Integrative Biosciences 10: 233-236, 2006

Integrative **Biosciences**

http://www.ibiosci.or.kr

Inhibitory Effects of Aqueous Extracts from *Nardostachys chinensis* on α-Melanocyte Stimulating Hormone-induced Melanogenesis in B16F10 Cells

Soo Jin Lee, Yung Hyun Choi[1] and Byung Tae Choi*

Departments of Anatomy and [1]Biochemistry, College of Oriental Medicine, Dongeui University, Busan 614-052, Korea

Abstract: For the purpose of the development of skin-whitening or therapeutic agents against hyperpigmentation, aqueous extract from *Nardostachys chinensis* (AENC) was evaluated for melanogenesis inhibitory activity in B16F10 melanoma cell. The treatment with AENC at the 0.2, 0.5 and 1.0 mg/ml level significantly inhibits the biosynthesis of melanin compared with untreated control. The tyrosinase activity also significantly decreased in AENC-treated cells at the 0.2 and 0.5 mg/ml level and inhibitory effects were more efficient than commercial arbutin at 0.1 mg/ml. The Western analyses confirmed the significantly decreased expression of tyrosinase and tyrosinase-related protein-2 by AENC treatment. These results indicate that AENC may contribute to the inhibition of melanin biosynthesis through regulating the expression as well as activity of tyrosinase and AENC may be useful as a new candidate in the design of new skin-whitening or therapeutic agents.

Keywords: *Nardostachys chinensis*, melanogenesis, tyrosinase, tyrosinase-related protein

Melanin is the major pigment for skin color and various dermatologic disorders arise from the accumulation of excessive levels of epidermal pigmentation (Wang et al., 2006). Tyrosinase inhibitors are important in skin-whitening because they catalyze melanin biosynthesis (Kim et al., 2005). Therefore, tyrosinase inhibitors such as arbutin have become increasingly significant in medical and cosmetic products and serve as a new paradigm in cosmetic or dermatologic agent research (Briganti et al., 2003; Roh et al., 2004).

A number of tyrosinase inhibitors are employed from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, because many inhibitors suffer from limitations such as safety concerns and low activity (Roh et al., 2004). The natural extracts have been employed as new agents for functional cosmetics to develop safe and effective skin-whitening (Aburjai and Natsheh, 2003; Wang et al., 2006). Recently, the use of traditional herbal medicines is increasing as a potential new agent or drug.

The *Nardostachys chinensis* has long been employed as a sedative and analgesic in Oriental Medicine. Several compound extracts which usually impact on the central nervous system have been isolated from *N. chinensis* including nardosinone, guaiane- and aristolane-type sesquiterpenoids (Tanitsu et al., 2002; Liu et al., 2005). However, the *N. chinensis* extract has been regarded as an effective constituent for skin care in Korea. In the present study, we investigated the effects of the aqueous extract from *N. chinensis* (AENC) on melanin synthesis and tyrosinase inhibition at the cellular level using B16 melanoma cells.

MATERIALS AND METHODS

Materials

Synthetic melanin, L-DOPA and alpha melanocyte stimulating hormone (α-MSH) were purchased from Sigma (St. Louis, MO). Antibody recognizing tyrosinase (H-109, sc-15341), tyrosinase related protein (TRP)-1 (G-17, sc-10443) and TRP-2 (G-15, sc-10452), were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Extraction

N. chinensis was purchased from a local herbal store, Kwang Myung Dang (Busan, Korea) and confirmed and authenticated by Professor Y. T. Lee, College of Oriental Medicine, Dongeui University. 100 g of *N. chinensis* was

*To whom correspondence should be addressed.
Tel: +82-51-850-8653; Fax: +82-51-853-4036
E-mail: choibt@deu.ac.kr

extracted at 90°C in deionized water 2 L for 2 hours. After water extraction, we collected the liquid layer separately from the deposits that were centrifuged at 3,000 rpm for 30 minutes. The supernatant was filtered with 0.45 μg pore sized filter paper and lyophilization. A voucher specimen (yield 1.3 g, number WC05-12) has been deposited at the College of Oriental Medicine, Dongeui University.

Cell culture

The B16F10 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Difco) and contained 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in 5% CO_2.

Measurement of melanin contents

The B16F10 cells were grown in 60 mm dishes and treated with the test extract for 5 days. The cells were washed with PBS and cenrifuged at 5,000 rpm for 1 min. Later the centrifuged cells were discarded supernatant, 1 N NaOH containing the 1% DMSO was added and incubated at 80°C for 1 hour. Intercellular melanin contents were measured for absorption at 475 nm. The absorbance values were compared to the standard curve obtained from synthetic melanin. Extracellular melanin contents were examined for absorption at 404 nm using the supernatant from the cultured media. The values were compared to absorption values of the control.

Tyrosinase activity

Tyrosinase activity was measured according to the modified method of Martinez-Esparza et al. (1998). Briefly, the B16F10 cells were grown in 100 mm dishes and treated with the test extract for 48 hours. Cells were washed with PBS, lysis buffer (1% Triton X-100, 0.1 M sodium phosphate, pH 7.0, 0.1 mM PMSF, 1 mM NaF) was added and lysed at 4°C for 30 minutes. Lysates were clarified by centrifugation. 50 μg of each protein extract was added to a 0.1 M sodium phosphate buffer (pH 7.0) and 0.05% L-DOPA and incubated at 37°C for 50 minutes. Enzymetic activity was then estimated by measuring at 405 nm.

Western blot analysis

The cells were harvested, washed, lysed and centrifuged at 14,000 rpm for 25 minutes. Protein concentration was quantified using the Bio Rad protein assay (BioTad Lab., Hercules, CA), following the manufacturer's method. An equal amount of protein was subjected to electrophoresis on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with the washed individual first antibody, incubated with a secondary antibody and then developed by the enhanced chemiluminescence according to the recommended procedure (Pierce Biotechnology, Inc.).

Flow cytometric analysis

The cells were removed from the dishes by trypsinization and washed with PBS. The pellets were centrifuged at a low speed, resuspended in a citrate buffer, and treated with RNase A. Nuclei were stained with a propidium iodide (PI, Sigma) solution. Afterwards, flow cytometric analyses were performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Data analysis

Data were expressed as mean ± SEM. Calculations of means, standard errors and Student's *t*-test were made using SigmaPlot. $p < 0.05$ was considered statically significant.

RESULTS

Inhibitory effect on melanogenesis

To determine the effects of AENC on cytotoxicity of B16F10 cells, we employed an MTT assay before further in vitro testing for melanogenesis. The AENC at 0.2, 0.5 and 1.0 mg/ml level did not show significant effects on cell toxicity compared with controls, thus we treated B16F10 cells with AENC at these doses. The melanin biosynthesis was strongly inhibited by AENC treatment compared with the untreated control. As shown in Fig. 1, the treatment with AENC showed a 28.9%, 76.2% and 93.1% inhibition of melanin synthesis at the 0.2, 0.5 and 1.0 mg/ml level respectively. AENC treatment also induced an increase of extracellular melanin content in a dose-dependent manner (Fig. 2).

Inhibitory effect on tyrosinase activity

The inhibitory effect of AENC on tyrosinase activity is shown

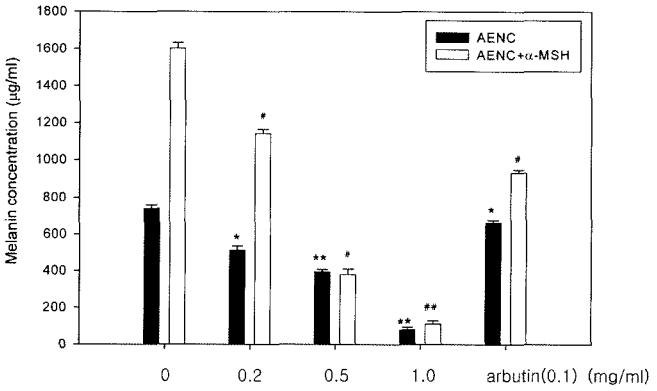

Fig. 1. Effects of AENC on melanin contents in α-MSH-treated B16F10 cells. Cells were seeded at 1×10^5 cells per dish. After 24 hours, cells were treated with several concentrations of AENC in presence or absence of 200 nM α-MSH, and cultured for 5 days. Then melanin contents were measured as Materials and Methods. Data was means ± SEM of three experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with the AENC absence group; $^{\#}p < 0.01$ and $^{\#\#}p < 0.005$ as compared with the α-MSH-treated group.

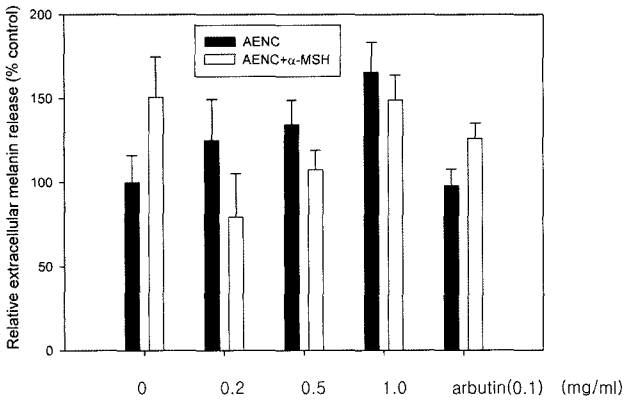

Fig. 2. Effects of AENC on extracellular melanin accumulation in α-MSH-treated B16F10 cells. Cells were seeded at 1×10^5 cells per dish. After 24 hours, cells were treated with several concentrations of AENC in the presence or absence of 200 nM α-MSH, and cultured for 5 days. Then extracellular melanin contents were measured as Materials and Methods. Data was means ± SEM of three experiments performed in duplicate.

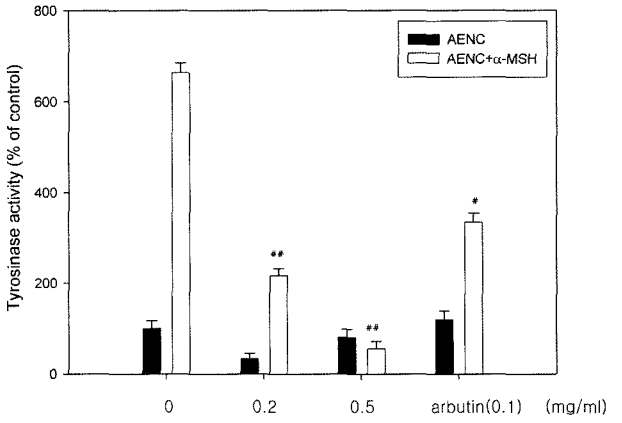

Fig. 3. Effects of AENC on tyrosinase activity in α-MSH-treated B16F10 cells. Cells were seeded at 1×10^5 cells per dish. After 24 hours, cells were treated with 0.2 and 0.5 mg/ml of AENC in the presence or absence of 200 nM α-MSH, and cultured for 3 days. Data are means ± SEM of three experiments performed in duplicate. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with the α-MSH-treated group.

in Fig. 3. AENC treatment at 0.2 and 0.5 mg/ml level markedly suppressed tyrosinase activity compared with the untreated control. The treatment with AENC showed a 67.3% and 91.8% inhibition of tyrosinase activity at the 0.2 and 0.5 mg/ml level, respectively. The treatment of AENC exhibited a greater inhibitory effect on tyrosinase activity than that of arbutin which showed a 49.8% inhibition.

Western blot analysis for tyrosinase, TRP-1 and TRP-2

To provide more direct evidence that AENC played a role in the regulation of melanogenesis through melanogenesis-related protein expressions, Western bolt analysis was performed. Western blot analysis showed that the treatment of AENC significantly decreased in tyrosinase and TRP-2

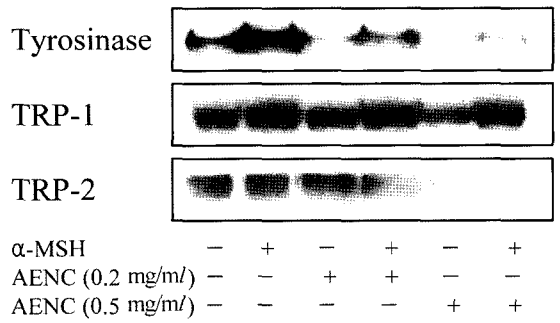

Fig. 4. Effects of AENC on the protein levels of tyrosinase, TRP-1, and TRP-2 in α-MSH-treated B16F10 cells. Cells were seeded at 1×10^5 cells per dish. After 24 hours, cells were treated with 0.2 and 0.5 mg/ml of AENC in the presence or absence of 200 nM α-MSH, and cultured for 5 days.

Table 1. Effects of AENC on the cell cycle distribution of B16F10 melanoma cells

Group	% of cell		
	G1	S	G2/M
Control	70.72	6.85	7.53
α-MSH	58.01	2.74	6.46
AENC (0.2 mg/ml)	72.24	9.10	6.61
AENC (0.2 mg/ml) + α-MSH	79.26	7.20	7.78
AENC (0.5 mg/ml)	53.20	10.27	12.07
AENC (0.5 mg/ml) + α-MSH	64.99	10.49	15.06

The data are the mean of two different experiments and shows representative examples for duplicate tests.

expression compared with untreated control. But TRP-1 expression was slightly decreased by AENC treatment (Fig. 4).

The effect on the cell cycle

To elucidate an alteration on the cell growth by AENC treatment, cell cycle analysis was employed using flow cytometry. The treatment of AENC at 0.5 mg/ml slightly decreased the population at the G1 phase in the cell cycle, but a low-dose of AENC caused non-specific cell death in B16F10 cells without any changes in the cell cycle.

DISCUSSION

Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis and protects the skin damage from UV or other sources. The inhibitory compounds for melanin biosynthesis are useful in cosmetics as skin-whitening agents and as a remedy for disturbances in pigmentation. The melanogenesis is closely related to hyperpigmentation and can be inhibited by an inhibition of tyrosinase (Nerya et al., 2003). The tyrosinase inhibitors have become increasingly important in medical and cosmetic products in relation to skin care (Wang et al., 2006).

Unfortunately, many inhibitors of tyrosinase suffer from limitation such as low activity and high cytotoxicity (Briganti et al., 2003; Kim et al., 2005). For example, hydroquinone was one of the most potent whitening agents first discovered, but since its introduction some toxic and mutagenic effects have been recognized. Safety is a primary consideration for tyrosinase inhibitors, especially for those used in cosmetic and therapeutic products. The ideal skin-whitening agent should inhibit the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and be non-mutagenic (Curto et al, 1999).

The isolation and the structural elucidation of the active constituents from natural products will provide useful leads in the development of skin-whitening agents. Many tyrosinase inhibitors such as proanthocyanidin, arbutin and ellagic acid have been isolated from plants and used in cosmetics for topical application (Yoshimura et al., 2005). The aqueous extract from Korean medicine displays skin-whitening efficacy. Although nardosinone isolated from *N. chinensis* has been used as an enhancer of nerve growth factor (Liu et al., 2005), *N. chinensis* has been employed in skin care in Oriental Medicine.

In the present study, B16F10 cells were exposed for 48 hours to various concentrations of AENC to determine the effects on cell proliferation. There were no significant AENC effects at 0.2, 0.5 and 1.0 mg/ml level on cell growth compared with controls. Then we examined the effects of AENC at these levels on the melanin biosynthesis. When B16F10 cells were treated with AENC, strongly decreasing melanin biosynthesis was observed. The inhibition rate of AENC at 0.5 and 1.0 mg/ml level was especially higher than that of the arbutin.

These results showed that AENC may have significant whitening effects and potential for practical application as whitening agents. To provide more direct evidence that AENC plays a role in the regulation of melanogenesis, we tested the effects on tyrosinase activity. Both 0.2 and 0.5 mg/ml levels of AENC showed significant inhibition of tyrosianse activity, higher than that of the arbutin.

Tyrosinase plays a key role in melanin biosynthesis, but two additional melanosomal enzymes including TRP-1 and TRP-2 are also involved in the eumelanogenesis pathways that produce brown and black pigments. Most whitening agents act specifically to reduce the function of tyrosinase, TRP-1 and TRP-2 (Briganti et al., 2003). Western blot analysis showed that treatment of AENC at 0.2 and 0.5 mg/ml level significantly decreased tyrosianse and TRP-2 expression in B16F10 cells.

It is also important to mention that AENC does not exhibit cytotoxicity against normal or cell lines including tumor cells. Thus we examined the cell cycle pattern to determine an alteration of cell cycle progression by AENC treatment. When we quantified the extent with cell cycle by measuring the fraction of nuclei, arrest of each cycle was almost negligible at both 0.2 and 0.5 mg/ml level. These imply that treatment of AENC with low-doses brought out similar cell cycle patterns as in untreated cells.

These results demonstrate that AENC has alleviating effect in melanin biosynthesis through inhibiting tyrosnianse activity and regulating tyrosinase and TRP-2 protein expression at a cellular level. Consequently, *N. chinensis* can be used as a new candidate in the design of skin-whitening or new therapeutic agents for skin hyperpigmenation.

ACKNOWLEDGMENT

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (106072-3).

REFERENCES

Aburjai T and Natsheh FM (2003) Plants used in cosmetics. *Phytother Res* 17: 987-1000.

Briganti S, Camera E, and Picardo M (2003) Chemical and instrumental approaches to treat hyperpigmentation. *Pigment Cell Res* 16: 101-110.

Curto EV, Kwong C, Hermersdorfer H, Glatt H, Santis C, Virador V, Hearing VJ Jr, and Dooley TP (1999) Inhibitors of mammalian melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. *Biochem Pharmacol* 57: 663-672.

Kim HJ, Seo SH, Lee BG, and Lee YS (2005) Identification of tyrosinase inhibitors from *Glycyrrhiza uralensis*. *Planta Med* 71: 785-787.

Liu JH, Yin F and Zheng XX (2005) *Nardostachys chinensis* glycoside induces characteristics of neuronal differentiation in rat pheochromocytoma PC12 cells. *Biol Pharmacol Bull* 28: 768-771.

Martinez-Esparza M, Jimenez-Cervantes C, Solano F, Lozano JA, and Garcia-Borron JC (1998) Mechanisms of melanogenesis inhibition by tumor necrosis factor-alpha in B16/F10 mouse melanoma cells. *Eur J Biochem* 255: 139-146.

Nerya O, Vaya J, Musa R, Izrael S, Ben-Arie R, and Tamir S (2003) Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. *J Agric Food Chem* 51: 1201-1207.

Roh JS, Han JY, Kim JH, and Hwang JK (2004) Inhibitory effects of active compounds isolated from safflower (*Carthamus tinctorius* L.) seeds for melanogenesis. *Biol Pharmacol Bull* 27: 1976-1978.

Tanitsu MA, Takaya Y, Akasaka M, Niwa M, and Oshima Y (2002) Guaiane- and aristolane-type sesquiterpenoids of *Nardostachys chinensis* roots. *Phytochemistry* 59: 845-849.

Wang KH, Lin RD, Hsu FL, Huang YH, Chang HC, Huang CY, and Lee MH (2006) Cosmetic applications of selected traditional Chinese herbal medicines. *J Ethnopharmacol* 106: 353-359.

Yoshimura M, Watanabe Y, Kasai K, Yamakoshi J, and Koga T (2005) Inhibitory effect of an ellagic acid-rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation. *Biosci Biotechnol Biochem* 69: 2368-2373.

[Received September 25, 2006; Accepted November 14, 2006]