Inhibitory Effect of Quercetin Isolated from Rose Hip (Rosa canina L.) against Melanogenesis by Mouse Melanoma Cells

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We investigated the effects of compounds isolated from a methanolic extract of rose hips on melanin biosynthesis in B16 mouse melanoma cells and the possible mechanisms responsible for the inhibition of melanin biosynthesis. We found that, among the isolated compounds, quercetin was a particularly potent melanogenesis inhibitor. To reveal the mechanism for this inhibition, the effects on tyrosinase of B16 mouse melanoma were measured. Quercetin decreased the intracellular tyrosinase activity as well as the tyrosinase activity in a cell culture-free system. We also examined the cellular level of tyrosinase protein and found that quercetin dose-dependently inhibited tyrosinase protein expression. We consider from these results that the inhibition of melanogenesis by quercetin was due to the inhibition of both tyrosinase activity and of the protein expression.

Key words: rose hip; quercetin; melanin; tyrosinase; melanoma cell

Melanin is a pigment with photoprotective properties that gives skin and hair its characteristic color. It is synthesized in melanosomes which are special organelles in specialized cells known as melanocytes. Melanin biosynthesis starts with a two-step conversion catalyzed by tyrosinase: hydroxylation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA), and subsequent oxidation of L-DOPA to dopaquinone.2) In the absence of a thiolic compound such as glutathione or cysteine, dopaquinone changes to dopachrome, this being followed by reactions catalyzed by dopachrome tautomerase (TRP-2) and 5,6-dihydroxyindol-2-carboxylic acid oxidase (TRP-1),3) and further polymerization reactions to yield the melanin pigment, eumelanin.

Melanocytes reside in the basal layer of the epidermis, with keratinocytes layered around and above them. Ultraviolet radiation directly stimulates melanin biosynthesis and the proliferation of melanocytes, and also the release of cytokines such as the α-melanocyte-stimulating hormone (MSH) from keratinocytes or melanocytes which up-regulate the tyrosinase level in melanocytes.4) Since tyrosinase is the key enzyme involved in melanogenesis, any substance exerting an inhibitory effect against tyrosinase could be expected to inhibit melanogenesis. Many tyrosinase inhibitors such as arbutin,5) kojic acid,6) and ellagic acid7) have been identified from natural products as skin-lightening agents, and some of these are used for cosmetics and/or functional foods. However, there is little information available about melanogenesis inhibition by rose hips.

We have found in a previous study that a methanolic extract of rose hips inhibited mushroom tyrosinase, and identified three kinds of flavonoids and four of their glycosides by an instrumental analysis from one of the column chromatographic fractions that showed the highest inhibitory activity against mushroom tyrosinase (Fig. 1).3) We investigate in the present study the effects of these isolated compounds on melanin biosynthesis in B16 mouse melanoma cells and consider the possible mechanisms responsible for the inhibition of melanin biosynthesis.

Materials and Methods

Materials. B16 mouse melanoma cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Theophylline, arbutin, and an RIPA buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified eagle’s medium (DMEM) and other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). The antibodies were polyclonal anti-tyrosinase (M-19, sc-7834), and horseradish peroxidase-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Preparation of the melanogenesis-inhibiting fractions. The procedures for preparing the rose hip extract, fractionating the mushroom tyrosinase-inhibiting fractions, and isolating and identifying the relevant compounds were as described in our previous paper.4) Compounds 1–7 (Fig. 1) were finally identified by an instrumental analysis.3)

Cell culture. B16 mouse melanoma cells were maintained in DMEM with 10% heat-inactivated fetal calf serum (FCS) and a 1% antibiotic-antimycotic at 37 °C in a humidified 5% CO2 atmosphere. Ten thousand cells in 500 μl of the medium were plated on a 24-well microplate. After 24 h of incubation, 500 μl of the medium with 2 mM theophylline and a test sample dissolved in a final concentration of 0.5% dimethyl sulfoxide (DMSO) were added. Various measurements were then performed after incubation for 72 h.

Measurement of cell viability. Cell viability was measured as described previously.8) After washing the cells with PBS, 500 μl of the medium with a 10% 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium solution (Tetra Color ONE, Seikagaku Corporation, Japan) was added. After 1 h of incubation, the formation of formazan was photometrically determined at 450 nm with a microplate reader. All analyses were performed in duplicate and each is expressed as a ratio relative to the value for the untreated control.

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Abbreviations: DMEM, Dulbecco’s modified Eagle’s medium; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; MSH, melanocyte-stimulating hormone; Mitf, microphthalmia-associated transcription factor; PKA, protein kinase A; ROS, reactive oxygen species; TRP-1, 5,6-dihydroxyindol-2-carboxylic acid oxidase; TRP-2, dopachrome tautomerase

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Measurement of melanin content. After the cells had been washed with PBS, they were lysed in 150 μl of 1 μm NaOH and boiled for 5 min to solubilize the melanin. A 100-μl amount of each lysate was put in a 96-well microplate, and the absorbance at 490 nm was measured with a microplate reader. All analyses were performed in duplicate and each is expressed as a ratio relative to the value for the untreated control.

Measurement of intracellular tyrosinase activity. Intracellular tyrosinase activity was measured as described previously with slight modifications.10 After the cells had been washed with PBS, they were lysed by freeze-thawing in 150 μl of PBS with 1% Triton-X. The lysate was clarified by centrifugation at 10000 × g for 5 min, before 100 μl of each lysate was put in a 96-well microplate and 100 μl of 2 mM l-DOPA as a substrate added. After incubating for 1 h at 37°C, the absorbance at 490 nm was measured with a microplate reader. All analyses were performed in duplicate and each is expressed as a ratio relative to the value for the untreated control.

Assay of tyrosinase inhibition in a cell culture-free system. The inhibition of tyrosinase obtained from a mouse melanoma cell lysate as a protein mixture was measured as described previously with slight modifications.11 Fifty microliters of the test material at different concentrations and 100 μl of 2 mM l-DOPA as a substrate for tyrosinase were added to a 96-well microplate and incubated at 37°C for 10 min. After this incubation, 50 μl of a B16 cell lysate (containing 400 μg/ml of protein) was added to the microplate, before incubating at 37°C for 10 min. The assay was also run using a reaction mixture without the substrate to correct for any potential interference by the sample. Dopachrome formation was estimated at 490 nm and corrected by the value obtained from the assay mixture devoid of the substrate. All analyses were performed in duplicate and each is expressed as a ratio relative to the value for the untreated control.

Western blotting. The cells were collected with the aid of a trypsin solution, and lysed with an RIPA buffer. The total cell protein concentration was measured by the Bradford protein assay. Equal amounts of protein from each lysate (25 μg/lane) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a PVDF membrane. The membrane was blocked in a washing buffer consisting of 0.1% Tween-20, 25 mM Tris, and 150 mM NaCl with a 10% blocking solution (EZBlock; Atto Co., Tokyo, Japan) for 1 h at room temperature with agitation. The membrane was then incubated with the primary antibody diluted in the washing buffer (anti-tyrosinase, 1:200) for 1 h at room temperature with agitation. The membrane was washed three times for 10 min each with the washing buffer, and then incubated for 1 h at room temperature with the horseradish peroxidase-conjugated bovine anti-goat IgG antibody diluted 1:1000 in the washing buffer. After this procedure, the membrane was again washed three times for 10 min each in the washing buffer. The antigen-antibody peroxidase complex was then detected by using a tetramethylbenzidine (TMB) solution (EZWestBlue; Atto Co., Tokyo, Japan) in accordance with the manufacturer’s instructions. A quantitative analysis of band density was conducted with Image J software.

Statistical analysis. Data were analyzed by Tukey-Kramer multiple-comparison test, using JMP 7.0.1 software.

Results

Effect of rose hip extract on the melanin content
The methanolic extract of rose hips, which reduced the melanin content of B16 mouse melanoma cells to 49.1% at 1 mg/ml (data not shown), was fractionated into its ethyl acetate and water fractions. The water fraction was fractionated by DIAION HP-20 column chromatography to give water and MeOH eluates. The ethyl acetate fraction showed a strong inhibitory effect against melanogenesis to 15.9% of the vehicle, DMSO, at a concentration of 250 μg/ml without any cytotoxicity (Table 1).

| Table 1. Effects of the Eluates (250 μg/ml) from Rose Hips on the Melanin Content and Melanoma Cell Viability |
|-------------------|-------------------|-------------------|
| Melanin content (% to DMSO) | Cell viability (% to DMSO) |
| DMSO | 100.0 ± 6.4 a | 100.0 ± 4.9 a |
| EtOAc layer | 15.9 ± 1.1 b | 107.8 ± 10 ab |
| H₂O eluate | 74.8 ± 7.8 c | 125.7 ± 6.4 b |
| MeOH eluate | 51.2 ± 7.9 d | 127.2 ± 8.8 b |
| Arbutin⁴ (735 μM) | 32.8 ± 3.0 e | 255.2 ± 13 c |

*Positive control
Each fraction was obtained as described in the Materials and Methods section. Each value is presented as the mean ± SD of three independent determinations. Values in a column not followed by a common letter are significantly different (p < 0.05).

Effect of compounds isolated from the EtOAc fraction of Rose Hips on the Melanin Content and Melanoma Cell Viability

| Table 2. Effects of Compounds (20 μM) Isolated from the EtOAc Fraction of Rose Hips on the Melanin Content and Melanoma Cell Viability |
|-------------------|-------------------|-------------------|
| Melanin content (% to DMSO) | Cell viability (% to DMSO) |
| DMSO | 100.0 ± 18.5 a | 100.0 ± 6.5 ab |
| 1 (+)-catechin | 94.6 ± 20.1 a | 97.3 ± 5.4 bc |
| 2 (+)-taxifolin | 90.1 ± 18.0 a | 88.8 ± 5.9 ab |
| 3-0-β-d-xlylopyparanoside | 71.6 ± 23.3 ab | 102.7 ± 8.3 ab |
| 4 (+)-taxifolin | 87.6 ± 13.0 a | 83.5 ± 5.5 bc |
| 5 queretin | 88.5 ± 15.2 a | 94.9 ± 5.7 bc |
| 6 queretin | 31.6 ± 3.1 bc | 127.1 ± 10.9 bc |
| 7 tiliroside | 114.0 ± 11.1 a | 69.4 ± 4.8 c |
| Arbutin⁴ (735 μM) | 21.8 ± 5.0 c | 249.7 ± 33.7 d |

*Positive control
Each value is presented as the mean ± SD of three independent determinations. Values in a column not followed by a common letter are significantly different (p < 0.05).

Effect of compounds isolated from the EtOAc fraction on the melanin content
To assess the effect on melanogenesis of the compounds (Fig. 1) isolated from the EtOAc fraction by column chromatography, we quantified the melanin content of B16 melanoma cells (Table 2). Glycosides of the flavonoids (compounds 2, 4, 5 and 7) and (+)-catechin (compound 1) did not significantly decrease the melanin content of the cells. (+)-Taxifolin (3) had a slight, but not significant, modifying effect, while queretin (6) had the highest inhibitory activity among the isolated compounds, significantly reduced the melanin content to 31.6%.

Dose-response effect of queretin on the melanin content
Quercetin was added to the culture medium at various concentrations (0, 10, 20, and 40 μM) in order to confirm its melanogenesis-inhibiting effect. As summarized in Fig. 2, quercetin reduced the melanin content in a concentration-dependent manner. The melanin content was significantly reduced to 64.0% at 10 μM, 34.5% at 20 μM and 17.0% at 40 μM, as compared with the non-treated control cells.
Dose-response effect of quercetin on tyrosinase activity

To investigate the mechanism for melanogenesis inhibition by quercetin, we measured the intracellular tyrosinase activity. As summarized in Fig. 3A, quercetin inhibited the intracellular activity of tyrosinase in a concentration-dependent manner. The intracellular tyrosinase activity was reduced to 30.9% at 20 µM and to 11.7% at 40 µM, as compared with the non-treated control cells. Moreover, quercetin also inhibited the tyrosinase activity in a cell culture-free system (Fig. 3B).

Dose-response effect of quercetin on tyrosinase protein expression

We performed western blotting against the cell lysate obtained from quercetin-treated B16 melanoma cells. As shown in Fig. 4, quercetin decreased the expression of tyrosinase protein in a concentration-dependent manner.

Discussion

*Rosa canina* L. (Rosaceae), known as the “dog rose,” is a prickly shrub distributed in Scotland and other areas of Europe. The fruits of this plant are known as “rose hips” and have been used as a diuretic, laxative, and anti-gout and anti-rheumatism medication in traditional European folk medicine. In the present study, B16 mouse melanoma cells were used to investigate the melanogenesis-inhibiting effect of the compounds previously isolated from rose hips. This method can evaluate both the incorporation of test substances into cells and their inhibition against melanogenesis, this being closer to an in vivo evaluation than employing mushroom tyrosinase.

We found that quercetin isolated from rose hips was a potent melanogenesis inhibitor in B16 mouse melanoma.
However, the glycosides of rose hip flavonoids (compounds 2, 4, 5 and 7 in Fig. 1) had no melanogenesis-inhibiting effect in B16 mouse melanoma cells. In general, these substances must be hydrolyzed by intestinal hydrolytic enzymes or by colonic microflora before they can be absorbed in vivo.\(^1\) Compound 5, a glycoside of quercetin, had no melanogenesis-inhibiting effect, as shown in Table 2, probably because it was not hydrolyzed, and thus not absorbed as quercetin in vitro.

We have demonstrated in a previous study\(^2\) that (+)-catechin, like quercetin, inhibited mushroom tyrosinase \textit{in vitro} but had no melanogenesis-inhibiting effect in B16 melanoma cells. Two previous studies have also demonstrated that (+)-catechin had no melanogenesis-inhibiting effect in B16 mouse melanoma cells.\(^3,4\) Moreover, (+)-catechin did not inhibit intracellular tyrosinase activity in the same cell line in the present study (data not shown). (+)-Catechin may not be incorporated into B16 mouse melanoma cells. It is presumed, therefore, that different results were obtained by each method. Since quercetin inhibited the intracellular tyrosinase activity concomitantly with a reduction in the melanin content, the inhibition of pigmentation may require suppression of the intracellular tyrosinase activity in B16 mouse melanoma cells.

Quercetin dose-dependently inhibited melanin production in B16 mouse melanoma cells (Fig. 2). This result could not be explained by cytotoxicity of quercetin, because there was no evident decrease in the number of viable cells up to a concentration of 40\(\mu M\). To reveal the mechanism responsible for the inhibitory effect of quercetin, its effects on tyrosinase were measured. Quercetin decreased the intracellular tyrosinase activity and also inhibited the tyrosinase activity in a cell culture-free system (Fig. 3). It is well documented that quercetin exerts an inhibitory effect on mushroom tyrosinase.\(^5-7\) We have also demonstrated that quercetin had a potent inhibitory effect on mushroom tyrosinase.\(^8\) Furthermore, we found that quercetin inhibited tyrosinase protein expression in a concentration-dependent manner (Fig. 4). It was presumed, therefore, that the suppression of intracellular tyrosinase activity was due to both the inhibition of activity and to the expression toward tyrosinase.

\(\alpha\)-MSH and theophylline have increased the cAMP concentration in cells, resulting in enhanced melanin production.

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**Fig. 3.** Inhibitory Effect of Quercetin on Tyrosinase Activity. A, The effects of quercetin and arbutin on the intracellular tyrosinase activity were measured as described in the Materials and Methods section. Each value is represented as the mean \(\pm\) SD of three independent determinations. Values not followed by a common letter are significantly different \((p < 0.05)\). B, The effects of quercetin and arbutin on tyrosinase activity in a cell culture-free system were measured as described in the Materials and Methods section. Each value is represented as the mean \(\pm\) SD of three independent determinations. Values not followed by a common letter are significantly different \((p < 0.05)\).

**Fig. 4.** Effect of Quercetin Concentration on Tyrosinase Protein Expression in Mouse Melanoma Cells. B16 melanoma cells were treated with quercetin and arbutin (ar) at the concentrations indicated in the figure and incubated for 72 h. Tyrosinase protein expression was determined by a western blotting analysis, as described in the Materials and Methods section. Top, original blot, bottom, densitometric analysis. Each value is represented as the mean \(\pm\) SD of three independent determinations. Values not followed by a common letter are significantly different \((p < 0.05)\).
production.\textsuperscript{18} cAMP has activated protein kinase A (PKA), which subsequently increased the expression of the microphthalmia-associated transcription factor (Mitf). Mitf effectively transactivates the expression of tyrosinase and its related genes by binding to their common promoters.\textsuperscript{19,20} Since quercetin has been shown to inhibit the PKA activity,\textsuperscript{21,22} it may inhibit melanogenesis in B16 mouse melanoma cells by suppressing the PKA activity.

Although our data suggest that quercetin attenuated melanogenesis in B16 mouse melanoma cells, the opposite effect that quercetin enhanced melanogenesis in human melanoma cells and normal epidermal melanocytes has previously been reported by Nagata et al.\textsuperscript{33} They have stated that the melanogenesis resulted from an increase in the activity of tyrosinase and decrease in the melanogenic inhibitor, and was concerned with genomic mechanisms involving both new messenger RNA and protein synthesis. However, quercetin did not significantly change the expression of tyrosinase protein and decreased tyrosinase mRNA in a dose-dependent manner. This discrepancy was not elucidated in their report. Our data that quercetin attenuated melanogenesis concomitantly with suppressed intracellular tyrosinase activity and its protein expression clearly explain the mechanism for melanogenesis inhibition by quercetin.

As already mentioned, the glycosides of rose hip flavonoids did not show any significant inhibitory effect on melanogenesis in B16 mouse melanoma cells. However, some of these compounds may have an inhibitory effect in vivo depending on their chemical structures because they are hydrolyzed to active aglycons by intestinal hydrolytic enzymes and/or colonic microflora. Moreover, it has been reported that a melanogenic inducer such as $\alpha$-MSH was produced from keratinocytes via stimulation by reactive oxygen species (ROS).\textsuperscript{4,24} Since flavonoids generally have antioxidative activity, they may inhibit melanogenesis in vivo by not only tyrosinase inhibition in melanocytes but also by ROS reduction. Further studies are underway to clarify the effects of rose hips on pigmentation in vivo.

In summary, using B16 mouse melanoma cells, we investigated the melanogenesis-inhibiting effect of compounds isolated from rose hips, and found that quercetin dramatically inhibited melanogenesis without exhibiting any cytotoxicity. We also demonstrated that the inhibition of cellular tyrosinase by quercetin was due both to the inhibition of tyrosinase activity and to down-regulation of tyrosinase protein expression.

\section*{References}