Combination of Amino Acids Reduces Pigmentation in B16F0 Melanoma Cells

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Amino acids, the building blocks of proteins, play significant roles in numerous physiological events in mammals. As the effects of amino acids on melanogenesis have yet to be demonstrated, the present study was conducted to identify whether amino acids, in particular alanine, glycine, isoleucine and leucine, influence melanogenesis in B16F0 melanoma cells. Glycine and L-isoleucine, but not D-isoleucine, reduced melanogenesis in a concentration-dependent manner without any morphological changes in B16F0 melanoma cells. L-Alanine and L-leucine, but not D-alanine and D-leucine, also reduced melanogenesis without any morphological changes in B16F0 melanoma cells. However these amino acids did not show a concentration-dependency. Combination of L-alanine and the other amino acids, particularly 4 amino acids combination, had an additive effect on the inhibition of melanogenesis compared with single treatment of L-alanine. None of the amino acids affected the activity of tyrosinase, a key enzyme in melanogenesis. These results suggest that L-alanine, glycine, L-isoleucine and L-leucine, but not the D-form amino acids, have a hypopigmenting effect in B16F0 melanoma cells, and that these effects are not due to the inhibition of tyrosinase activity. Combination of these 4 amino acids had the additive effect on hypopigmentation that was as similar as that of kojic acid.

Key words amino acid; melanogenesis; B16F0 melanoma cell; tyrosinase

Pigmentation due to synthesis and dispersion of melanin protects the skin from harmful effects of sunlight, but unwanted hyperpigmentation can also produce a significant psychologic stress. Therefore, many trials to induce hypopigmentation using melanin synthesis inhibitors, chemical peels and lasers have been conducted. Melanin is synthesized in the melanosomes of melanoma cells, which produce melanin by a process that involves the transformation of tyrosine into 3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosinase and the subsequent transformation of L-DOPA into melanin. Tyrosinase is the key enzyme in the pathway of melanogenesis, and plays a regulatory role in the production of melanin. Accordingly, the regulation of tyrosinase may not only control melanin production but also provide a strategy for hypopigmentation. A number of compounds have been screened for their effectiveness in reducing melanogenesis, such as hydroquinone and kojic acid, which have been reported as tyrosinase inhibitors. However, these agents have various side effects, such as the adverse cutaneous toxicity of hydroquinone and tumor-promoting effect of kojic acid. Thus, there has been increasing impetus to find alternative hypopigmenting agents.

Amino acids are important as they participate in multiple biochemical processes in mammals. In human, alanine and glycine are not considered essential amino acids, since they can be synthesized de novo from a non-amino acid source of nitrogen (e.g., ammonium ions) and an appropriate carbon source. On the other hand, branched-chain amino acids, such as isoleucine and leucine, are considered essential amino acids in mammals, and supplemental dietary values of these amino acids have been established from nitrogen balance studies, and are the basis of the current FAO/WHO/UNU 1985 recommendation for amino acid intake. It has been reported that dietary intake of these amino acids may have beneficial effects, since L-alanine improves the survival rate of rats in a model of acute liver failure induced by administration of d-galactosamine, glycine prevents increases in hepatocyte replication caused by potent peroxisome proliferators and tumor promoter, and isoleucine and leucine are utilized in various metabolic situations ranging from fatigue to liver cirrhosis and encephalopathy. However, no experimental data is available on the effects of amino acids on melanogenesis. Thus, we examined whether amino acids, in particular alanine, glycine, isoleucine and leucine, influence melanogenesis in B16F0 melanoma cells.

MATERIALS AND METHODS

Materials B16F0 melanoma cells (CRL-6322) were obtained from ATCC (Manassas, VA, U.S.A.). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM) and other supplements were purchased from SIGMA (St. Louis, MO, U.S.A.). L-Alanine, D-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, D-isoleucine, L-leucine, D-leucine, D-l-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine were obtained from Wako (Tokyo, Japan). Kojic acid was obtained from ALPS Pharmaceutical Ind. Co., Ltd (Gifu, Japan). Melanin, L-DOPA and mushroom tyrosinase were obtained from SIGMA (St. Louis, MO, U.S.A.). BCA™ Protein Assay Kit was purchased from PIERCE (Rockford, IL, U.S.A.).

Cell Culture B16F0 melanoma cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37℃ under 5% CO₂ atmosphere. The cells were detached from culture bottles using 0.025% trypsin and 0.5 mM ethylenediaminetetra acetic acid in phosphate-buffered saline (PBS). B16F0 melanoma cells were seeded into 6-well plates at a density of 1×10⁵ cells per well and allowed to attach overnight. Each

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dose of sample reagent was added to a well and incubated for another 72 h. After each incubation, cells were washed with cold PBS. The cells were then dissolved in 400 μl of 1 N NaOH at 80°C for 1 h and their melanin content measured.

**Melanin measurement** Melanin content was measured as described previously with slight modifications. The absorbance of each lysate of B16F0 melanoma cells was measured at 405 nm and compared with a standard curve of known concentration of synthetic melanin. The amounts of protein in the above cell lysates were determined using BCA™ Protein Assay Kit. Each value was calculated as the melanin content per mg protein (μg/mg protein) to compensate for differences between samples.

**Mushroom Tyrosinase Assay** Mushroom tyrosinase activity was determined as described previously with slight modifications. A 105 μl aliquot of 100 mM phosphate buffer (pH 6.8), 10 μl of various concentrations of samples and 20 μl of 200 U/ml mushroom tyrosinase were mixed and incubated at room temperature. After a 10-minute incubation, 65 μl of 2.5 mM L-DOPA was added and the mixture incubated for another 10 min at room temperature. Absorbance was then measured at 490 nm to determine the production of dopachrome.

**Statistical Analysis** Data are expressed as mean±S.E.M. Significant differences between treatment groups were determined by the F-test, followed by Student's (equal variances) or Aspin–Welch (unequal variances) t-test (comparison between two groups), or the Bartlett test, followed by parametric (equal variances) or nonparametric (unequal variances) Dunnett’s test (comparison between multiple groups).

**RESULTS**

**Effects of 20 α-Amino Acids on Melanogenesis in B16F0 Melanoma Cells** To screen the effects of 20 α-amino acids on melanogenesis in B16F0 melanoma cells, B16F0 melanoma cells were treated with various concentrations of α-amino acids for 72 h. Table 1 showed a result of primary screening on melanogenesis in B16F0 melanoma cells. Among the 20 α-amino acids, L-alanine, glycine, L-isoleucine and L-leucine showed an inhibitory effect, but the other α-amino acids did not inhibit melanogenesis in B16F0 melanoma cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mM)</th>
<th>Inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>2</td>
<td>Yes</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>8</td>
<td>No</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>Glycine</td>
<td>4</td>
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</tr>
<tr>
<td>L-Histidine</td>
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<tr>
<td>L-Isoleucine</td>
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</tr>
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<td>L-Leucine</td>
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<td>Yes</td>
</tr>
<tr>
<td>L-Valine</td>
<td>16</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> Growth inhibition of cells. N.T.: not tested.

**Combination of L-Alanine and the Other Amino Acids Showed Further Inhibition of Melanogenesis in B16F0 Melanoma Cells** To determine the effects of combination of L-alanine and the other amino acids, glycine, L-isoleucine and L-leucine, on melanogenesis in B16F0 melanoma cells, B16F0 melanoma cells were treated with a combination of the 2 to 4 amino acids at 4 mM each for 72 h. Although the treatment of L-alanine at 4 mM showed an inhibition of melanogenesis, combination of the 2 to 4 amino acids showed further decrement of melanogenesis in B16F0 melanoma cells (Fig. 2). In particular, combination of the 4 amino acids showed the strongest decrement of melanogenesis to a similar as kojic acid (Fig. 2). None of combination treatment of amino acids had any significant changes in morphology of B16F0 melanoma cells (data not shown). Kojic acid (2.5 mM) reduced melanin production to 32.5±1.2 μg/mg protein without any morphological changes in B16F0 melanoma cells (Figs. 1A—D).

**Combination of L-Alanine and the Other Amino Acids Did Not Inhibit the Tyrosinase Activity of Mushroom Origin** To determine the inhibitory effects of L-alanine, glycine, L-isoleucine and L-leucine on tyrosinase activity, mushroom tyrosinase was incubated with L-DOPA as the substrate and the formation of dopachrome was measured. None of the amino acids (1—16 mM each) had any significant effect on mushroom tyrosinase activity (Fig. 3). However, kojic acid (0.016—1 mM) inhibited mushroom tyrosinase activity in a concentration-dependent manner (Fig. 3).
DISCUSSION

Melanogenesis plays an important role in protecting skin from sun-related injury, however sometimes it can produce undesirable problems, such as lentigo, seborrheic keratosis and melasma. Tyrosinase plays a critical regulatory role in melanin biosynthesis and it has been suggested that tyrosinase activity is pivotal. Thus, many studies have focused on the regulation of tyrosinase activity in melanoma cells. First, we examined the primary effect of 20 α-amino acids on melanogenesis in B16F0 melanoma cells. We did not examine the effect of L-tyrosine, because of the poor solubility. In the primary screening, although L-alanine, glycine, L-isoleucine and L-leucine showed an inhibitory effect on melanogenesis in B16F0 melanoma cells, the other α-amino acids did not. Therefore, next we examined the effect of D and L-alanine, glycine, D and L-isoleucine and D and L-leucine on melanogenesis in B16F0 melanoma cells, using various concentrations. These L-form amino acids and glycine, but not the D-forms, produced a hypopigmenting effect without any changes in morphology of B16F0 melanoma cells. Therefore, our findings suggest that L-alanine, glycine, L-isoleucine and L-leucine are unique among the amino acids as potential melanogenesis inhibitor.

The contents of amino acids within a skin, in particular in the stratum corneum, plays an important role in regulating the hydration state of the skin surface. Some amino acids are popular ingredients in cosmetics. However, the hypopigmenting effects of amino acids have not been studied. Interestingly, L-alanine, glycine, L-isoleucine and L-
leucine, but not the d-forms, inhibited melanogenesis in B16F0 melanoma cells. Moreover, combination of the 4 amino acids had an additive inhibitory effect on melanogenesis in B16F0 melanoma cells. In addition, combination of the 4 amino acids, which induced hypopigmentation by themselves, had the additive effect on hypopigmentation that was as similar as that of kojic acid. Thus, L-alanine, glycine, L-isoleucine and L-leucine are good candidates for safe effective melanogenesis inhibitors.

In summary, we found that L-alanine, glycine, L-isoleucine and L-leucine have an inhibitory effect on melanogenesis without influencing cell morphology of B16F0 melanoma cells. Therefore, it is likely that the inhibition of tyrosine transport could also be involved in the hypopigmenting effect of amino acids. Although further examination is required to clarify the mechanism in detail, we can at least exclude the direct inhibition of tyrosinase activity by amino acids.

the supplementation of tyrosine to melanosomes by some mechanism, such as transmembrane transport, is known to be a critical element limiting melanin synthesis. Therefore, it is likely that the inhibition of tyrosine transport could also be involved in the hypopigmenting effect of amino acids. Although further examination is required to clarify the mechanism in detail, we can at least exclude the direct inhibition of tyrosinase activity by amino acids.

REFERENCES