INTRODUCTION

The biosynthesis of melanin pigment, or melanogenesis, is a major defense mechanism against ultraviolet radiation (UVR). Melanin is produced in specialized membrane-bound organelles (melanosomes) in melanocytes of the skin (Riley, 1997), and the melanosomes, which contain the melanin pigments, are then transferred to neighboring keratinocytes through the dendrites. The melanin granules accumulate above the nuclei of the keratinocytes and absorb harmful UVR. Therefore, these compounds protect the skin from UVR-induced DNA damage (Kobayashi et al., 1998).

Melanogenesis is associated with several skin disorders, such as vitiligo. Vitiligo is a common disorder characterized by white spots appearing on the skin of the body, and more than 1% of the general population suffers from this disease. Vitiligo is caused by the loss of functional melanocytes (Kovacs, 1998), and its treatment is often difficult because of the lack of effective methods for restoring the pigmentation (Jeon et al., 2007). Therefore, agents that can induce melanogenesis may play important roles in damaged and pathogenic skin.

Several studies have reported not only hyperpigmentary factors, including stem cell factor (Grabbe et al., 1994) and basic fibroblast growth factor (Halaban et al., 1987), but also melanogenic inducers, including bovine pituitary extract (Wilkins et al., 1985), 12-O-tetradecanoylphorbol-13-acetate (Krasagakis et al., 1993), bee venom (Jeon et al., 2007), and cAMP elevating agents, such as forskolin, 3-isobutyl-1-methylxanthine, α-melanocyte stimulating hormone, and glycyrrhizin (Wong and Pawelek, 1975; Halaban et al., 1984; Hunt et al., 1994; Lee et al., 2005). These factors induce melanin biosynthesis in in vitro system, though they are rarely used clinically.

Melanin biosynthesis in melanocytes is associated with several melanogenic factors, including tyrosinase, tyrosinase-related protein 1 (TRP-1), DOPAchrome tautomerase (DCT), and microphthalmia-associated transcription factor (MITF). Tyrosinase is the key enzyme in pigment synthesis, being responsible for the first two rate-limiting steps of melanogenesis: (1) the hydrolysis from the metabolic precursor of melanin, L-tyrosine, to L-dihydroxyphenylalanine (L-DOPA) and (2) the oxidation from L-DOPA to dopaquinone (del Marmol and Beermann, 1996). TRP-1 and DCT play important roles in modifying...
ing the melanin into different types. They are responsible for the production of brownish 5,6-dihydroxyindole-2-carboxylic acid-rich eumelansins. Moreover, TRP-1 plays important roles in the correct trafficking and stabilization of tyrosinase (Hearing, 2000; Toyofuku et al., 2001), and DCT is known to be involved in tyrosinase detoxification (Jimenez-Cervantes et al., 1994). MITF is a transcriptional regulator that plays a key factor in melanin synthesis by activating the gene expression of tyrosinase, TRP-1, and DCT (Bertolotto et al., 1998a; 1998b).

Recently, antimelanogenic activity of diosgenin in B16 melanoma cells was reported (Lee et al., 2007). After confirming that diosgenin also decreased melanin synthesis in a murine melanocyte melan-a cell line, we screened various steroidal compounds that have structural similarity to diosgenin with melan-a cells. We found that sarsasapogenin (SAR) increased melanin production significantly. SAR is a spirostan-3-ol type of steroidal sapogenin found in the rhizome of Anemarrhena asphodeloides (Liliaceae). It has been used as starting material for the industrial synthesis of steroids. Its pharmacological activities are known as anticancer (Bao et al., 2007), antidepressant (Ren et al., 2007), improvement of learning ability and memory (Hu et al., 2005), and antioxidant (Ma et al., 2001). The effect of SAR on melanogenesis has not been reported in the literature. Therefore, we confirmed the effect of SAR on melanin synthesis in a murine melanocyte melan-a cell line and investigated the precise mechanism of SAR in this study.

MATERIALS AND METHODS

Materials

RPMI1640 media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-streptomycin (PS) was purchased from Invitrogen (Grand Island, NY, USA). All other chemicals, including SAR, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of the highest purity grade. Primary and secondary antibodies were purchased from Santa Cruz Biotech (Delaware Ave, CA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture

Murine melanocyte melan-a cells were kindly supplied from Dr. Byeong Gon Lee at the Skin Research Institute, Amore-Pacific Co. (Yongin, Korea). Melan-a cells were maintained in RPMI1640 supplemented with 10% FBS, 1% PS, and 200 nM 12-O-tetradecanoylphorbol-13-acetate and incubated at 37°C in a humidified incubator with 5% CO2.

Melanin contents and cell viability assay

The melan-a cells were seeded with 1×105 cells/well in a 24-well plate and incubated for 72 h after being treated with SAR and diosgenin. After 72 h, the melanin contents were measured using a modification of the methods reported by Hosoi et al. (1985). In brief, after removing the media, the cells were washed twice with phosphate-buffered saline. Then 1 N sodium hydroxide solution (1 ml) was added to each well to dissolve the melanin contents. The melanin absorbance was measured at 405 nm using a microplate reader.

The percentage of viable cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. After treatment and incubation of the samples for 72 h, as described in the previous paragraph, the medium was removed and 100 μl of 0.1 mg/ml MTT solution was added to each well and incubated at 37°C for 1 h. The MTT solution was then removed and 100 μl dimethyl sulfoxide was added to each well. The formazan formation was measured by absorbance at 570 nm in a microplate reader.

Cell-derived tyrosinase activity assay

Melan-a cells were seeded with approximately 5×105 cells in 100-mm culture dishes. When the cells were confluent, they were detached from the dishes and gathered. The cells pellets were disrupted with tyrosinase buffer (1% Triton-X100 and 100 μl/ml phenylmethylsulfonyl fluoride in 80 mM phosphate buffer) via an ultrasonicator in an ice bath. The cells were centrifuged, and the supernatants were used for the enzyme assay. The cell-derived tyrosinase activity assay required 150 μg of protein (Fuller et al., 2000). The tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA (Takahashi and Parsons, 1990). For the DOPA oxidation assay, 40 μl of each sample and 120 μl L-DOPA (8.3 mM in 80 mM phosphate buffer, pH 6.8) were mixed with proteins and incubated in a dark place for 20 min at 37°C. The tyrosinase activity was calculated at 490 nm with a microplate reader. The tyrosinase specific inhibitor, kojic acid, was used as a positive control (Cabanes et al., 1994).

Cell-derived DCT activity assay

Melan-a cells were seeded with approximately 5×105 cells in 100-mm culture dishes. When the cells were confluent, they were detached from the dishes and gathered. The cells were then disrupted with a hypotonic medium (80 mM phosphate buffer containing 1% Brij 35, pH 6.8) via an ultrasonicator in an ice bath. After 1 h, the cells were centrifuged and the supernatants were used for the enzyme assay. The cell-derived DCT activity assay used 7 mg/ml of protein (Fuller et al., 2000). DOPAchrome was obtained by mixing cold L-DOPA and silver oxide. L-DOPA (0.5 mg) was dissolved in 1 ml 80 mM phosphate buffer (pH 6.8), and 3 mg silver oxide was dissolved in 1 ml L-DOPA solution. After 3 min of mixing, the mixtures were filtered through a 0.22-μm filter. To remove all impurities from the silver, the filtrate was treated with 30% (v/v) Chelex 100 with gentle agitation. For examination of SAR’s inhibitory effect on DCT activity, 20 μl of 1, 5, and 10 μM SAR, 50 μl of 80 mM phosphate buffer (pH 6.8), and 50 μl DOPAchrome substrate were mixed with 25 μl crude DCT. DCT activity was calculated at 340 nm with a microplate reader after 20 min of incubation.

Western blot analysis

To detect the protein levels of tyrosinase, TRP-1, DCT, and MITF, melan-a cells were treated with SAR for 72 h. The cells were then lysed in cold lysis buffer (0.1% sodium dodecyl sulfate, 150 mM sodium chloride, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 100 μg/ml phenylmethane-sulfonylfluoride, 1 μg/ml aprotinin, and phosphatase inhibitor in 50 mM Tris-Cl, pH 8.0). Samples of the resulting protein (40 μg) were separated on 10% acrylamide gels and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% non-fat skim milk in Tris-buffered saline-T and incubated overnight with the primary antibodies at 4°C. The membranes
were then incubated with secondary antibodies for 1 h at room temperature. The membranes were developed using ECL Reagents (Amersham Pharmacia Biotech). Densitometry analysis of the bands was performed with the ImageMaster™ 2D Elite software, version 3.1 (Amersham Pharmacia Biotech).

**Statistical analysis**

Data were expressed as means ± S.D. from at least three independent experiments. A Student’s t-test was used for statistical analyses, and only p-values <0.05 were reported as significant. *p<0.05 and **p<0.001 indicate statistically significant differences from the control group.

**RESULTS**

**Effects of SAR on melanin synthesis and cell viability in melan-a cells**

Recently, a study found out that inhibitory activity of diosgenin on melanogenesis in B16 melanoma cells was reported (Lee et al., 2007). We already confirmed that it had reduced melanin contents in melan-a cells. We also screened the effect of various steroidal compounds including diosgenin on melanogenesis in melan-a cells. On the contrary of diosgenin, we found that SAR (Fig. 1A) increased melanin production significantly compared with diosgenin. Therefore, to compare the effects on melanogenic regulation between the two substances, we tried to perform melanin content and cell viability assays after treatment of SAR and diosgenin in melan-a cells for 72 h. As shown in Fig. 1B, 10 μM of diosgenin decreased melanin content. However, SAR significantly stimulated melanogenesis. SAR increased melanin contents by 128.0 ± 6.1%, 207.2 ± 17.0%, and 224.6 ± 10.0% compared to the control at concentrations of 1, 5, and 10 μM, respectively. Both SAR and diosgenin had no influence on cell viability in the same concentration range in melan-a cells (Fig. 1C). Therefore, SAR strongly increased melanin synthesis without cell toxicity in the tested concentrations.

**Fig. 1.** Effects of SAR on melanin synthesis and cell viability in melan-a cells. (A) The chemical structure of SAR. (B) Melanin contents and (C) cell viability. SAR (1, 5, and 10 μM) and diosgenin (DG, 10 μM) were tested in melan-a cells. Melanin contents and cell viabilities of melan-a cells were tested after 72 h of daily treatment. The results are averages of three independent experiments, and data are expressed as means ± SD. *p-value <0.05 and **p-value <0.001).

**Fig. 2.** Effects of SAR on melan-a-originated tyrosinase and DCT activities. (A) Tyrosinase activity. To determine the effect of SAR on tyrosinase activity, 150 μg of proteins from the melan-a cell extracts were reacted with each sample. Kojic acid (KA, 100 μg/ml) was used as a positive control. (B) DCT activity. Protein from the melan-a cell extracts (7 mg/ml) were treated with SAR and DOPA-chrome substrate for 20 min. The results are averages of three independent experiments, and data are expressed as means ± SD. ***p-value <0.001).
pigmentary agents. Following in vitro screening of various steroid compounds from natural resources in melan-a cells, we confirmed the strong effect of SAR on the stimulation of melanin production. SAR is a steroidal sapogenin that has been used as starting material for the industrial synthesis of various steroids. Further study will be needed to investigate the relationship between steroidal structure and regulation of melanogenesis.

Pigmentation may result from the stimulation of melanocyte proliferation, melanogenesis, and migration, or dendritogenesis (Lan et al., 2005; Scott et al., 2006). Among them, this study focused on the materials that can promote melanogenesis. Tyrosinase is the key enzyme in melanin production, initiating a cascade of reactions that convert the precursor of melanogenic pathway, L-tyrosine, to the melanin polymer (del Marmol and Beermann, 1996). MITF is the key transcriptional regulator in melanogenesis. It activates the gene expression of melanogenic enzymes, and it is a nuclear component of the signal transduction pathway (Bertolotto et al., 1998a). Therefore, the stimulation of tyrosinase and MITF levels induces melanogenesis. In this study, SAR increased the expression of tyrosinase and MITF. This indicates that SAR may stimulate melanin production through the increase of tyrosinase and MITF protein levels. Moreover, as shown in Fig. 1C, SAR treatment has no influence on the percentage of viable cells. Therefore, our reports suggest that the increase in melanin levels by SAR may not result from the stimulation of melanocyte proliferation. In this study, we did not examine the effects of SAR on melanocyte migration and dendritogenesis. Further studies will be needed to investigate not only melanogenesis-related signal transduction, such as mitogen-activated protein kinases activation and cyclic AMP, but also the effects of SAR on melanocyte migration and dendritogenesis.

In this study, SAR significantly increased protein level of tyrosinase (Fig. 3). However, Fig. 2A shows that SAR had no influence on cell-originated tyrosinase activity. The inconsistency between the enzyme activity and protein expression level of tyrosinase might have been caused by tyrosinase enzyme activity assay system. Tyrosinase enzymatic activities can be measured in cell system and/or cell-free system (Kim et al., 2005). To investigate the direct effect of SAR on tyrosinase activity, we performed tyrosinase enzymatic activity assay in cell-free system using melan-a cell lysates in this study. Further study is needed to measure the change of tyrosinase activity in melan-a cells after treatment with SAR.

A previous study reported that diosgenin decreased melanogenesis in B16 melanoma cells (Lee et al., 2007). We also confirmed that diosgenin reduced melanin contents in melan-a cells. Although, the structure of SAR (255-β-spirostan-3β-ol) is similar to diosgenin (25R-spirostan-5-β-ol), SAR increased melanin production in melan-a cells. The difference of effects seems to be specific to a cis-linkage between rings A and B of the steroid nucleus due to a 5β-H configuration and a (S)-configuration at C-25. We suggest that the A/B-cis linkage via a 5β-H configuration may be more important than the (S)-configuration at C-25 for various pharmacological activities. Previous studies reported that SAR had anti-diabetic effects, such as reducing blood glucose and reversing weight gain in diabetic animal models; however, diosgenin, with a Δ3 double bond, had a much lower anti-diabetic activity than did SAR.

**DISCUSSION**

As the most external portion of the body, the skin often suffers from various environmental stresses, such as UVR. Melanogenesis is considered a protective mechanism against UVR-induced DNA damage (Kobayashi et al., 1998). Therefore, melanin production in the skin is very important to human health. Moreover, depigmenting diseases, such as vitiligo, can lead to depression and similar mood disorders in patients who are stigmatized for their hypopigmentary condition. Recently, we focused on determining the compound that induces melanin production in melanocyte cells. Many previous studies have reported the role of steroids in stimulating melanogenesis. Cholesterol (Schallreuter et al., 2009), vitamin D analogs (Birlea et al., 2009), ovarian hormones, including estradiol and estriol (Maeda et al., 1996), and glucocorticoids, such as dexamethasone (Ito et al., 1991), have been known as hyperpigmentary agents. Following in vitro screening of various steroid compounds from natural resources in melan-a cells, we confirmed the strong effect of SAR on the stimulation of melanin production. SAR is a steroidal sapogenin that has been used as starting material for the industrial synthesis of various steroids. Further study will be needed to investigate the relationship between steroidal structure and regulation of melanogenesis.

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**Fig. 3.** Effects of SAR on expression of melanogenic proteins in melan-a cells. To determine the protein levels of tyrosinase, TRP-1, DCT, and MITF, melan-a cells were treated with 1, 5, and 10 μM of SAR for 72 h. Protein cell lysates (40 μg) were used for Western blot analysis. Equal protein loadings were confirmed using anti-tubulin antibody. Densitometry analysis of the bands was performed as described in the methods section. The results are averages of three independent experiments, and data are expressed as means.
(Appelzweig, 1987). Moreover, SAR had the ability to stop the decline in muscarinic acetylcholine receptors (mACHRs) in animal models of Alzheimer’s disease (Hu et al., 2005). However, tigogenin, with a 5α-configuration, showed no effect on mACHRs. Therefore, A/B-cis linkage and 5β-H configuration in spirostan-type steroids may also be related to melanogenic regulation. In further studies, we will investigate the influence of various spirostan-type steroids with 5α- or 5β-configuration on melanogenesis.

In this study, SAR significantly induced melanin synthesis in murine melanocytes melan-a cells. Although SAR did not influence melanogenic enzyme activity, the protein expressions of tyrosinase and MITF were strongly stimulated by treatment with SAR. These results suggest that SAR treatment may induce hyperpigmentation through the increase of tyrosinase and MITF protein expression in melan-a cells. Therefore, this study supports the hypothesis that SAR from the rhizomes of A. asphodeloides may be used for the treatment of hypopigmentation-related diseases, such as vitiligo. We are currently working on indicating the molecular mechanisms of SAR for the induction of hyperpigmentation in melanocytes from different origins.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2010-0025362).

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