Inhibitory Effects of Whisky Congeners on Melanogenesis in Mouse B16 Melanoma Cells

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We examined the effect of whisky congeners, substances other than ethanol in whisky, on melanogenesis in mouse B16 melanoma cells. Treatment with whisky congeners significantly blocked melanogenesis. Our results indicate that the inhibitory effects of whisky congeners on melanogenesis is due to direct inhibition of tyrosinase activity and to suppression of tyrosinase protein levels.

Key words: whisky congeners; melanin; melanogenesis; tyrosinase

Melanin production is principally responsible for skin color, and it plays an important role in the prevention of sun-induced skin injury.1–3 Melanin is produced by melanocytes in the basal layer of the epidermis.1–3 Synthesis of melanin starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, the enzyme catalyzing the rate-limiting step in melanin biosynthesis.4 This tyrosinase process is involved in the abnormal accumulation of melanin pigments, known as hyperpigmentation. Hence, many reports have described pharmacologic and cosmetic agents that inhibit tyrosinase activity or that block melanogenic pathways, leading to skin lightening.5,6

Studies of the effects of polyphenols contained in daily beverages on human health have focused mainly on flavonoids.7 Whisky polyphenols are composed of ellagic acid derived from ellagitannin (hydrolysable tannin), phenylpropanoids, vanillin, and lignan.8–10 Polyphenols exude from the oak cask into whisky during the years of aging.8–10 Some of them go through degradative and oxidizing reactions, turning into a variety of substances,8–10 but the effects of the polyphenols in whisky on health remain unclear. A recent report indicates that whisky congeners have a protective effect on ethanol-induced gastric mucosal damage,11 but other biological activities are not understood. In this study, we investigated the effects of whisky congeners on melanin biosynthesis in mouse B16 melanoma cells.

To determine the effects of whisky congeners on melanogenesis, mouse B16 melanoma cells were cultured in the presence of whisky congeners. Mouse B16 melanoma 4A5 cells (Riken Cell Bank, Tsukuba, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified, CO2-controlled (5%) incubator. Cells (n = 4 × 10⁴) were seeded on 24-well plates. Treatment with whisky congeners began 24 h after seeding. Whisky congeners (2.5 mg/whisky L), prepared by evaporating a single-malt whisky, followed by lyophilization and dissolving in DMSO, was added to the cell cultures. Melanogenesis in B16 melanoma cells was initiated by the addition of 1 M α-MSH, and was assessed by determination of the intracellular melanin content. For determination of the melanin content, the B16 cells were washed with PBS and dissolved in 2 N NaOH for 1 h at 60°C. The absorbance at 470 nm was measured, and the melanin content was measured using the accepted standard of synthetic melanin.

When B16 melanoma cells were treated with α-MSH in the presence of whisky congeners, significant decreases in α-MSH-induced melanogenesis were observed as compared with that in the absence of whisky congeners (Fig. 1A). As shown in Fig. 1A, the levels of melanin content were reduced in a concentration-dependent manner by whisky congeners, with the maximal level at 100 µg/ml. To establish the relative efficacy of whisky congeners, its inhibitory effects were compared with a melanogenesis inhibitor, kojic acid.
The inhibitory effect of whisky congeners was significantly stronger than that of kojic acid (Fig. 1A).

To exclude the possibility that the inhibitory effects of whisky congeners on melanogenesis was caused by inhibition of cell growth, we determined the number of cells grown in the presence of whisky congeners. Cell viability was determined by the trypan blue exclusion test. Cell numbers were not significantly change at 100 \( \mu \text{g/ml} \) of whisky congeners (Fig. 1B).

As shown in Fig. 1C, there was a time-dependent increase in melanin content in cells stimulated with \( \alpha \text{MSH} \), but melanogenesis was repressed when the cells were treated with whisky congeners. Melanogenesis is dependent on the activity of tyrosinase, a rate-limiting enzyme in melanin biosynthesis. We examined the effect of whisky congeners on \( \alpha \text{MSH} \)-mediated increases in tyrosinase activity. For measurement of tyrosinase activity, the cells were washed with ice-cold PBS and then lysed by incubation at 4 °C for 30 min in RIPA buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitors (Complete™ protease inhibitor mixture, Roche, Switzerland). The lysates were centrifuged at 15,000 × g for 30 min to obtain the supernatant as a source of tyrosinase. The reaction mixture contained 50 mM phosphate buffer, pH 6.8, 0.05% L-dopa, and the supernatant. After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring the absorbance at a wavelength of 475 nm. Relative tyrosinase activity (%) was expressed as the absorbance at 475 nm. Although the tyrosinase activity in the absence of whisky congeners was enhanced by \( \alpha \text{MSH} \) in time-dependent manner, activity was not increased in whisky congener-treated cells (Fig. 1D).

To identify of the mechanism by which whisky congeners reduce melanin contents B16 melanoma cells, we examined the inhibitory effect on murine tyrosinase activity using L-dopa as substrate. As shown in Fig. 2, tyrosinase activity was strongly suppressed in a concentration-dependent manner by whisky congeners. One hundred \( \mu \text{g/ml} \) of whisky congeners exhibited a similar potency to 100 \( \mu \text{g/ml} \) kojic acid. These results indicate that the inhibitory effect of whisky congeners on melanogenesis, might be due to at least in part to direct inhibition of tyrosinase activity.

To investigate further the mechanism of whisky congener-induced suppression of melanogenesis, we examined the effect of whisky congeners on the expression of tyrosinase by Western blot analysis. The
antibody to tyrosinase was from Santa Cruz Biotechnology (Santa Cruz, CA). Although expression of tyrosinase protein was greatly enhanced by treatment with /\_\_\_\_\_\_\_MSH, expression was not increased in the presence of whisky congeners (Fig. 3A). Inhibition of melanogenesis by whisky congeners correlated well with the decrease in tyrosinase activity which corresponds to its expression level.

To determine whether the decreased protein level of tyrosinase was responsible for the decrease in mRNA expression in whisky congener-treated B16 cells, we examined the levels of tyrosinase mRNA by RT-PCR. Total RNA was extracted from B16 melanoma cells using TRIZOL-Reagent according to manufacturer’s protocol. RNA samples (2 \mu g/reaction) were reverse-transcribed with Superscript in the presence of oligo-dT, and the RT reaction was used for amplification with Taq polymerase. The resulting cDNA was amplified using specific primers. The primers used were as follows; for tyrosinase 5’-GGCCAGCTTTACGGCAGT and 5’-TGTTGCTTACATGGGCAAATC. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Takara bio, Japan) were added as a control for the same reverse transcriptase product. Amplification conditions were 94 °C (30 s), 60 °C (30 s), 72 °C (40 s) for 21 cycles (tyrosinase), and 18 cycles (GAPDH). The PCR products were electrophoresed on 1.3% agarose gel containing ethidium bromide. As shown in Fig. 3B and C, whisky congeners caused a significant decrease in the level of tyrosinase protein, whereas the level of

**Fig. 2.** Effects of Whisky Congeners on Tyrosinase Activity in Cell-Free System.

Tyrosinase activity was determined by measuring the formation of dopachrome. Data represent the mean ± S.D. of two different experiments, each carried out in duplicate.

**Fig. 3.** Effects of Whisky Congeners on Expression of Tyrosinase in B16 Melanoma Cells.

The cells were treated with αMSH (1 \mu M) and whisky congener (50 \mu g/ml). The levels of tyrosinase protein after treatment for (A) the indicated times and (B) 48 h were determined by Western blot analysis using specific antibody. C, The levels of tyrosinase mRNA after treatment for 24 h were determined by RT-PCR analysis.
tyrosinase mRNA was unchanged. These results suggest that down-regulation of tyrosinase expression by whisky congeners occurred at the post-transcriptional level, but the exact mechanisms remain to be discovered.

In summary, the present study indicates that whisky congeners suppress melanogenesis by direct inhibition of tyrosinase activity and modulation of the protein amounts of tyrosinase in mouse B16 melanoma cells. Hence, whisky congeners should be useful for application in the cosmetic field. The whisky congeners originate mainly in oak barrels, and polyphenols and produced during the aging of spirits, including whisky, in oak casks. Further studies are needed to identify the effective components in whisky congeners to determine the exact mechanism underlying the effect of whisky congeners. They are in progress in our laboratory.

References