Sulforaphane Inhibited Melanin Synthesis by Regulating Tyrosinase Gene Expression in B16 Mouse Melanoma Cells

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Sulforaphane is a compound widely present in consumed vegetables, particularly broccoli. Previous studies have demonstrated that sulforaphane has many physiological effects including anti-cancer, anti-oxidation, and detoxification. In this study, we found that sulforaphane inhibited melanogenesis and tyrosinase expression. The inhibitory effect of 5 μM sulforaphane on melanogenesis was determined to be equivalent to that of 100 μM arbutin. In addition, sulforaphane induced phosphorylated extracellular signal-regulated kinase (ERK) and inhibited phosphorylated p38. It has been reported that the phosphorylated mitogen-activated protein (MAP) kinase family (ERK and p38) controls tyrosinase expression. Our data indicate that sulforaphane inhibited melanogenesis and tyrosinase expression by affecting the phosphorylated MAP kinase family. These findings indicate that sulforaphane might be an effective skin-whitening agent.

Key words: sulforaphane; B16 melanoma cell; melanin; tyrosinase; mitogen-activated protein (MAP) kinase family

Melanin is the biological pigment that forms the skin color and protects skin cells from UV irradiation.1) Tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) are key enzymes involved in melanogenesis.2,3) It has been reported recently that the MAP kinase family controls melanogenesis.4–6) Phosphorylated ERK inhibits the activation of microphthalmia-associated transcription factor (MITF), a melanocyte specific transcription factor.5) Phosphorylated p38 can activate MITF, promoting melanin synthesis.4,5)

When skin is exposed to UV, α-melanocyte stimulating hormone (α-MSH) is secreted from keratinocytes. α-MSH then binds to the receptor, MC1R, and induces the expression of tyrosinase by activating MITF.7) The synthesis of melanin begins with the conversion of tyrosine to dopaquinone by tyrosinase. TRP-1 and TRP-2 are involved in the downstream reactions of the biosynthetic pathway.

Overproduction and accumulation of melanin might occur in melasma, freckles, and other forms of melanin hyperpigmentation.8,9) To remedy these problems, melanogenesis inhibitors have been developed as agents for use in medications, and cosmetics. For example, tyrosinase inhibitors such as arbutin, kojic acid, and ellagic acid have been included in some cosmetic products.10–12)

In this study, we examined the inhibitory effect of sulforaphane on tyrosinase. Sulforaphane, a compound found in broccoli and other vegetables, belongs to the isothiocyanate group. It is derived from the hydrolysis of sulforaphane glucosinolate by myrosinase, which is activated in masticated broccoli.13) It has been reported that sulforaphane can help remove ROS by inducing the expression of detoxification enzymes.14) ROS contributes to melanin synthesis and DNA damage. Thus, inhibitors of tyrosinase or scavengers of ROS as antioxidants might suppress melanogenesis in the epidermal layer of the skin.15) We report here the effects of sulforaphane on melanin synthesis and on tyrosinase activity in B16 melanoma cells.

Materials and Methods

Materials. Sulforaphane was purchased from Funakoshi (Tokyo). Dulbecco’s modified Eagle’s medium (DMEM) was from Nissui Pharmaceutical (Tokyo). Fetal bovine serum (FBS) was from Nichirei Biosciences (Tokyo). Trypsin-EDTA, penicillin-streptomycin, trypsin inhibitor, melanin, arbutin, kojic acid, mouse anti-β-actin antibody (A-5411), and rabbit anti-goat antibody conjugated with horseradish peroxidase were from Santa Cruz Biotechnol-

3.4-Dihydroxyphenylalanine (l-DOPA) was from Kanto Chemical (Tokyo). Rabbit anti-phosphorylated p38 (Thr180/Tyr182) rabbit anti-phosphorylated p38 (9212), rabbit anti-phosphorylated ERK (Thr202/Tyr204) (9101), rabbit anti-phosphorylated ERK (9102) antibodies, goat anti-rabbit antibody conjugated with horseradish peroxidase, and horse anti-mouse antibody conjugated with horseradish peroxidase were from Cell Signaling Technology.
Cell culture. B16 murine melanoma cells (JCRB0202) were purchased from the JCRB Cell Bank (Osaka). B16 cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin (100 IU/50 µg/ml) in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were seeded into 60-mm tissue culture dishes, and sample treatment began 24 and 72 h after seeding.

Measurement of melanin contents. Melanin contents were measured based on a previously established procedure, with slight modifications. Briefly, cells were incubated at a density of 2.5 x 10⁵ cells in a 60-mm dish. Sulforaphane or arbutin treatment began 24 and 72 h after seeding. After 96 h of treatment, the cells were washed with phosphate-buffered saline (PBS) and detached by trypsin-EDTA treatment. The cell suspension was centrifuged at 1,000 x g for 5 min. The cell pellets were resuspended in 1 ml of PBS, and 0.9 ml of cell suspension was again centrifuged at 1,000 x g for 5 min. The cell pellets were dissolved in 1 ml of 1 M NaOH. The absorbance at 475 nm was determined and compared with a standard curve prepared using synthetic melanin.

Cell viability assay. A cell viability assay was performed by the trypan blue staining method. After incubation of cells with samples for 4 d, the culture medium was removed and the cells were detached with trypsin-EDTA from the dishes. One hundred µl of cell suspension was added 100% of 0.4% trypan blue in a 96-well plate. We counted viable cells with an electron microscope.

Tyrosinase activity assay. Tyrosinase activity in the B16 melanoma cells was determined by measuring the rate of oxidation of L-DOPA by a method described previously. The cells were cultured in individual wells of a 24-well plate at a density of 1 x 10⁵ cells/ml. On the fifth day, the cells were washed and lysed in 100 µl of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and protease inhibitors (Complete™ protease inhibitors mixture), frozen at -80°C for 30 min, and then thawed on ice. The lysates were centrifuged at 10,000 x g for 30 min at 4°C. The protein concentration of the supernatant was measured by the Bradford assay method. Eighty µl of each lysate protein preparation (8 µg of protein) and 20 µl of 2 mM L-DOPA were added to individual wells of a 96-well plate. The absorbance was measured for 5 h at 490 nm using a microplate reader at 37°C.

Western blotting analysis. The cells were lysed in 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and protease inhibitors. The proteins in cell lysate were separated by 7.5% SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. The blotted membrane was treated with 5% non-fat milk in PBS containing 0.05% Tween 20 for 1 h. For analysis of tyrosinase, the membrane was incubated for 1 h with anti-tyrosinase antibody (1:1,000), and further incubated with anti-rabbit antibody conjugated with horseradish peroxidase (1:1,000). For analysis of phospho-tyrosinase, the membrane was incubated for 1 h with anti-phospho-tyrosinase antibody (1:1,000) and further incubated for 1 h with anti-rabbit antibody conjugated with horseradish peroxidase (1:1,000).

Statistical analysis. Data were expressed as mean ± SD, and were analyzed by Dunnett’s test to evaluate significant differences of the means as compared with control.

Results

Effects of sulforaphane on melanogenesis using B16 melanoma cells
To investigate the effects of sulforaphane on melanogenesis, the melanin contents of the B16 cells without and with treatment with sulforaphane were measured. Arbutin was used as a positive control. At doses of 0.1, 0.5, 1, 2.5, and 5 µM sulforaphane, melanin production were compared with the untreated control (Fig. 1). Sulforaphane decreased melanin production 89.8 ± 3.2%, 82.4 ± 5.8%, 70.6 ± 11.8%, 48.5 ± 5.8%, and 35.9 ± 4.4% in a dose-dependent manner without noticeable cytotoxicity up to a concentration of 5 µM (Fig. 2). However, there was a significant cytoxic effect at 10 µM (data not shown). Nevertheless, at 0.5 µM, sulforaphane significantly decreased melanin production as compared with the untreated control.

The inhibitory action of 5 µM sulforaphane on melanogenesis was at a level equivalent to that of 100 µM arbutin.

Effects of sulforaphane on tyrosinase expression in B16 melanoma cells
Since tyrosinase is a major regulator in melanin synthesis, we were interested in examining the direct inhibitory effect of sulforaphane on tyrosinase expression. At 0.1, 0.5, 1, 2.5, and 5 µM of sulforaphane, the tyrosinase activities in the B16 melanoma cells were

Fig. 1. Effects of Sulforaphane on Melanin Production in B16 Melanoma Cells.
Cells were treated with various concentrations (0.1–5 µM) of sulforaphane. The data shown represent the mean ± SD derived from three determinations. Significant difference versus control; * p < 0.05 (Dunnett’s test).
activity directly, kojic acid, at 100 µM, sulforaphane showed no inhibitory effect on tyrosinase and sulforaphane on tyrosinase activity (Fig. 3A). Figure 3B shows the effects of kojic acid and sulforaphane for the durations indicated (0–720 min). After 24 h of serum starvation, the cells were treated with 5 µM sulforaphane for the durations indicated (0–720 min).

Effects of sulforaphane on MAP kinase activation in B16 melanoma cells

It is known that phosphorylated p38 activated MITF induces melanin synthesis in B16 melanoma cells.5,6 In contrast, activation of the ERK pathway suppresses melanin synthesis.6,7 Our results indicate that phosphorylation of p38 decreased from 360 until 720 min after treatment with 5 µM sulforaphane (Fig. 5). At the same time, ERK was activated by phosphorylation from 60 min to 720 min following treatment with sulforaphane. Inhibition of p38 phosphorylation and induction of ERK phosphorylation both resulted, in a reduction in melanin synthesis. These results suggest that sulforaphane might modulate MAP kinase pathways, thereby exerting its control on tyrosinase expression.

Discussion

Sulforaphane, a member of the isothiocyanate family, is present abundantly in broccoli and broccoli sprouts. The concentration of sulforaphane in broccoli sprouts (1,153 mg/100 g, dry weight) is about 10 times that of mature broccoli (44–171 mg/100 g, dry weight).19) Isothiocyanates are sulfur-containing compounds including allyl-, benzyl-, phenylethyl-, isopropyl-, and methyl-isothiocyanate.20) They are widely distributed among cruciferous vegetables such as broccoli, cauliflower, watercress, and cabbage.21) With regard to their biological functions, it has been reported that phenylethyl isothiocyanate has cancer chemopreventive activity.22) Sulforaphane is a compound of the methyl isothiocyanate group. It has been reported that it might activate phase-II detoxification enzymes such as aldo-keto reductase 1C1 (AKR1C1).23) Additionally, it has been shown to induce apoptosis of cancer cells.24) In this study, we discovered that sulforaphane is capable of reducing melanogenesis and tyrosinase activity in a dose-dependent manner. Moreover, we found that the inhibitory action of 5 µM sulforaphane on tyrosinase expression level of tyrosinase in B16 melanoma cells. Western blot analysis was performed to determine the expression level of tyrosinase in B16 melanoma cells. The results indicated that the level of tyrosinase protein was decreased strongly by treatment with 2.5 and 5 µM sulforaphane (Fig. 4). Based on the band intensity, the level of tyrosinase expression in the B16 cells treated with 2.5 and 5 µM sulforaphane was approximately 80% of that expressed in the untreated control cells.

### Western Blot Analysis

Tyrosinase activity (% of control) Sulforaphane (µM) Activity of Tyrosinase in B16 Melanoma Cells

<table>
<thead>
<tr>
<th>Sulforaphane (µM)</th>
<th>Tyrosinase Activity (% of Control)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
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<tr>
<td>0.1</td>
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<td>0.5</td>
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<td>1</td>
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<td>2.5</td>
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<td>5</td>
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<td>Arbutin (100 µM)</td>
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* *p < 0.05 (Dunnett’s test).*

### Effects of Sulforaphane on Tyrosinase Activity in B16 Melanoma Cells

Fig. 4. Effects of Sulforaphane on Tyrosinase Expression in B16 Melanoma Cells.

Expression levels of tyrosinase were analyzed by Western blot. β-Actin levels were served as control.

### Fig. 5. Effects of Sulforaphane on MAP Kinase Pathways in B16 Melanoma Cells.

After 24 h of serum starvation, the cells were treated with 5 µM sulforaphane for the durations indicated (0–720 min).

**Discussion**

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melanogenesis was equivalent to that of 100 μM arbutin. In an enzyme inhibition experiment, sulforaphane failed to inhibit tyrosinase activity directly. Subsequent Western blot analysis indicated that it suppressed melanogenesis, probably by modulating tyrosinase gene expression.

It was reported recently that the molecular mechanism of melanogenesis is regulated by MAP kinase pathways. The phosphorylated p38 MAP kinase cascade can activate MITF, which in turn upregulates the expression of tyrosinase, TRP-1, and TRP-2 inducing melanin production.4–6) Hence, melanin production can be inhibited by p38 MAP kinase inhibitors such as SB203580,4) whereas a 90-kDa ribosomal S6 kinase inhibited by p38 MAP kinase inhibitors such as (RSK-1) and ERK cascade negatively regulate MITF expression.6) In general, the ERK cascade plays a main role in cell growth control, but in B16 melanoma cells, ERK and RSK-1 induce MITF phosphorylation and lead to its ubiquitination. As a result, ubiquitinated MITF is downregulated by proteasome-mediated degradation.6)

In our study, sulforaphane treatment inhibited p38 phosphorylation in B16 cells. Sulforaphane might act as an inhibitor for MITF activation via inhibition of p38 phosphorylation. Because of this, melanin production in B16 melanoma cells treated with sulforaphane decreased as compared with the untreated control. Moreover, phosphorylated ERK was induced by sulforaphane treatment for as long as 60 min. Phosphorylated ERK persisted until 720 min after sulforaphane treatment. Based on this finding, it is thought that sulforaphane might depress MITF expression by activating the ERK cascade. Thus, it appears that sulforaphane might inhibit melanogenesis through inhibition of p38 phosphorylation and the induction of ERK phosphorylation.

However, at earlier stages following sulforaphane treatment, p38 phosphorylation was induced and ERK phosphorylation was inhibited. It has been reported that isothiocyanate-related antioxidants such as sulforaphane modulate MAP kinase pathways and induce Nrf2 accumulation in the nucleus. Nrf2 induces expression of antioxidation enzymes and reduces oxidative stress.25) It has also been reported that sulforaphane can elevate the expression levels of the phase-II detoxification enzymes that prevent intracellular accumulation of reactive oxygen species (ROS) after UV exposure.14)

In summary, sulforaphane appeared to play an interesting role inhibiting melanin production in human skin cells by downregulating tyrosinase gene expression. The anti-melanogenic mechanism, which appears to involve suppression of tyrosinase expression by modulating MAP kinase pathways, was partially elucidated.

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References