Inhibitory Effects of Cinnamic Acid on Melanin Biosynthesis in Skin

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Cinnamic acid is a wildly distributed phenylpropanoid component naturally occurring in plants, and is mainly found in Cinnamomum cassia Blume and Panax ginseng. Cinnamic acid was recently reported to exert a tyrosinase inhibitory effect. However, research on melanocytes and animal bodies was not reported until now. In this study, we examined the effects of cinnamic acid on melanin biosynthesis within the melanocytes and brown guinea pigs. Melan-a cells were used to examine the effects of cinnamic acid in the melanocytes. Treatment with 100 ppm of cinnamic acid resulted in a significant reduction of melanin production in the melan-a cells at 29.0%. This compound also exhibited a potent inhibitory effect on tyrosinase activity and reduced tyrosinase expression in the melan-a cells. Moreover, cinnamic acid exhibited depigmenting activity on the UV-B-induced hyperpigmentation of brown guinea pig skin. Our results suggest that cinnamic acid might act as a skin whitening agent via inhibition of tyrosinase activity and expression within melanocytes.

Key words cinnamic acid; melanin; depigmenting agent

Melanin is essential for skin protection against UV-light, but the over-production of melanin causes serious skin disorders such as freckles, discoloration, and pigmented age spots. Melanogenesis is carried out in the melanocytes, which are mainly located in the basal layer of the epidermis. The degree of melanization is a major factor for skin color in animal bodies. Melanin biosynthesis within the melanocytes is controlled by several enzymes. In the melanin biosynthesis pathway, tyrosinase catalyzes the first rate-determining step, and is considered to be the key enzyme in melanin production. Therefore, searching for potent tyrosinase inhibitors is valuable in the development of skin whitening agents.

Cinnamic acid is one of the major components of Cinnamomum cassia Blume. This compound is reported to have various biological activities such as anti-oxidant, anti-inflammatory, and anti-cancer properties. Moreover, Lee et al. recently reported the inhibitory effect of cinnamic acid on tyrosinase activity. However, its effects on melanocytes and animal skin were not yet reported.

This study examined the depigmenting ability of cinnamic acid using melan-a cells and brown guinea pigs; and its effects on the expression of melanin biosynthesis-related enzymes were examined. Furthermore, cinnamic acid's UV-protecting effect and cell extracted-tyrosinase inhibitory effect were investigated.

MATERIALS AND METHODS

Materials Mushroom tyrosinase, L-dopa, kojic acid, and cinnamic acid were purchased from Sigma-Aldrich Co. (U.S.A). FBS, RPMI, and PS were purchased from Gibco BRL (U.S.A).

Tyrosinase Extraction The melan-a cells were disrupted by resuspension in a tyrosinase buffer (80 mM PO4 buffer + 1% Triton-X 100 + 100 μg/ml of PMSF), which was followed by sonication in an ice bath. After centrifugation at 12500 rpm for 15 min, the supernatant was used for the enzyme assay; 150 μg of the proteins were required for each reaction.

Measuring Tyrosinase Activity Tyrosinase activity was measured by its dopa oxidase activity, using a slightly modified version of the method reported by Shono and Toda. Each concentration (1 mM, 500 μM, 100 μM, 10 μM) of the test substance was dissolved in MeOH. Next, 120 μl of L-dopa (5 mM, dissolved in a 67 mM phosphate buffer, pH 6.8), and 40 μl of either the same buffer or the test sample, were added to a 96-well microplate; then 40 μl of tyrosinase was added. The amount of dopachrome in the reaction mixture was measured after incubation at 37 °C for 30 min. Based on the optical density at 490 nm (OD 490), the inhibitory activity of the sample was expressed as the concentration that inhibited 50% of the enzyme activity (IC50). Kojic acid was used as the reference.

Cell Line and Culture Procedures The melan-a cells were cultured in RPMI 1640 medium under 10% FBS and 200 nm phobol 12-myristate 13-acetate (TPA) conditions. To a 100μl tissue culture dish, 10 ml of medium was added and then seeded with 5×105 cells. If the cells were confluent after 3 to 4 d at 37 °C and 5% CO2, they were seeded with 105 cells/well in a 24-well plate, and then incubated for 24 h. Each well was changed with 990 μl of medium everyday, as well as treated with 10ul of sample [solvent (v/v): propylene glycol/ EtOH/H2O=5/3/2] for 3 d; then the plate was incubated for 1 d.

Cell Viability The percentage of viable cells was determined by staining the cell population with crystal violet. After media removal from each well, the wells were washed with PBS. Then 200 μl of crystal violet (0.1% CV, 10% EtOH, and the remaining volume as PBS) was added. The plate was incubated at room temperature for 5 min and washed with water two times. After adding 1 ml of EtOH, it was shaken at room temperature for 10 min. The UV absorption was measured at 590 nm.

Fig. 1. Chemical Structure of Cinnamic Acid

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Determining Melanin Content in the Melan-a Cells
After media removal from each well, the plate was washed with PBS, followed by the addition of 1 ml of 1 N NaOH to dissolve the melanin. The UV absorption was measured at 400 nm. Phenylthiourea (PTU) was used as a positive control.

Western Immunoblotting Analysis
The melan-a cells were harvested and extracted in a triple-detergent lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml of PMSF, and 1 μg/ml of aprotinin]. The protein content was measured using a protein assay kit (Bio-rad, Hercules, CA, U.S.A.). Next, 50 μg of the protein was separated on 8% SDS-polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Animals
SLC brown guinea pigs weighing 450 g were purchased from Shizuoka Co. (Japan), and housed individually in a temperature and moisture controlled animal room with a 12 h light/dark cycle.

UVB-Induced Hyperpigmentation in Brown Guinea Pigs
The UVB-induced hyperpigmentation was performed on the backs of brownish guinea pigs, using methods slightly modified from those reported by Ando et al. and Imokawa et al. The guinea pigs were anesthetized with pentobarbital (30 mg/kg), and separate areas (1 cm diametrical circles) on the back of each animal were exposed to UV-B radiation (Waldmann UV 800, Herbert Waldmann GmbH, Philips TL/12 lamp emitting 280—305 nm). The total UV-B dose was 500 mJ/cm² per exposure. Groups of 4 animals were used in the experiments. The animals were exposed to the UV-B radiation once a week for 8 weeks from the day after birth. After six weeks we found that the degree of pigmentation was assessed by the L-value, measured using a chromameter (CR-300, Minolta, Japan).

Table 1. Tyrosinase Inhibitory Effects of Cinnamic Acid

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (μM)</th>
<th>Inhibition (%)</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kojic acid</td>
<td>10</td>
<td>6.1±2.9</td>
<td>544.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>33.3±3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>77.6±3.9</td>
<td></td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>10</td>
<td>0.3±3.8</td>
<td>693.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>13.1±2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>70.6±3.1</td>
<td></td>
</tr>
</tbody>
</table>

The tyrosinase was extracted from melan-a cells. Kojic acid was used as a positive control.

Table 2. Effects of Kojic Acid and Cinnamic Acid on Melan-a Cell Growth and Melanin Production

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (μM)</th>
<th>Cell viability (%)</th>
<th>Melanin production (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kojic acid</td>
<td>5</td>
<td>96.6±4.8</td>
<td>97.1±2.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>98.6±2.9</td>
<td>91.0±5.0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>84.8±3.7</td>
<td>81.9±5.2</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>5</td>
<td>101.9±3.4</td>
<td>98.1±2.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>101.0±4.5</td>
<td>84.3±3.3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>91.2±2.7</td>
<td>71.0±4.3</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. of three experiments.

Inhibitory Effects against Melan-a Cell Tyrosinase Activity
Tyrosinase is believed to be a key enzyme in the melanin biosynthesis pathway. This enzyme catalyzes the oxidation of dopa to dopachrome. In this study, we investigated the inhibitory effects of cinnamic acid on tyrosinase within melan-a cells. This tyrosinase was extracted from the melan-a cells using a tyrosinase extraction buffer with sonication. Based on the results, cinnamic acid showed 13.1% and 70.6% tyrosinase inhibitory activity at 100 and 1000 μM, respectively (Table 1). Although the inhibitory effect was lower than that of kojic acid at the same concentration, the cinnamic acid exhibited inhibition in a dose dependent manner.

Effects on Melanin Production and Cell Viability in Melan-a Cells
Here, melan-a cells were used to measure the inhibitory effects of cinnamic acid on melanin production within the melanocytes. The melan-a cells were derived from the normal epidermal melanoblasts of embryos from inbred C57BL mice. The treatment results for cinnamic acid are presented in Table 2. After 3 d, cinnamic acid (500 μM) reduced 29% of the melanin content without cell toxicity. However, the inhibitory effect of kojic acid was much lower than that of cinnamic acid at the same concentration.

Intracellular Analysis of Tyrosianase and Dopachrome Tautomerase Levels
Tyrosinase and dopachrome tautomerase are important melanin generation-related proteins. To investigate the regulation of protein expression in melan-a cells following cinnamic acid treatment, Western immunoblotting analysis was performed. After treatment at concentrations of 5 μM, 50 μM, and 500 μM of cinnamic acid to the melan-a cells for 3 d, the proteins in the melan-a cells were extracted for quantitative analysis. The results show that cinnamic acid significantly reduced the intracellular tyrosinase level in a dose dependent manner. However, a change in the level of intracellular dopachrome tautomerase was not observed at any of the cinnamic acid concentrations (Fig. 2).

Depigmenting Effects on the Hyperpigmentation of Brown Guinea Pig Skin
The hyperpigmentation of brown guinea pig skin was induced by UV-B radiation to investigate the depigmenting effects of cinnamic acid. Vehicle and cinnamic acid were applied to the hyper-pigmented skin areas once a day. After six weeks we found that the degree of pigmentation was reduced by treatments of 0.5% and 1% cinnamic acid as compared to the vehicle; the ΔL-values were 0.59 and 1.73 respectively. In addition, visible edema and flare were not observed at any of the cinnamic acid treated sites of the dorsal skin during the experiment (Fig. 3).
According to our results, cinnamic acid exhibited a potent inhibitory effect on melan-a cells. Therefore, cinnamic acid acts as a depigmenting agent via regulation of the starting step in the melanin biosynthesis pathway. Moreover, this compound exhibited de-pigmenting effects on the UV-B-induced hyperpigmentation of brown guinea pig skin. The skin of the animals returned to its original color after treatment with cinnamic acid, without visible edema or flare. Overall, these results suggest that cinnamic acid may be useful as a de-pigmenting agent for skin. Further studies toward identify effect on alpha-melanocyte stimulating hormone and microphthalmia transcription factor (Mitf) expression to explore the depigmenting pathway.

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