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Article

Effect of Hydroxycinnamic Acid Derivatives from Corn Bran on Melanogenic Protein Expression

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A recent study has demonstrated that polyamine conjugates from corn (*Zea may* L.) bran and related hydroxycinnamic acids have antioxidant and antimelanogenic activities. To verify the ability of hydroxycinnamic acid derivatives (HCAD) from corn bran to reduce skin pigmentation as cosmetic active ingredients, we assessed the effect on melanogenic proteins expression in murine B16 melanoma cells and on skin pigmentation levels in clinical trials. We investigated the effect of HCAD on melanogenic protein expression using Western blot analysis. Furthermore, we assessed the effects of a cream containing 0.1% by weight corn bran extract (CBE) with HCAD such as *N*,*N*'-dicoumaroylputrescine, *N*-*p*-coumaroyl-*N*'-feruloylputrescine and *N*,*N*'-diferuloylputrescine in clinical trials. HCAD reduced the forskolin-induced increase in melanin content of cultured melanoma cells. To elucidate the effect of HCAD on melanogenesis, we performed a Western blot analysis of melanogenic proteins, such as tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2 and microphthalmia-associated transcription factor (MITF). HCAD reduced levels of tyrosinase and MITF, however, it did not change TRP-1 and TRP-2 levels. The 0.1% CBE cream significantly reduced skin pigmentation with no irritation. The present study indicates that HCAD have good potential to be useful antimelanogenic agents.

Key words: antimelanogenic agent, corn (*Zea may* L.), hydroxycinnamic acid derivatives, microphthalmia-associated transcription factor, polyamine conjugates, tyrosinase, tyrosinase-related protein

Skin pigmentation results from melanin synthesis by melanocytes, which is induced by exposure to UV radiation. In mammals, melanin synthesis is stimulated by a large number of effectors, including UV-B radiation [Friedmann and Gilchrest, 1987] and cAMP-elevating agents (forskolin, 3-isobutyl-1-methylxanthine, α-melanocyte stimulating hormone (α-MSH), glycyrrhizin) [Wong and Pawelek, 1975; Halaban *et al*., 1984; Hunt *et al*., 1994; Lee *et al*., 2005].

The first step in melanin synthesis is hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (DOPA), followed by the oxidation of DOPA to DOPA quinine [Jimenez-Cervantes *et al*., 1993]. These two reactions are catalyzed by tyrosinase enzymatic activity and constitute major regulatory points common to melanogenic pathways. The conjugation of DOPA quinine with cysteine or glutathione yields 5-S-cysteinyl DOPA and glutathionyl DOPA, which are progressively transformed into the reddish-yellow pheomelanins. The spontaneous oxidation of DOPA quinine in the absence of thiol compounds yields DOPA chrome, which is used for the synthesis of the brownish-black eumelanins. Melanin-containing melanosomes are then transferred to keratinocytes through the dendritic tips of melanocytes, resulting in the even distribution of melanins throughout the epidermis.

Naturally occurring hydroxycinnamic acid derivatives, such as *p*-coumaric acid (CA), ferulic acid (FA), and caffeic acid, are known to have a variety of biological activities, including anticancer, antiinflammation [Zhang and Ji, 1992], antihepatoxicity [Perez-Alvarez *et al*., 2001], antibacterial [Ramos-Nino *et al*., 1996], antimutation [Yamada and Tomita, 1996], and antioxidation [Natella *et al*., 1999]. In particular, phenolic acid amides (polyamine conjugates) containing a parent moiety of CA and FA, including *N*,*N*'-dicoumaroylputrescine (DCP), *N*-*p*-coumaroyl-*N*'-feruloylputrescine (CFP) and *N*,*N*'-diferuloylputrescine (DFP) have been found to possess strong antioxidant activities as radical scavengers against reactive oxygen species and to inhibit tyrosinase activity

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[Iwai *et al*., 2004]. Previous studies have reported that hydroxycinnamic acid derivatives (HCAD) from corn bran, possess antimelanogenic activity in B16 melanoma cells [Choi *et al*., 2007; Kim *et al*., 2009]. However, the other effects of these compounds, such as their effects on tyrosinase, TRP-1, TRP-2 and MITF are not known. In the present study, we investigated the effects of HCAD on the levels of melanogenic related proteins, as well as skin pigment levels *in vivo*.

Materials and Methods

Materials and chemicals. Corn bran was purchased from Goksan, Korea. The Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Lab. (USA). CA, FA and forskolin were obtained from Sigma Chemical Co.. The goat anti-tyrosinase, mouse anti-microphthalmia-associated transcription factor (MITF), goat anti-TRP-1, rabbit anti-tyrosinase-related protein (TRP)-2, goat anti-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA). All other reagents used for this study were analytical or HPLC grade.

Preparation of corn bran extract (CBE) and HCAD. Corn bran was extracted with 80% aqueous ethanol by ultrasonicatioin. The extract was filtered and evaporated and subsequently, defatted by solubilizing in *n*-hexane and partitioning with dichloromethane. The dichloromethane fraction was evaporated to obtain the final CBE. As stated in a previous study, CBE included several HCAD, CA, FA, DCP, CFP and DFP as predominant constituents accounting for 1.81±0.13%, 1.56±0.05%, 1.32±0.17%, 5.84±0.06% and 25.11±0.81%, respectively [Kim *et al*., 2009]. HCAD, CFP and DFP were isolated and purified from the CBE by a combination of solvent fractionation and chromatography using silica gel, ODS-A and Sephadex LH-20 column chromatography.

Cell culture. B16F1 murine melanoma cells were obtained from the American Type Culture Collection (USA) and cultured in DMEM supplemented with 10% FBS in an humidified atmosphere containing 5% CO_2 in air at 37°C.

Measurement of cellular melanin contents. B16 melanoma cells, pretreated with the indicated concentrations of HCAD for 1 h, were treated for 48 h with forskolin (10 μM). After treatment, the melanin content was measured using a slight modification of a previously reported method [Oka *et al*., 1996]. Cells were detached by incubation in trypsin/EDTA. After precipitation, the color of the cell pellets was evaluated visually, and pellets were solubilized in 1 N NaOH containing 10% dimethyl sulfoxide (DMSO). Spectrophotometric analysis of melanin content was performed by quantifying absorbance at 405 nm. Three independent experiments were performed in triplicate.

Western blotting. B16 melanoma cells, pretreated with the indicated concentrations of HCAD for 1 h, were treated for 48 h with forskolin (10 μM). B16 melanoma cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% skim milk in Tris buffered saline containing 0.05% Tween 20. The blots were incubated with antibodies specific for tyrosinase (1:200 dilution), MITF (1:200), TRP-1 (1:200), TRP-2 (1:200), or actin (1:500), followed by horseradish peroxidase-conjugated secondary antibodies. Bound anti-bodies were detected using an enhanced chemilumine-scence kit (Amersham, Little Chalfont, UK) following the manufacturer's instructions. Actin was used as the loading control.

Clinical assessment. Female volunteers (N=21), between 18-60 years of age with normal healthy skin, were recruited for the study. Using UV irradiation, 2 tanned areas were made on the back. In a double-blind fashion, each tanned area was treated with (test) or without (control) 0.1% CBE cream twice a day, for 8 weeks. In order to evaluate the changes in skin pigmentation, a Mexameter MX 18 (Courage and Khazaka, Koln, Germany) and Spectrophotometer CM-2500d (Konica Minolta, Osaka, Japan) were used. The so-called individual typology angle (ITA°) [Piérard, 1998] was derived using the following equation: ITA°=[Arc Tangent {(L*-50)/ b*}] $180\pi^{-1}$. The values of ITA represent the skin typology. This evaluation was performed at Dermapro Co. Ltd. (Seoul, Korea).

Statistical analysis. Correlations among the results were analyzed using the statistical software SPSS (SPSS Science, Chicago, IL). Statistical significance was defined as $p< 0.05$.

Results

The effect of HCAD on melanin production. To assess the effect of HCAD on melanogenesis, B16 cells were treated with HCAD prior to treatment with forskolin. The melanin content of B16 melanoma cells that had not been treated with stimulants such as forskolin was very low. Treatment with individual HCAD inhibited the effects of forskolin, DFP (57% reduction in pigment) and CFP (48% reduction in pigment) at 50 μg/mL exhibited considerable inhibitory activity, but CA (25% reduction in pigment) at the same concentration was less

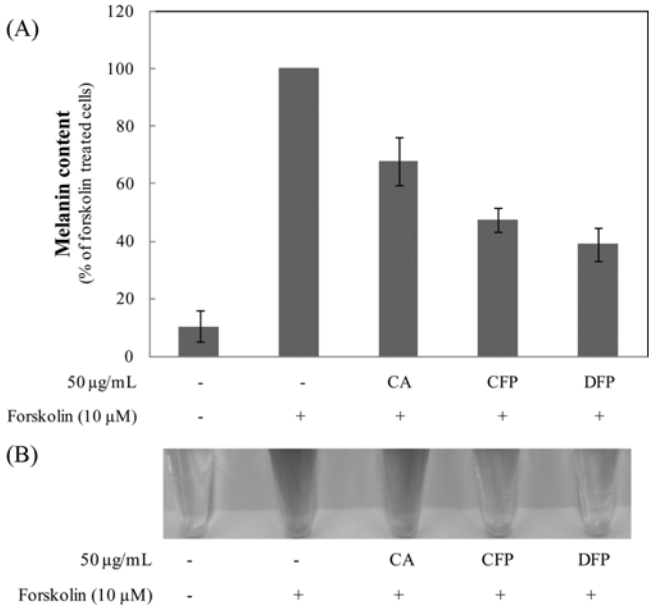

Fig. 1. Effect of HCAD on melanin production in B16 melanoma cells. (A), melanin content; (B), photograph of B16 melanoma cell suspensions.

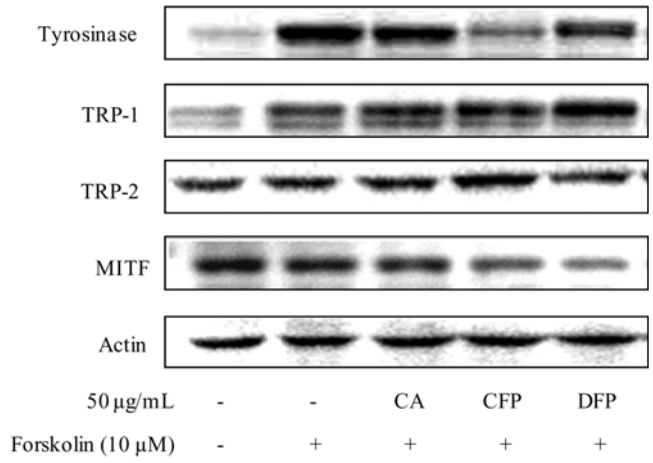

Fig. 2. Effect of HCAD on the expression of melanogenic proteins in B16 melanoma cells.

effective than the polyamine conjugates (Fig. 1A). These results confirmed the antimelanogenic activity of the HCAD [Choi *et al*., 2007]. Moreover, the effects were clearly visible in the color of the suspensions of cells treated with HCAD, which was lighter than the forskolin treated cells (Fig. 1B).

Western blotting of B16 melanoma proteins treated with HCAD. To investigate whether the inhibitory activity of HCAD is related to the melanogenesis pathways involving the expressions of tyrosinase, TRPs and MITF, B16 melanoma cells were treated with HCAD before forskolin stimulation. Cell lysates were subjected to SDS-PAGE and Western blot analysis (Fig. 2). When cells were stimulated by forskolin alone, a significant

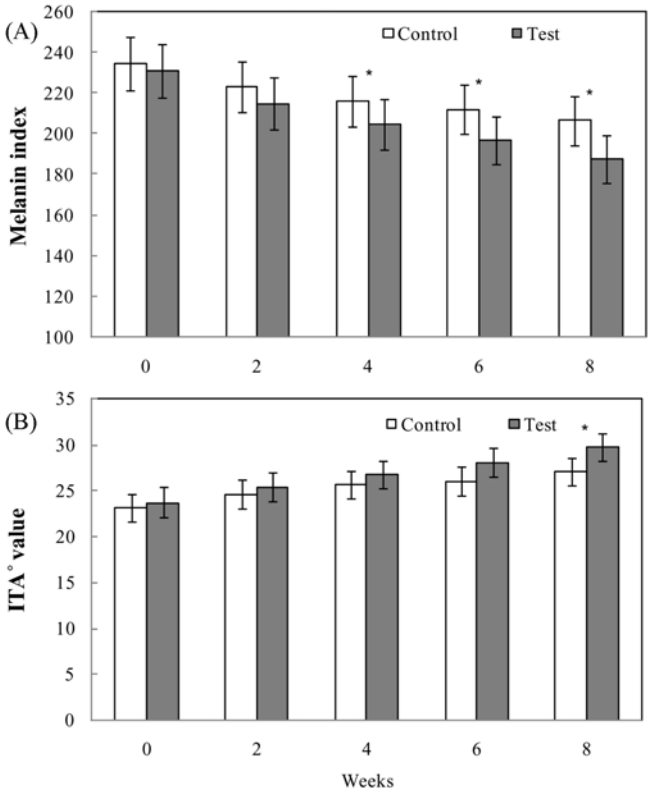

Fig. 3. Changes in skin pigmentation after application of cream with (test) or without (control) 0.1% CBE. (A), Mexameter MX 18; (B), Spectrophotometer CM-2500d.

increase in tyrosinase levels were detected, and TRP-1 levels also increased. Compared to treatment with forskolin alone, CFP and DFP treatment inhibited forskolin-stimulated tyrosinase and MITF expression in B16 melanoma cells. The inhibition of tyrosinase expression in CFP-treated cells is due to a decrease in MITF expression. Although DFP significantly reduced the protein level of MITF, there was little decrease in that of tyrosinase. TRP-1 and TRP-2 levels showed no significant changes in the HCAD -treated B16 melanoma cells.

Efficacy of cream containing CBE. To assess the efficacy of a skin cream containing the CBE, an 8 weeks double-blind control-test study was performed with 21 female volunteers, with the cream being applied twice a day. A melanin index generated using the Mexameter MX 18 indicated the melanin content (Fig. 3A). The application of cream containing CBE resulted in a significant reduction in melanin content compared to controls. After 8 weeks, the reduction in the melanin index in the test group was significant: 19%. The 4-week and 6-week results also showed significant reductions in the test group: 11% and 15%, respectively. In the control group the reductions were 8% (4 weeks), 10% (6 weeks) and 12% (8 weeks).

After treatment, colorimetric changes were recorded with a Spectrophotometer CM-2500d. After 8 weeks, the test group had significantly increased ITA values, compared with the control group (Fig. 3B).

Discussion

In the present study, we have demonstrated the antimelanogenic mechanism of HCAD from corn bran in B16 murine melanoma cells. To stimulate melanogenesis, we used the adenylate cyclase activator forskolin.

Tyrosinase, TRP-1, TRP-2 and MITF are well characterized proteins involved in melanin synthesis. To investigate the effects of HCAD on melanogenic proteins level, we performed a Western blotting analysis to examine the amounts of tyrosinase, TRP-1, TRP-2 and MITF. B16 melanoma cells that had been stimulated by forskolin had higher levels of tyrosinase and TRP-1 than unstimulated cells. HCAD reduced levels of tyrosinase and MITF, but had no influence on TRP-1 and TRP-2 levels. Forskolin induces tyrosinase gene expression through the activation of MITF gene expression. In particular, the inhibition of tyrosinase expression in CFP-treated cells is due to a decrease in MITF expression. Although DFP significantly reduced the levels of MITF, there was a little decrease in tyrosinase levels. Increased levels of cAMP activate protein kinase A (PKA), which subsequently phosphorylates the cAMP response binding protein (CREB). CREB binds the cAMP response element (CRE) motif of the MITF promoter and activates MITF gene transcription [Steingrimsson *et al*., 2004]. The inhibition of the extracellular signal-regulated kinase (ERK) signaling has been reported to induce hyperpigmentation by increasing tyrosinase activity, suggesting that the activation of ERK signaling would downregulates melanogenesis by inhibiting tyrosinase activity [Lee *et al*., 2007]. Further studies are necessary to clarify the mechanism of cAMP-mediated and ERK signaling pathway-mediated suppression of MITF and its downstream signal pathway, including expression of tyrosinase and TRPs.

We have demonstrated that HCAD inhibited the effects of forskolin in B16 melanoma cells. To investigate the possibility of using these compounds to reduce skin pigmentation, the cream containing CBE was applied to volunteers. The levels of skin pigmentation of the test and control groups were measured at two-week intervals using the Mexameter and Spectrophotometer, melanin index and ITA, respectively. The test group had significantly less pigmentation than the control group.

In conclusion, these studies demonstrate that HCAD from corn bran may be useful inhibitors of melanogenesis and that corn bran may be an effective component in cosmetics designed to lighten skin.

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