried, dissolved in 1 ml of 0.85 M KOH, and boiled for 15 min. After cooling, absorbance was measured with a spectrophotometer at 400 nm. The amount of cellular melanin was corrected by the protein contents of the samples. Protein concentration was determined by the method of Bradford (Bradford, 1976).

Assay of tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity using a modified method described by Lerch (Lerch, 1987). Cells were washed twice with PBS and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). Tyrosinase activity was analyzed spectrophotometrically by following the oxidation of DOPA to DOPAchrome at 475 nm. The reaction mixture containing 100 μl of freshly prepared substrate solution [0.1% L-DOPA in 0.1 M sodium phosphate (pH 6.0)] and 50 μl of enzyme solution was incubated at 37°C. The absorbance change was measured during the first 10 min of the reaction while the increase of the absorbance was linear, and corrections for auto-oxidation of L-DOPA in controls were made. The specific activity of tyrosinase in control cells was 0.37 ± 0.01 mmol L-DOPA oxidized/min/mg protein and activities were expressed as percentage of control cells.

MTT assay

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mosmann, 1983). After treatment, cells were washed twice with PBS. MTT (100 μg/0.1 ml PBS) was added to each well. Cells were incubated at 37°C for 1 h, and dimethyl sulfoxide (100 μl) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer (Spectra MAX PLUS, Molecular Devices, Sunnyvale, U.S.A.).

Western blotting

Cells were homogenized in 100 μl of ice-cold lysis buffer (20 mM HEPES; pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenates containing 20 μg of protein were separated by SDS-PAGE with 10% resolving and 3% acrylamide stacking gel (Laemmli, 1970), and transferred to nitrocellulose sheets (Schleicher & Schuell, Germany) in a Western blot apparatus (Bio-Rad, Hercules, U.S.A.) run at 50 V for 2 h. The nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 h with 1 μg/ml goat anti-murine tyrosinase IgG (Santa-Cruz Biotechnology Inc., Santa-Cruz, U.S.A.). The binding of antibody was detected with anti-goat IgG conjugated with alkaline phosphatase (Sigma, St. Louis, U.S.A.). Immunoblots were developed using a BCIP/NBT solution (Pierce, Rockford, U.S.A.).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was prepared using Tri-zol solution (Gibco-BRL, Paisley, UK) according to the manufacturer's instructions. After the preparation of cDNA with oligo $d(T)^{16}$ as a reverse transcriptase primer from the extracted RNA, amplification with PCR was performed using GeneAmp kit (Perkin Elmer, Foster City, U.S.A.) according to the manufacturer's manual. The oligonucleotide primers used for PCR are as follows: tyrosinase upstream 5'-GGC CAG CTT TCA GGC AGA GGT-3'; downstream 5'-TGG TGC TTC ATG GGC AAA ATC-3': TRP-1 upstream 5'-GCT GCA GGA GCC TTC TTT CTC-3'; downstream 5'-AAG ACG CTG CAC TGC TGG TCT-3': TRP-2 upstream 5'-GGA TGA CCG TGA GCA ATG GCC-3'; downstream 5'-CGG TTG TGA CCA ATG GGT GCC-3': actin upstream 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; downstream 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. cDNA amplification used the product of about 1 μg of total RNA. The reaction was cycled 20 times through 60 s at 94°C, 60 s at 56°C, and 60 s at 72°C. Fifty percent of reaction mixture was analyzed by electrophoresis on 1.5% agarose gels and stained by ethidium bromide. In order to check the reproducibility of the results, each experiment was carried out more than three times.

Results

To investigate the effect of GR on melanogenesis, B16/F10 murine melanoma cells were incubated with various concentrations of GR for 72 h. The cellular content of melanin was increased by GR in a dose-dependent manner (Figure 1). At the concentration of 0.5 mM, the

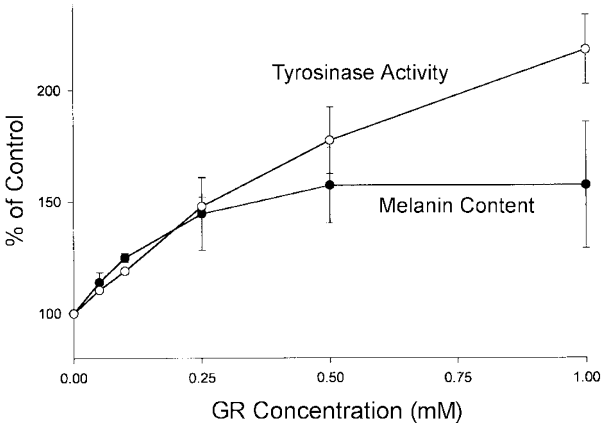

Figure 1. Effect of GR on the melanin content and tyrosinase activity. After incubation of B16 murine melanoma cells with various concentrations of GR for 72 h, melanin contents and tyrosinase activities were determined as described in "Materials and Methods". Data are expressed as a percentage of control and are mean ± SE of three separate experiments.

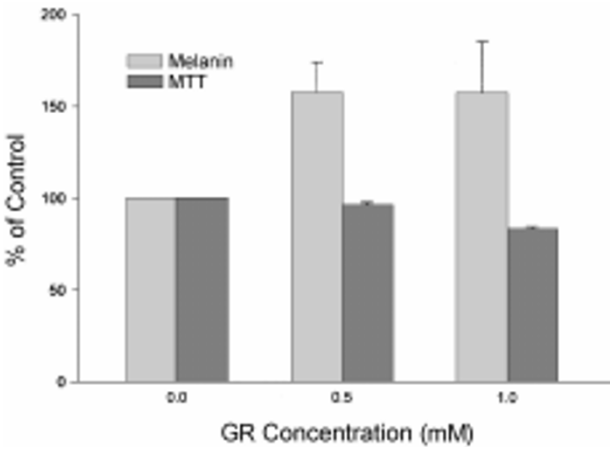

Figure 2. Effect of GR on the melanin content and cytotoxicity. After incubation of B16 murine melanoma cells with various concentrations of GR for 72 h, determination of melanin contents and MTT assay were performed. Data are expressed as a percentage of control and are mean ± SE of three separate experiments.

cellular melanin content reached nearly to plateau, about 160% of control cells. The cellular tyrosinase activity was also increased dose-dependently by GR, reaching to 220% of control cells at the concentration of 1 mM (Figure 1). No cytotoxicity was observed with less than 0.5 mM of GR. Even at 1 mM, only 15% decrease in cell number estimated by MTT assay was observed (Figure 2).

GR has the structure of triterpenoid saponin composed of one GA and two glucuronic acids. To determine whether the stimulatory effect of GR on melanogenesis was due to the component of GA, cells were incubated with GA and its effect was investigated. As shown in Figure 3, GA showed no effect on the cellular melanin content and tyrosinase activity till 10 μM. Rather, it

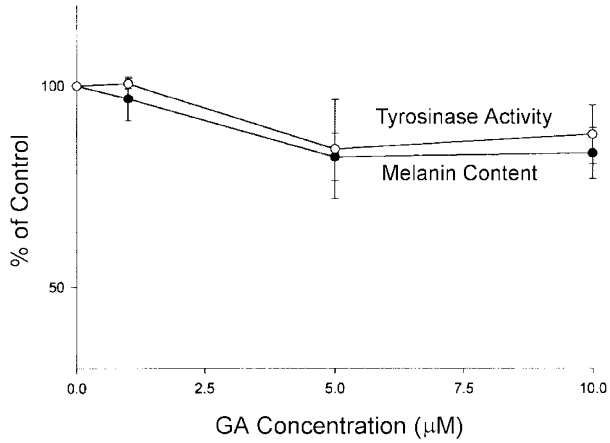

Figure 3. Effect of GA on the melanin content and tyrosinase activity. After incubation of B16 murine melanoma cells with various concentrations of GA for 72 h, melanin contents and tyrosinase activities were determined. Data are expressed as a percentage of control and are mean ± SE of three separate experiments.

Figure 4. Western blotting of tyrosinase. B16 murine melanoma cells were incubated with none (lane 1), 1 mM GR (lane 2) or 0.1 mM IBMX (lane 3) for 72 h. After the lysis of cells, proteins were separated on SDS-PAGE and transferred to nitrocellulose paper. Immunoblotting with murine tyrosinase antibody was performed.

showed a tendency of suppression for melanogenesis. Over the concentration of 10 μM, it was impossible to assay due to strong cytotoxicity.

The amount of tyrosinase protein in the cell was visualized by Western blotting. When cells were treated with 1 mM of GR for 72 h, significant increase of tyrosinase protein was observed (Figure 4, lane 2). As a positive control, isobutylmethylxanthine (IBMX) was used to induce the tyrosinase expression (Figure 4, lane 3).

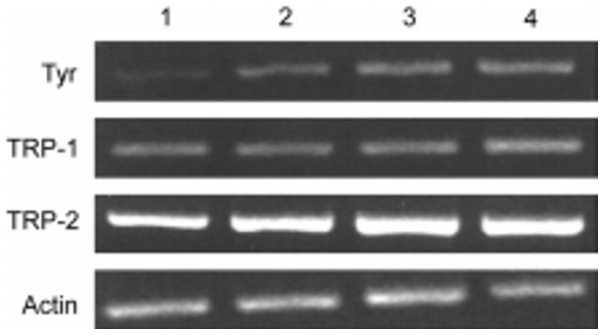

Figure 5. RT-PCR profiles of tyrosinase, TRP-1 and TRP-2. B16 murine melanoma cells were incubated with none (lane 1), 0.5 mM GR (lane 2), 1 mM GR (lane 3) or 0.1 mM IBMX (lane 4) for 72 h. RT-PCR for tyrosinase, TRP-1 and TRP-2 gene was performed on RNA extracts.

The expressions of mRNA for tyrosinase, TRP-1 and -2 were investigated through the RT-PCR. Cells were treated with 0.5 and 1 mM of GR for 72 h and the total cellular RNA was extracted. Specific mRNAs were amplified, after reverse transcription, with PCR using specific primers for tyrosinase, TRP-1 and -2. As shown in Figure 5, mRNAs of tyrosinase and TRP-2 were increased by the treatment of GR. No significant change was observed on TRP-1. All of the three components were increased by IBMX (Figure 5, lane 4). These results indicate that GR-induced stimulation of melanogenesis occurs at the transcriptional level.

Discussion

In this study, we observed that GR stimulates the melanogenesis in B16 murine melanoma cells, and GR-induced stimulation occurs through the increased expression of tyrosinase and TRP-2 at the transcriptional level.

The hydrophobic fraction of licorice extracts, which contains various flavonoids, has been known to have the inhibitory effects on melanogenesis possibly due to its inhibition of tyrosinase activity (Yokota *et al*., 1998). As shown in the results, it was impossible to assess the effect of GA on melanogenesis due to its high cytotoxicity at the concentrations over 10 μM. Even at the non-cytotoxic levels, GA showed nearly no or slight suppressive effect on melanogenesis in contrast to the previous report of Abe *et al.* (1987). The difference of drug preparation might be one of the possible explanations. GR becomes hydrophilic by two attached glucuronic acids to the hydrophobic core, GA. GR has stimulatory effect indicating that glycoside structure of GR is important in the stimulation on melanogenesis. GR showed no direct effect on tyrosinase activity (data not shown).

Melanin is a unique pigmented biopolymer synthesized by specialized cells known as melanocytes, dendritic cells that exist as relatively minor populations in the skin, hair, eyes and other locations. Melanin has a number of important functions, ranging from its role in the determination of phenotypic appearance, to protective coloration, to balance and auditory processing, to absorption of toxic drugs and chemicals, and to neurologic development during embryogenesis (Hearing, 1998).

Melanogenesis itself is a complex process. In fact, the number of genes involved in regulating mammalian pigmentation is quite large, and at least 80 genetic loci are known to regulate melanogenesis either directly or indirectly (Hearing, 1999). Mutations of these genes have been shown to be associated with different pigmentary diseases, including various forms of ocular and oculocutneous albinism, piebaldism, Hirschsprung's disease, and Waardenberg's syndrome (Hearing, 1999).

Among those, tyrosinase gene family had been known to play an important role in the regulation of melanogenesis (Pawelek and Chakraborty, 1998). Tyrosinase gene family consists of tyrosinase, TRP-1 and TRP-2. They are glycoproteins embedded in the melanosome membrane that share 70-80% nucleotide sequence homology with 30-40% amino acid identity, and share common functional motifs such as epidermal growth factor receptor and copper binding sites (del Marmol and Beermann, 1996).

GR increased the gene expression and protein contents of tyrosinase. Expression of TRP-2, but not TRP-1, was also increased by GR. Recent works on molecular mechanisms regulating pigmentation suggest that cAMP and protein kinase C (PKC) are the two major intracellular signaling molecules critical for pigmentation (Busca and Ballotti, 2000). Extracellular signals increase cAMP levels, activating protein kinase A (PKA). PKA then presumably alters the transcription rate of key genes including tyrosinase. Inactive PKC may also be activated by extracellular signal. Active PKC then phosphorylates tyrosinase, leading to its activation (Park and Gilchrest, 1999). Recently, there are reports that cGMP signal transduction pathway stimulates melanogenesis (Romero-Graillet *et al*., 1996; Romero-Graillet *et al*., 1997). The signal transduction pathway in GR-mediated stimulation of melanogenesis requires further study.

Stimulatory effect of GR on melanogenesis might be applied to various conditions of hypopigmentation-related disorders as an adjunctive therapy. It would also be possible to produce effective agents available for such purpose by modification of chemical structure of GR.

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