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Methyl gallate from *Acer barbinerve* decreases melanin synthesis in Mel-Ab cells

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Methyl gallate (MG) was isolated from the bark of *Acer barbinerve*, which has traditionally been used in Oriental medicine. In the present study, we examined the effects of MG on melanin synthesis in Mel-Ab melanocyte cells. MG decreased melanin pigmentation in a concentration-dependent manner, but did not directly inhibit tyrosinase activity. Further analysis showed that MG had no effect on extracellular signal-regulated kinase (ERK) activation, but induced phosphorylation of glycogen synthase kinase (GSK)3β, which is known to increase β-catenin accumulation. Accordingly, the β-catenin level was increased by MG. However, a specific GSK3β inhibitor did not rescue the MG-induced inhibition of melanogenesis. Additionally, MG decreased the protein expression of microphthalmia-associated transcription factor (MITF) and tyrosinase, which regulate melanin synthesis. Based on these results, we conclude that MG inhibits melanogenesis by decreasing the expression of MITF and tyrosinase.

1. Introduction

Methyl gallate (MG) is a derivative of gallic acid. Recently, MG was isolated from the stem bark of *Acer barbinerve* (Lee et al. 2013). *A. barbinerve* is usually found in the fields of Korea, Japan, and China and its bark has traditionally been used in Oriental medicine. MG possesses several biological properties including anti-oxidant, anti-inflammatory, and anti-microbial activities (Choi et al. 2008; Choi et al. 2009; Hsieh et al. 2004; Kang et al. 2009). MG also exerts anti-tumor effects by inhibiting tumor infiltration of $CD4^+/CD25^+$ regulatory T cells (Lee et al. 2010). In addition, MG shows an inhibitory effect on glioma proliferation and migration through inhibition of Akt and extracellular signal-regulated kinase (ERK) (Lee et al. 2013). However, the effects of MG on melanogenesis have not been investigated.

The color of human skin, hair, and eyes is determined by the production and distribution of melanin. Melanin pigments are produced in melanocytes, epidermal pigment cells. Melanin synthesis is induced by various stimuli, including UV irradiation, cytokines, and hormones (Hearing and Tsukamoto 1991) and is catalyzed by three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2. Tyrosinase, the rate-limiting enzyme in this process, controls melanin synthesis by catalyzing hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and oxidation of DOPA to DOPAquinone (Hearing and Tsukamoto 1991). The expression of these three enzymes is regulated by microphthalmia-associated transcription factor (MITF) expression, which correlates with pigmentation and survival of melanocytes (Yasumoto et al. 1997).

The Wnt pathway is known to be involved in MITF expression. Binding of Wnt to its receptors induces phosphorylation of

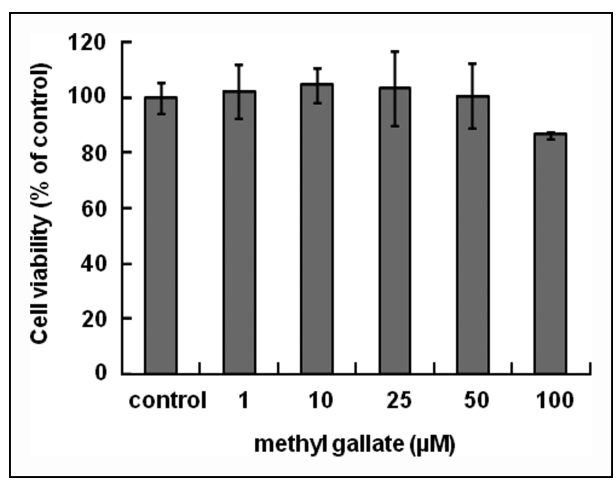

Fig. 1: Effects of MG on Mel-Ab cell viability. After serum starvation for 24 h, the cells were treated with MG at 1-100 μM for another 24 h. Cell viability was determined by crystal violet assay. Each measurement was made in triplicate and data represent the means ± S.D.

Abbreviations: GSK3β, glycogen synthase kinase 3β; ERK, extracellular signal-regulated kinase; MG, methyl gallate; MITF, microphthalmia-associated transcription factor; PVDF, polyvinylidene fluoride; TRP, tyrosinase-related protein; DOPA, dihydroxyphenylalanine; LEF/TCF, lymphoid-enhancing factor/T cell factor; CT, cholera toxin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

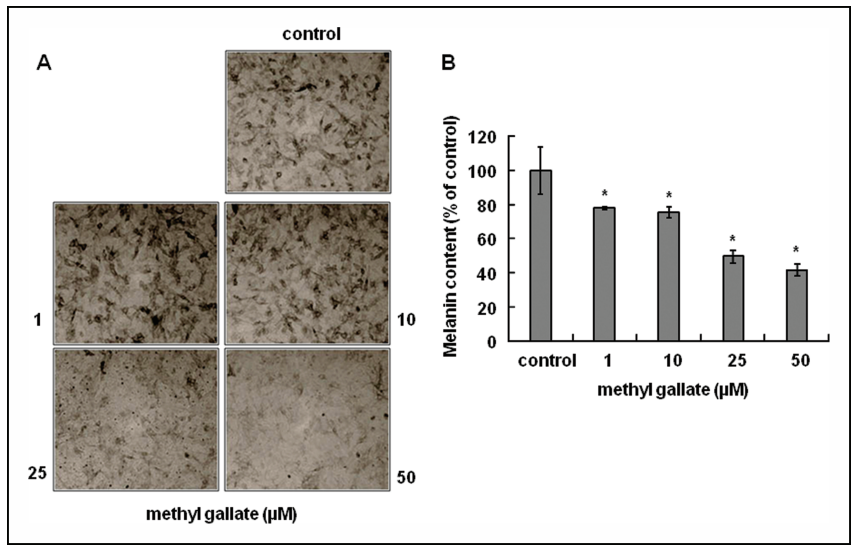

Fig. 2: Effects of MG on melanin synthesis. Mel-Ab cells were incubated with 1-50 μM MG for 3 days. (A) Phase-contrast pictures were taken using a digital video camera. (B) Melanin content was measured as described in the Experimental section. Each determination was measured in triplicate, and data represent the means ± S.D. **P*< 0.05 compared to the untreated control.

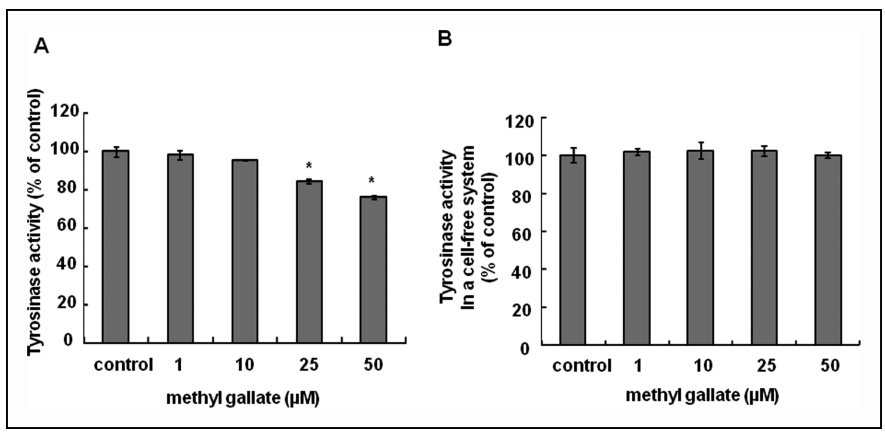

Fig. 3: Effects of MG on tyrosinase activity. (A) Mel-Ab cells were treated with 1-50 μM MG for 3 days, and tyrosinase activity was measured as described in the Experimental section. (B) Tyrosinase activity in a cell-free system was measured to test the direct effect of MG on tyrosinase. Each measurement was measured in triplicate, and the data represent mean ± S.D. **P*< 0.05 compared to the untreated control.

glycogen synthase kinase 3β (GSK3β). Phosphorylated GSK3β cannot degrade β-catenin, and instead β-catenin is translocated into the nucleus (Aberle et al. 1997; Liu et al. 2002) where it forms a complex with lymphoid-enhancing factor/T cell factor (LEF/TCF) (Behrens et al. 1996; Peifer and Polakis 2000; Wu et al. 2003). Activity of this complex increases MITF expression. Therefore, GSK3β may be a target for the regulation of melanogenesis. On the other hand, it has also been reported that ERK activation inhibits melanogenesis (Kim et al. 2003, 2006). In the present study, we examined the effects of MG on melanogenesis in Mel-Ab cells. We also investigated the underlying mechanisms of the hypopigmentary effect induced by MG.

2. Investigations and results

2.1. Effects of MG on cell viability

Mel-Ab cells were treated with MG at various concentrations (1-100 μM) and cell viability was determined by crystal violet assay. MG had no effect on Mel-Ab cell viability at concentrations of 1-50 μM (Fig. 1). However, a higher concentration of MG (100 μM) was associated with 80% cell viability. Therefore, MG was used at concentrations from 1-50 μM for the subsequent experiments.

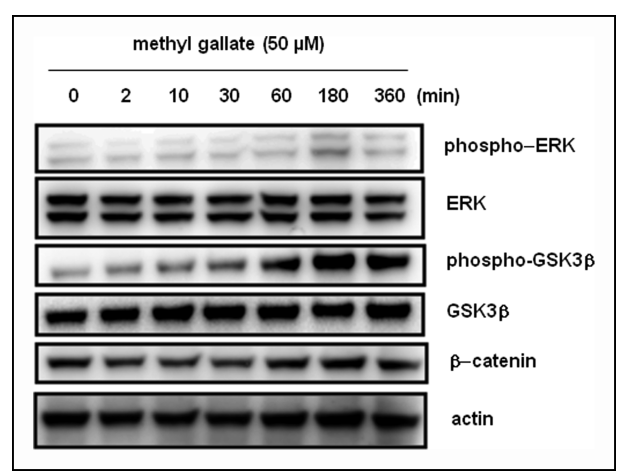

Fig. 4: Effects of MG on various signal transduction pathways in Mel-Ab cells. After serum starvation for 24 h, the cells were treated with 50 μM MG for the indicated times. Whole cell lysates were subjected to Western blot analysis with antibodies against phospho-GSK3β, phospho-ERK, and β-catenin. Equal protein loading was confirmed by actin expression level.

2.2. Effects of MG on melanin synthesis in Mel-Ab cells

To examine the effect of MG on melanogenesis in Mel-Ab cells, cells were treated with MG at concentrations of 1-50 μM for

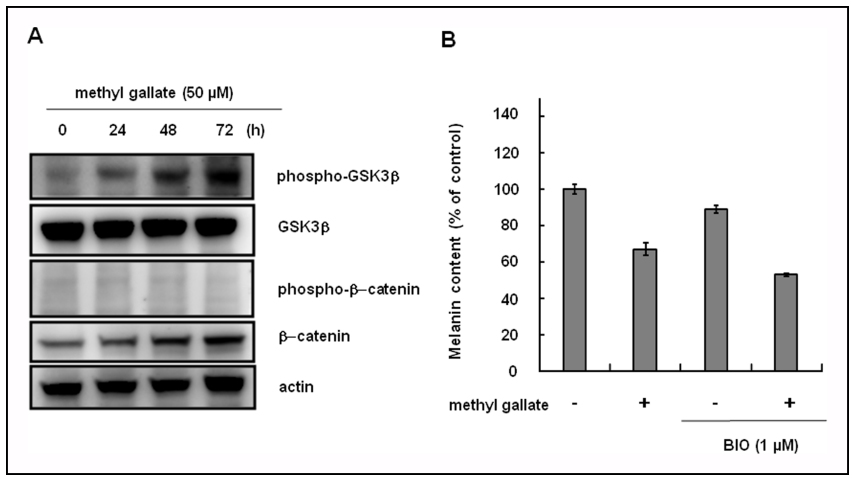

Fig. 5: Effects of MG on the Wnt pathway (A) Mel-Ab cells were incubated with 50 μM MG for 24-72 h. Whole cell lysates were analyzed by Western blotting with antibodies against phospho-GSK3β, phospho-β-catenin, and β-catenin. Equal protein loading was confirmed by actin expression. (B) Mel-Ab cells were treated with MG for 3 days in the presence and the absence of BIO, a specific GSK3β inhibitor. Melanin content was then measured. Each determination was measured in triplicate, and data represent the means ± S.D.

3 days and then photographed under a phase contrast microscope (Fig. 2). The MG-treated cells showed decreased melanin pigmentation compared with untreated cells. Moreover, we measured melanin content after MG treatment. As shown in Fig. 2B, MG decreased the melanin content of cells in a concentration-dependent manner.

2.3. Effects of MG on tyrosinase activity in Mel-Ab cells

Mg-Ab cells were exposed to MG and tyrosinase activity was measured. Tyrosinase activity was inhibited by MG in a concentration-dependent manner (Fig. 3A). Next, we examined tyrosinase activity in a cell-free system using mushroom tyrosinase. MG showed no direct inhibitory effect on tyrosinase (Fig. 3B) suggesting that MG may inhibit the expression of tyrosinase rather than its activity.

2.4. Effects of MG on signaling pathways related to melanin synthesis

It is known that ERK activation regulates melanin synthesis. Therefore, we first investigated whether ERK is affected by MG in a time-course experiment and found that MG had no effect on ERK phosphorylation. GSK3β and β-catenin, which are associated with the Wnt pathway, also regulate melanogenesis. Our results showed that GSK3β was phosphorylated after 60-360 min of MG treatment (Fig. 4). However, the level of β-catenin did not change over this time period.

2.5. Effects of GSK3β inhibitor on the MG-induced decrease in melanin synthesis

Because GSK3β was phosphorylated by MG after 60-360 min of treatment, we investigated GSK3β phosphorylation after 24-72 h of MG treatment. As shown in Fig. 5A, GSK3β was strongly phosphorylated (inactivated) after 24-72 h. Accordingly, β-catenin accumulated as a result of GSK3β inactivation. Therefore, we further examined the involvement of GSK3β using BIO, a specific GSK3β inhibitor. Cells were treated with MG for 3 days in the absence and presence of BIO, and melanin synthesis was measured. However, BIO could not rescue the decrease in melanin synthesis induced by MG. These data suggest that the phosphorylation of GSK3β induced by MG was not related to the reduction in melanogenesis.

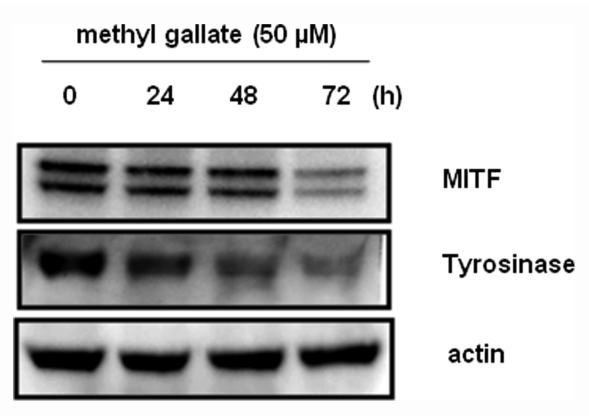

Fig. 6: Effects of MG on MITF and tyrosinase protein expression. Mel-Ab cells were incubated with 50 μM MG for 24-72 h. Western blot analysis was performed with antibodies against MITF and tyrosinase. Equal protein loading was confirmed by actin expression level.

2.6. Effects of MG on MITF and tyrosinase level

We next examined MITF and tyrosinase protein levels after 24-72 h of MG treatment. As shown in Fig. 6, MITF and tyrosinase protein levels were reduced after 24 h of MG treatment.

3. Discussion

The overproduction of melanin can lead to hyperpigmentary disorders such as melasma, freckles, post-inflammatory melanoderma, and solar lentigo. Research into new skin whitening agents to improve hyperpigmentation has received great attention. In the present study, we showed that MG inhibited melanin synthesis. However, MG had no effect on tyrosinase activity in a cell-free system indicating that MG inhibits melanin synthesis indirectly by modulating the intracellular signaling pathways that regulate expression of tyrosinase. Therefore, we investigated the molecular mechanisms involved in MG-regulated melanin synthesis.

Although the ERK pathway is known to be highly involved in melanogenesis, we found that MG had no effect on this pathway. In contrast, we found that GSK3β was phosphorylated (and thereby inactivated) by MG. In accordance with these results, the level of β-catenin protein was increased in a time-dependent manner after treatment with MG (Fig. 5). It has been reported that accumulation of β-catenin induces MITF expression (Bellei et al. 2011). The citrus flavanone naringenin enhances melanin synthesis by increasing the intracellular accumulation of β-catenin (Huang et al. 2011). Moreover, FTY720, a sphingolipid analogue, inhibits melanogenesis by decreasing the expression of β-catenin (Lee et al. 2011). Therefore, β-catenin is deeply involved in the regulation of melanogenesis. However, our results showed that MG increased the β-catenin level, indicating that β-catenin may not be involved in the MG-induced regulation of melanogenesis.

We also showed that MG decreased the protein levels of MITF and tyrosinase. In a previous study, GSK3β was shown to phosphorylate MITF at Ser298, thereby enhancing its binding activity to the tyrosinase promoter (Li et al. 2012). It was also reported that diosgenin, a steroidal saponin, increased the phosphorylation level of GSK3β, leading to the inhibition of melanin synthesis via reduction of tyrosinase expression (Lee et al. 2007). We previously reported that *Xanthium strumarium L.* (Asteraceae), inhibited melanogenesis by decreasing tyrosinase expression through GSK3β phosphorylation (Li et al. 2012). Therefore, we hypothesized that MG-induced GSK3β phosphorylation decreases the binding activity of MITF to the tyrosinase promoter. To examine the possible involvement of GSK3β in MG-induced hypopigmentation, we used a specific GSK3β inhibitor, BIO. In a previous study, BIO was shown to restore the reduction in melanin induced by KHG25855 (2-cyclohexylamino-1,3-thiazolehydrochloride) (Kim et al. 2011). Unexpectedly, we found that BIO did not recover MG-induced melanin reduction (Fig. 5) suggesting that the GSK3β-mediated pathway is not responsible for the MG-induced hypopigmentation. Although the exact signaling pathways underlying the mechanism of MG activity should be clarified, the reduction of MITF and tyrosinase expression appears to be responsible for the hypopigmentation.

In conclusion, we found that MG decreased melanin synthesis through the reduction of MITF and tyrosinase protein expression, which was not related to GSK3β and β-catenin activity. We propose that MG could be pursued as a new skin whitening agent.

4. Experimental

4.1. Materials

MG was isolated from the stem bark of *A. barbinerve* (Lee et al. 2013). Cholera toxin (CT), 12-O-tetradecanoylphorbol-13-acetate (TPA), and mushroom tyrosinase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). BIO and antibodies specific for phospho-ERK1/2 (Thr202/Tyr204, #9101S), phospho-GSK3β (#9336), and β-catenin (#9581) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and microphthalmia Ab-1 (C5, MS-771-P0) was obtained from NeoMarkers (Fremont, CA, USA). Secondary antibodies specific for anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA).

4.2. Cell culture

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that synthesizes large quantities of melanin. Mel-Ab cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37 °C in 5% CO_2.

4.3. Cell viability assay

Cell viability was measured using a crystal violet assay. After incubation with MG for 24 h, the culture medium was removed and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed four times with distilled water. The crystal violet retained by adherent cells was extracted with 95% ethanol, and absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Measurement of melanin content and microscopy

Melanin content was measured as described previously (Smalley and Eisen 2000) with slight modification. Briefly, cells were treated with MG in DMEM containing 10% FBS for 3 days. Cells were then dissolved in 550 μL of 1 N NaOH at 100 °C for 30 min and centrifuged at 13,000 rpm for 5 min. The optical density (OD) of the supernatants was measured at 400 nm with an ELISA reader. Before measuring melanin content, the cells were observed under a phase contrast microscope (Olympus IX50, Tokyo, Japan) and photographed using a DCM300 digital microscope camera (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.).

4.5. Tyrosinase activity

Tyrosinase activity was analyzed as described previously (Busca et al. 1996) with slight modification. Briefly, Mel-Ab cells were seeded in 6-well plates and incubated with MG for 3 days. The cells were then washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. Cells were disrupted by freezing and thawing, and lysates were clarified by centrifugation at 15,000 rpm for 10 min. After quantification of the protein levels of the lysate and adjusting the protein concentrations with lysis buffer, 90 μL of each lysate, adjusted to contain the same amount of protein, was placed in each well of a 96-well plate, and 10 μL of 10 mM L-DOPA was added to each well. The control wells contained 90 μL of lysis buffer and 10 μL of 10 mM L-DOPA. Following incubation at 37 °C, absorbance was measured every 10 min for at least 1 h at 475 nm using an ELISA reader. A cell-free assay system was used to examine the direct effects of MG on tyrosinase activity. For this assay, 60 μL of phosphate buffer containing MG was mixed with 20 μL of 53.7 U/mL mushroom tyrosinase, and 20 μL of 10 mM L-DOPA was then added. After incubation at 37 °C, absorbance was measured at 475 nm.

4.6. Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete™; Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. A total of 20 μg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then saturated with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with a horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

The statistical significance of the differences between groups was assessed by analysis of variance (ANOVA), followed by the Student's *t*-test. P values < 0.05 were considered significant.

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