O-methylated Flavone Wogonin Specifically Reduces Melanosome Transport through Downregulation of Melanophilin

Kumiko Kobayashi-Nakamura[1], Michiko Kudo[1], Tatsuya Ogawa[2], Masahiko Asahi[2], and Kentaro Naito[1]

[1]DHC Corporation Laboratories, Division 2, 2-24 Hamada, Mihama-ku, Chiba 261-0025, Japan
[2]DHC Corporation, Fragrance and Cosmetics Department, 2-8-21 Minami-Azabu, Minato-ku, Tokyo 106-0047, Japan

Abstract

Melanocytes produce dense pigment-containing organelles termed melanosomes that are transported to neighboring keratinocytes for screening against ultraviolet radiation. Before release from melanocytes, melanosomes are intracellularly transported from the perinuclear region to the cell periphery along cytoskeletons. Although several recent reports have shown that the downregulation of intracellular melanosome transport provides skin lightening, few studies have attempted to identify an effective inhibitor against melanosome transport. Our primary work has shown that *Scutellaria baicalensis* has a potent inhibitory effect on melanogenesis and that wogonin is the active component among several flavones contained in *S. baicalensis*. In this study, we show the inhibitory effect of wogonin on melanosome transport in melanocytes. Upon culturing, we found that melanosomes in mouse melanoma cell line B16F10 cells after wogonin treatment were localized to the perinuclear region and were conspicuously absent in the cell periphery, suggesting that wogonin inhibits melanosome transport. To investigate the effect of wogonin on melanosome transport, melanosome distribution assays were performed in B16F10 cells. To define the structural specificity of wogonin (5,7-dihydroxy-8-methoxyflavone) responsible for melanosome transport disorder, B16F10 cells were cultured with several wogonin analogs. Demethylation of the 8-*O*-methyl group in wogonin completely failed to generate the intracellular aggregation of the melanosomes. However, 7-*O*-methylation of baicalein (5,6,7-trihydroxyflavone) resulted in the acquired ability to induce impaired melanosome transport. In addition, oroxylin A, a 6-*O*-methyl positioned isomer of wogonin, was shown to inhibit melanosome dispersion. Thus, we found that the mono-*O*-methyl group in the flavone

A-ring is essential for inhibiting melanosome transport, and that wogonin is the most potent melanosome transport inhibitor among wogonin analogs. Analysis of protein levels in B16F10 cells showed that wogonin significantly reduces in melanophilin (MLPH), one of the key molecules in melanosome transport. These results suggest that mono-*O*-methyl flavones, such as wogonin, inhibit melanosome transport through the downregulation of MLPH, thus proposing a new approach for the treatment of pigmentation disorders.

Introduction

Melanocytes in the basal layer of the epidermis produce various amounts and types of melanins within specific organelles termed melanosomes, which are transported to neighboring epidermal keratinocytes (1). Mature melanosomes are intracellularly transported along two cytoskeletons, including microtubules and actin filaments, attached to the plasma membrane and subsequently released into the extracellular matrix (2). The activation of melanocytes (such as by ultraviolet irradiation) causes skin-pigmentation, including freckles, melasma, and age spots.

Several ingredients derived from plants have been identified as skin depigmentation agents. For example, arbutin, a glycosylated hydroquinone, has been identified as to be an inhibitor against melanogenesis from the *Arctostaphylos uva-ursi* (3). *Scutellaria baicalensis*, a Chinese medicinal plant, has been widely used in the clinical treatment of inflammatory diseases, including atopic dermatitis, hyperlipemia, and atherosclerosis (4, 5, 6). Its root contains several functional flavonoids, such as baicalein and baicalin. These compounds exhibit anti-inflammatory activity (7). Wogonin (5,7-dihydroxy-8-methoxyflavone) is a flavonoid found in wogon extract (Figure 1).

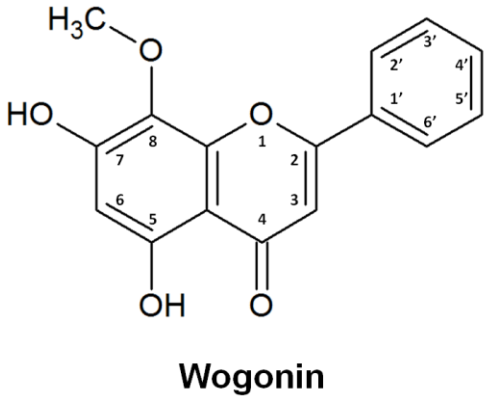

Figure 1. Structure of wogonin.

When applied to the skin, wogonin reduced levels of cyclooxygenase (COX)-2 in both UVB-treated HaCaT cells and a mouse model of inflammatory skin disease (8, 9). However, the effect of wogonin on skin pigmentation is unclear. Upon culturing, we first found that wogonin induces the maldistribution of melanosomes in mouse melanocytes, increasing the possibility that wogonin inhibits melanosome transport. Recently, the molecular mechanism of actin-based melanosome transport has been well documented by genetic and biochemical analyses of three gene products. The first key regulator of actin-based melanosome transport is myosin Va, and the other two are Rab27A and melanophilin (MLPH) (10). Appropriate downregulation of melanosome transport contributes to controlling melanogenesis, which is helpful for esthetic application and treatment of skin hyperpigmentation. Therefore, we investigated whether wogonin downregulates melanosome transport.

Materials and Methods

Melanosome distribution assay

B16F10 cells were cultured on caver glasses at 37°C for 24 h and then treated with 50 μM wogonin for 72 h. Cells were fixed in 4% (w/v) paraformaldehyde for 15 min and permeabilized with 0.1% (v/v) Triton X-100 for 10 min. Thereafter, cells were incubated with alexafluor 594 phalloidin for 30 min. 4′ -6-Diamidino-2-phenylindole was used for nuclear staining. Images were recorded on a Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany) confocal microscope. Melanosome distribution assays were performed as previously described with several modifications (11). Cells in which >50% of melanosomes were located around the nucleus were judged to be melanosome-aggregated. Using >100 cells, melanosome aggregation was checked and measured. This experiment was repeated three times independently. Statistical significance was determined using a Tukey–Kramer test.

Western blot analysis

Equal protein amounts of each sample (10 μg) were run on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 1% (w/v) nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), membranes were incubated with an anti-MLPH antibody overnight at 4°C with gentle agitation, then incubated with a horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature and

visualized by enhanced chemiluminescence.

Results and Discussion

In B16F10 cells treated with wogonin, melanosomes were localized at the periphery of the nucleus compared with the control cells (Figure 2). The perinuclear localization of melanosomes implies that wogonin inhibits the transport of melanosomes from the ER towards the plasma membrane.

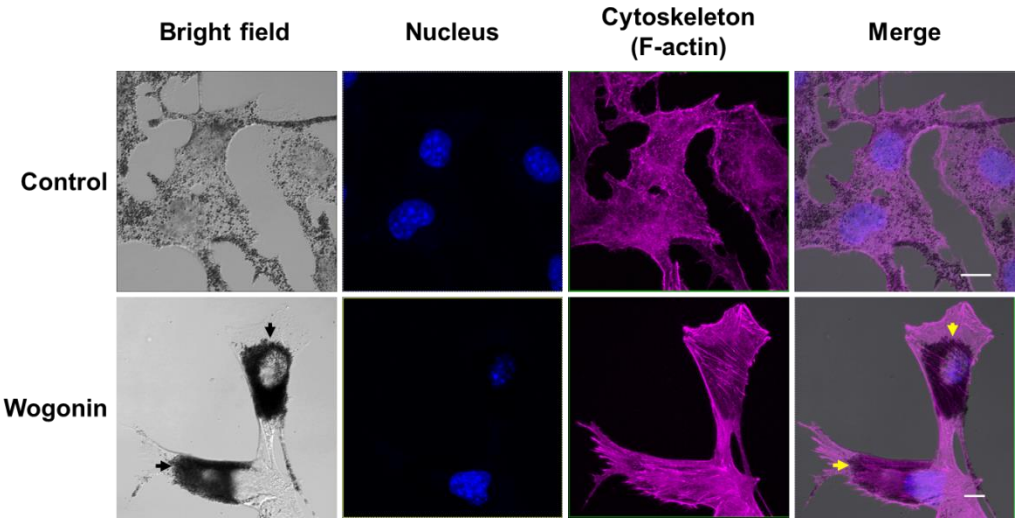

Figure 2. Wogonin induces inhibition of normal melanosome transport. B16F10 cells were treated with 50 μM wogonin for 72 h. Black pigments: melanosome, blue: nucleus, and magenta: cytoskeletal actin filament. Scale bars indicate 10 μm. Melanosomes in the control cells were distributed over the entire cell. However, wogonin-treated cells were observed in the perinuclear melanosome aggregation (arrow head).

To define the structural specificity of wogonin, which is responsible for melanosome transport disorder, B16F10 cells were cultured with several wogonin analogs; i.e., the 8-demethyl wogonins. The demethylated compound of the 8-*O*-methyl group in wogonin, called norwogonin or 5,7,8-trihydroxyflavone, did not induce intracellular melanosome aggregation (Figure 3), suggesting the 8-methoxy group of wogonin is an essential component for the inhibition of melanosome transport. Wogonoside, the 7-*O*-glycoside form of wogonin, was shown to attenuate perinuclear melanosome aggregation (Figure 3).

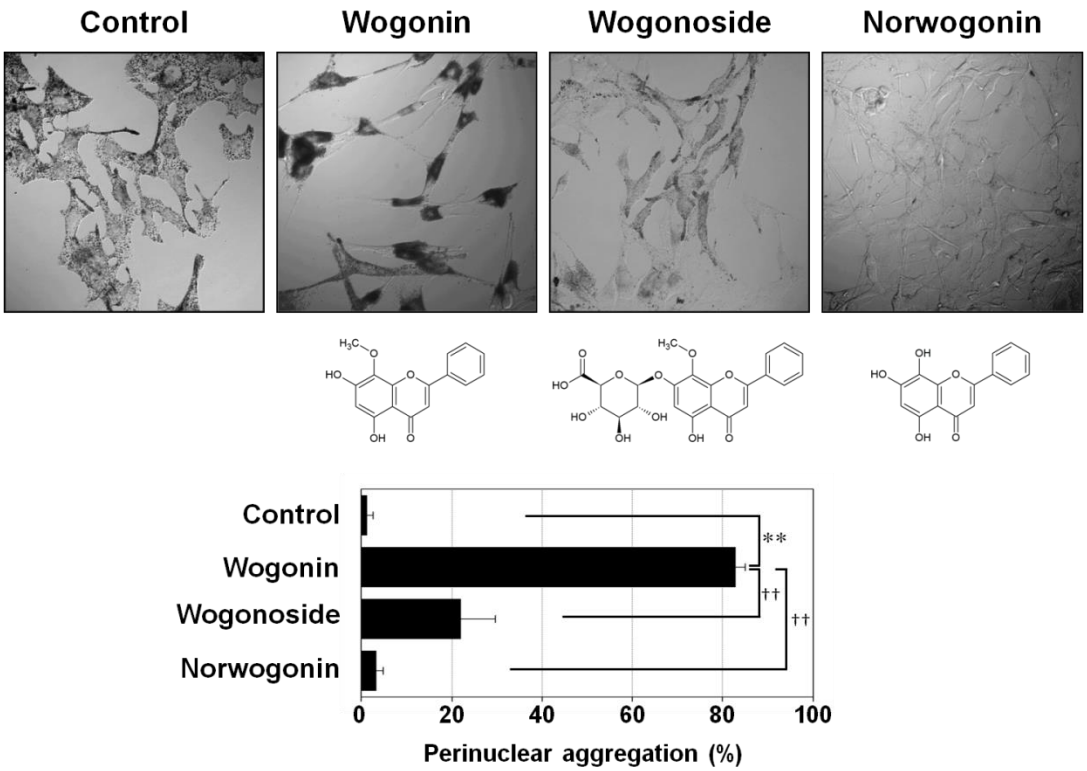

Figure 3. Glycosidation or 8-hydroxylation of wogonin shows diminished effects on perinuclear melanosome aggregation.

Wogoniside is the glycoside of wogonin. Norwogonin (5,7,8-trihydroxyflavone) is a demethylated product of the 8-*O*-methyl group in wogonin. B16F10 cells were cultured for 3 days with wogonin, wogonoside, or norwogonin at a concentration of 50 μM. Values represent mean ± SD of triplicate determinations [**$p < 0.01$ versus the control group; ††$p < 0.01$ versus the indicated group (Tukey–Kramer test)].

Conversely, the 7-*O*-methylation of baicalein (5,6,7-trihydroxyflavone) resulted in the acquired ability to induce impaired melanosome transport (Figure 4). In addition, oroxylin A, a 6-*O*-methyl-positioned isomer, was shown to inhibit melanosome dispersion (Figure 4). Upon comparing the three mono-*O*-methyl isomers (wogonin, 7-*O*-methyl baicalein, and oroxylin A), wogonin was the most potent melanosome transport inhibitor (Figure 4). These results indicate that the mono-*O*-methyl group on the flavone A-ring involves the disruption of melanosome transport. Moreover, with regard to wogonoside, a large substituent at the 7-position, such as glycoside in this study, cannot show enough activity for the inhibition of the normal melanosome transport, probably because of reduced interaction with wogonin's target molecule.

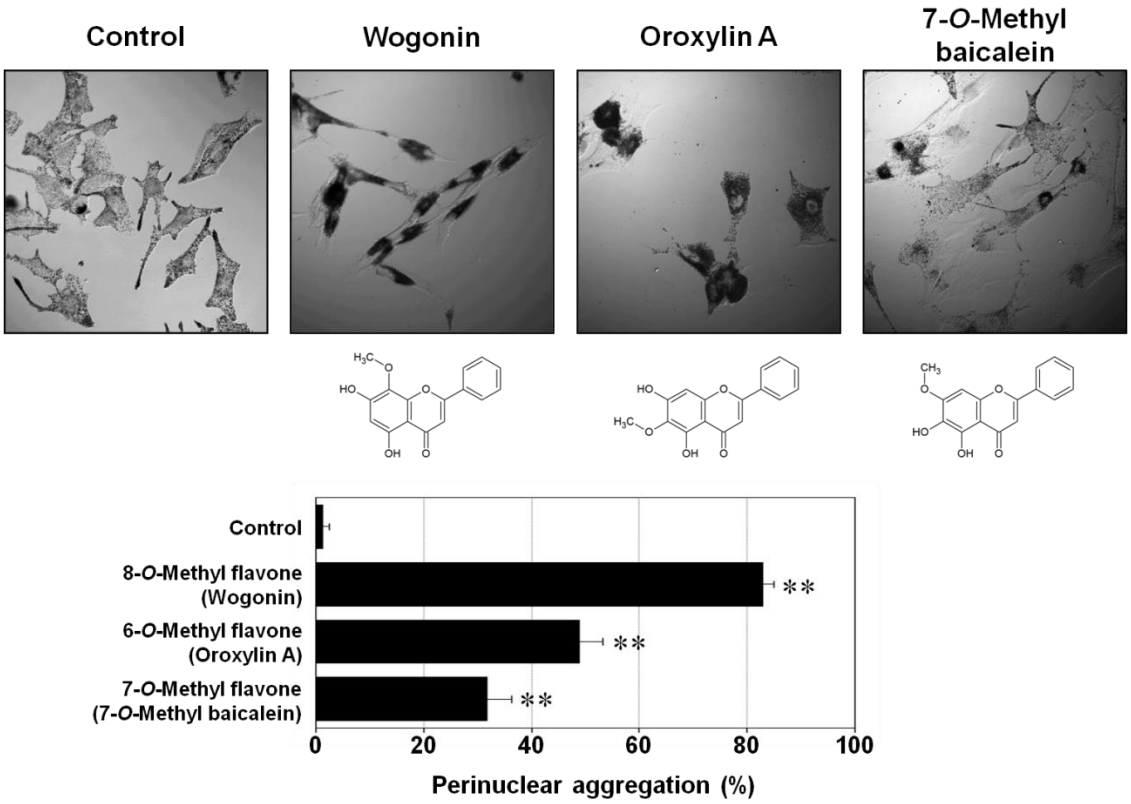

Figure 4. *O*-methyl group in flavone A-ring plays a key role in downregulating melanosome transport. B16F10 cells were cultured for 3 days with wogonin, oroxylin A, or 7-*O*-methyl baicalein at a concentration of 50 μM. Values represent mean ± SD of triplicate determinations [*$p < 0.05$, **$p < 0.01$ versus the control group (Tukey–Kramer test)].

We focused on the similarity in melanosome distribution between wogonin-treated cells (Figure 2) and a leaden-like phenotype, which has been reported to be created by the *mlph* mutation (12). To examine the effects of wogonin on MLPH, we analyzed protein levels of MLPH by immunoblotting. The level of MLPH was significantly reduced by wogonin in a dose-dependent manner (Figure 5), suggesting that wogonin may downregulate the actin-based melanosome transport system by altering MLPH levels.

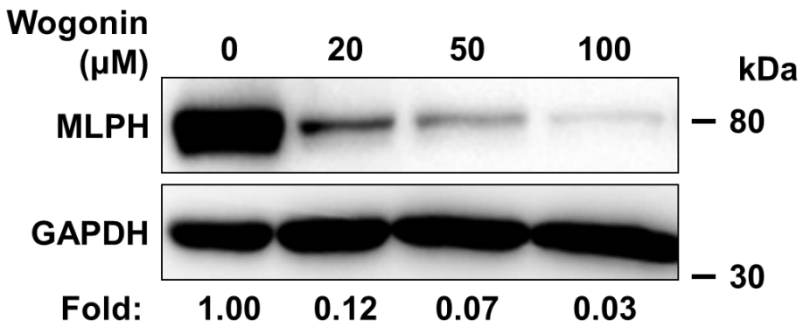

Figure 5. MLPH expression was attenuated by wogonin.

B16F10 cells were treated with 0, 20, 50, and 100 μM of wogonin for 72 h. The MLPH level was determined by western blotting with the anti-MLPH antibody. The expression of the housekeeping protein GAPDH was measured as the control.

Conclusion

For the first time, we show that wogonin induces perinuclear melanosome aggregation, indicating that wogonin inhibits melanosome transport and accumulation in the vicinity of the nucleus. Furthermore, we clarified the structural specificity of wogonin responsible for inhibiting melanosome transport; i.e., the mono-*O*-methyl group in wogonin is essential for the suppression of melanosome transport through the downregulation of MLPH. These findings enable us to propose a new approach for the treatment of pigmentation disorders using mono-*O*-methyl flavones.

References

1. Duval C, Cohen C, Chagnoleau C, Flouret V, Bourreau E, and Bernerd F: Key regulatory role of dermal fibroblasts in pigmentation as demonstrated using a reconstructed skin model: impact of photo-aging. *PLoS One* **9**: e114182, 2014

2. Raposo G and Marks MS. Melanosomes: dark organelles enlighten endosomal membrane transport. *Nat Rev Mol Cell Biol* **8**: 786-797, 2007

3. Maeda K and Fukuda M: Arbutin: mechanism of its depigmenting action in human melanocyte culture. *J Pharmacol Exp Ther* **276**: 765-769, 1996

4. Chang YL, Shen JJ, Wung BS, Cheng JJ, and Wang DL: Chinese herbal remedy

wogonin inhibits monocyte chemotactic protein-1 gene expression in human endothelial cells. *Mol Pharmacol* **60**: 507-13, 2001

5. Polier G, Ding J, Konkimalla BV, Eick D, Ribeiro N, Kohler R, Giaisi M, Efferth T, Desaubry L, Krammer PH, and Li-Weber M: Wogonin and related natural flavones are inhibitors of cdk9 that induce apoptosis in cancer cells by transcriptional suppression of mcl-1. *Cell Death Dis* **2**: e182, 2011

6. Lucas CD, Allen KC, Dorward DA, Hoodless LJ, Melrose LA, Marwick JA, Tucker CS, Haslett C, Duffin R, and Rossi AG: Flavones induce neutrophil apoptosis by down-regulation of mcl-1 via a proteasomal-dependent pathway. *Faseb J* **27**: 1084-1094, 2013

7. Lee W, Ku SK, and Bae JS: Anti-inflammatory effects of Baicalin, Baicalein, and Wogonin *in vitro* and *in vivo*. *Inflammation* **38**: 110-125, 2015

8. Kimura Y and Sumiyoshi M: Effects of baicalein and wogonin isolated from *Scutellaria baicalensis* roots on skin damage in acute UVB-irradiated hairless mice. Eur *J Pharmacol* **661**: 124-132, 2011

9. Lim H, Park H, and Kim HP: Inhibition of contact dermatitis in animal models and suppression of proinflammatory gene expression by topically applied flavonoid, wogonin. *Arch Pharm Res* **27**: 442-448, 2004

10. Ohbayashi N and Fukuda M: Role of Rab family GTPases and their effectors in melanosomal logistics. *J Biochem* **151**: 343-351, 2012

11. Kuroda TS, Ariga H, and Fukuda M: The actin-binding domain of Slac2-a/melanophilin is required for melanosome distribution in melanocytes. *Mol Cell Biol* **23**: 5245-5255, 2003

12. Hume AN, Collinson LM, Hopkins CR, Strom M, Barral DC, Bossi G, Griffiths GM, and Seabra MC: The leaden gene product is required with Rab27a to recruit myosin Va to melanosomes in melanocytes. *Traffic* **3**: 193-202, 2002