Downregulation of Melanocyte-Specific Facultative Melanogenesis by 4-Hydroxy-3-Methoxycinnamaldehyde Acting as a cAMP Antagonist

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TO THE EDITOR

The second messenger cAMP upregulates gene expression of microphthalmia-associated transcription factor (MITF) or tyrosinase in skin pigmentation (Busca and Ballotti, 2000). α-Melanocyte-stimulating hormone (α-MSH) and forskolin are cAMP elevators and stimulate melanin production in melanocytes (Busca and Ballotti, 2000). Intracellular cAMP activates protein kinase A (PKA) from an inactive holoenzyme complex consisting of a regulatory subunit dimer bound to two catalytic subunits (Kim *et al.*, 2007). The regulatory subunit of PKA cooperatively binds with two cAMP molecules, concomitantly releasing the catalytic subunit and thereby allowing phosphorylation of PKA substrates including the Ser-133 residue of cAMP-responsive element-binding protein (CREB; Taylor *et al.*, 2008). The level of phospho (p)-CREB protein can be used as an active index in the upregulation of MITF, a basic helix-loop-helix transcription factor crucial for melanocyte pigmentation (Vachtenheim and Borovansky, 2010). In turn, MITF binds to the M box or E box in the promoter region of the tyrosinase gene for transcriptional activation (Vachtenheim and Borovansky, 2010).

Abnormal accumulation of melanin pigments is responsible for pigmented disorders such as melasma, freckles, and senile lentigo, which can be substantially ameliorated by treatment with arbutin or other tyrosinase inhibitors (Maeda and Fukuda, 1996; Ortonne and Passeron, 2005). Cinnamaldehyde displays an anti-aging potential in skin treatment and inhibits tyrosinase activity in mushrooms (Lee, 2002; Takasao *et al.*, 2012), but its effect on melanocyte pigmentation remains to be defined. Here, 4-hydroxy-3-methoxycinnamaldehyde (4H3MC, Supplementary Figure S1a online) inhibited α-MSH-induced melanin production in B16 mouse melanoma cells and primary human melanocytes with more effectiveness than cinnamaldehyde, 4-hydroxy-3-methoxycinnamic acid, 4-hydroxycinnamic acid, and cinnamic acid (Figure 1a and b, Supplementary Figure S1b online). Moreover, 4H3MC inhibited melanin production in B16 cells stimulated with other cAMP elevators, such as N^6,2′-*O*-dibutyryl (db)-cAMP and forskolin, in which Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS) and H-89 were also effective (Supplementary Figure S2a and b online). Rp-cAMPS is a cell-permeable cAMP antagonist that interrupts PKA activation, and H-89 is a PKA inhibitor that targets catalytic activity after dissociation and activation from the holoenzyme complex (Wu *et al.*, 2004; Lochner and Moolman, 2006). However, 4H3MC at the concentrations with antimelanogenic activity did not affect the viability of B16 cells (Supplementary Figure S2c online), excluding nonspecific cytotoxicity. To understand the *in vivo* efficacy of 4H3MC, we carried out UVR–induced skin pigmentation in guinea pigs (Supplementary Figure S3a online). Topical treatment with 4H3MC decreased melanin contents in the basal layer of the epidermis and ameliorated visual skin pigmentation in UV-irradiated dorsal skins of guinea pigs (Figure 1c, Supplementary Figure S3b online).

Treatment with 4H3MC itself did not affect cAMP levels in α-MSH-activated B16 cells (Supplementary Figure S4a online). To elucidate an antimelanogenic mechanism of 4H3MC, we tested whether it could affect cAMP-induced proximal signaling events. Treatment with 4H3MC or Rp-cAMPS inhibited the dissociation of catalytic subunit-α (PKA-Cα) from the regulatory subunit IIβ of PKA (PKA-RIIβ) in cAMP-elevated B16 cells or primary human melanocytes, whereas H-89 was not effective (Figure 2a and b, Supplementary Figure S4b online). Moreover, 4H3MC inhibited α-MSH-induced PKA activation in B16 cells but did not affect *in vitro* kinase activity of cell-free PKA with catalytic subunit only (Supplementary Figure S5a and b online). These results indicate that 4H3MC directly interrupted cAMP-induced dissociation and activation of PKA holoenzyme.

We next performed a fluorescence analysis with 8-[φ-575]-cAMP, specifically probing the cAMP-binding A and B sites on the regulatory subunit of PKA (Moll *et al.*, 2008). 8-[φ-575]-cAMP increased fluorescence values upon binding to PKA-RIIβ polypeptide in cell-free reactions (Figure 2c). Treatment with non-fluorescent 4H3MC dose dependently decreased the fluorescence intensity of 8-[φ-575]-cAMP (Figure 2c), indicating the displacement of 8-[φ-575]-cAMP from the complex with PKA-RIIβ polypeptide. This mechanism of action was reversible, as fluorescence intensity was recovered in the pre-treatment with 4H3MC followed by competitive titration with excess 8-[φ-575]-cAMP (Supplementary Figure S6 online). Consistent with this, 4H3MC was well fitted into the cAMP-binding A or B site on the crystal structure of

Abbreviations: CREB, cAMP-responsive element-binding protein; db-cAMP, N^6,2′-O-dibutyryl-cAMP; 4H3MC, 4-hydroxy-3-methoxycinnamaldehyde; MITF, microphthalmia-associated transcription factor; α-MSH, α-melanocyte-stimulating hormone; PKA, protein kinase A; PKA-RIIβ, regulatory subunit IIβ of PKA; Rp-cAMPS, Rp-adenosine 3′,5′-cyclic monophosphorothioate

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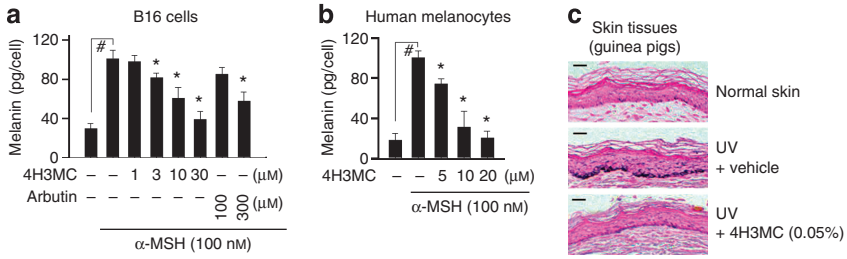

Figure 1. 4-Hydroxy-3-methoxycinnamaldehyde (4H3MC) inhibits melanin production in α-melanocyte-stimulating hormone (α-MSH)-activated melanocyte cultures and UV-irradiated dorsal skins of guinea pigs. B16 cells were stimulated with α-MSH in the presence of 4H3MC or arbutin for 3 days (**a**) and primary human melanocytes for 8 days (**b**). Melanin contents were measured with synthetic melanin as a standard and then normalized to cell numbers. Data are represented as mean ± SD from three separate experiments. $^{\#}P<0.05$ vs. media alone–added group. $^{*}P<0.05$ vs. α-MSH alone-stimulated group. (**c**) Dorsal skins of guinea pigs were topically treated with 4H3MC followed by UVR. Skin tissues biopsied at the 4th week after starting UVR were sectioned and then stained with Fontana–Masson stain (silver nitrate). Bar = 100 μm.

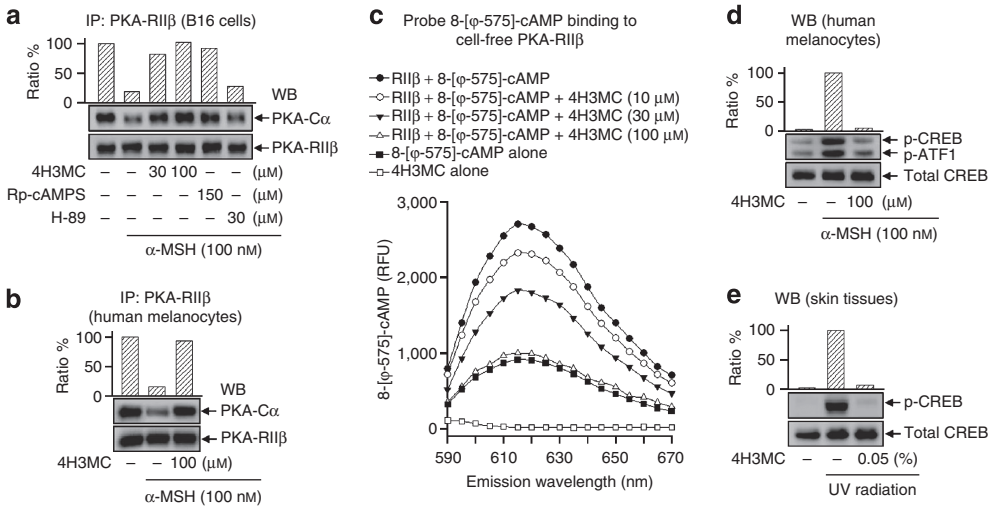

Figure 2. 4-Hydroxy-3-methoxycinnamaldehyde (4H3MC) inhibits α-melanocyte-stimulating hormone (α-MSH)- or UV-induced dissociation and activation of protein kinase A (PKA) holoenzyme. B16 cells (**a**) or primary human melanocytes (**b**) were pre-treated with 4H3MC for 2 hours and stimulated with α-MSH for 10–20 minutes in the presence of 4H3MC. Whole cell extracts were subjected to immunoprecipitation (IP) with antibody against regulatory subunit IIβ of PKA (PKA-RIIβ), followed by western blot analysis (WB) with antibody against PKA-Cα or PKA-RIIβ to detect the co-precipitation. (**c**) Fluorescence probe 8-[φ-575]-cAMP (5 μM) was incubated with cell-free PKA-RIIβ polypeptide (2 μg ml^{-1}) for 2 hours to reach stable fluorescence values and then treated with 4H3MC for 2 hours. Relative fluorescence units (RFU) were measured under emission at 590–670 nm and excitation at 575 nm. (**d**) Primary human melanocytes were pre-treated with 4H3MC for 2 hours and stimulated with α-MSH for 30–40 minutes in the presence of 4H3MC. Cell extracts were subjected to WB with anti-p-CREB or anti-CREB antibody. (**e**) Dorsal skins of guinea pigs were topically treated with 4H3MC followed by UVR. Skin tissues were biopsied at the 4th week after starting UVR. Protein extracts from skin specimens were subjected to WB with anti-p-CREB or anti-CREB antibody.

PKA-RIIβ under the most energetically favorable simulation (Supplementary Figure S7a and b online). Thereby, 4H3MC mimicked Rp-cAMPS or bisabolangelone in the antimelanogenic mechanism, even though its skeleton structure is not analogous to those of the agonist cAMP and the antagonist Rp-cAMPS or bisabolangelone (Roh *et al.*, 2013).

4H3MC consequently suppressed downstream melanogenic pathways. Treatment with 4H3MC inhibited PKA-catalyzed CREB phosphorylation in α-MSH- or db-cAMP-activated melanocyte cultures, whereas no significant change was observed in total levels of CREB (Figure 2d, Supplementary Figure S8a and b online). In this experiment, anti-p-CREB antibody also recognized the phosphorylated form of activating transcription factor-1, closely relating to p-CREB in structure and function (Hummler *et al.*, 1994). Moreover, topical treatment with 4H3MC diminished CREB phosphorylation in UV-irradiated dorsal skins of guinea pigs (Figure 2e). CREB-responsive CRE motifs appear to be essential for maximal MITF induction in cAMP-elevated melanocytes, and MITF has an important role in the melanocyte-specific expression of tyrosinase gene, even though the promoter of MITF or tyrosinase gene contains several *cis*-acting elements (Vachtenheim and Borovansky, 2010). Treatment with 4H3MC suppressed α-MSH-induced gene expression of MITF or tyrosinase in B16 cells at the transcription level, as did Rp-cAMPS (Supplementary Figures S9a–c and S10a–c online). Moreover, topical treatment with 4H3MC attenuated the protein or mRNA levels of both MITF and tyrosinase in UV-irradiated dorsal skins of guinea pigs (Supplementary Figure S11a and b online).

In conclusion, the cAMP-binding site of PKA is a molecular target of 4H3MC

in the antimelanogenic action down-regulating the expression of MITF or tyrosinase gene in cAMP-elevated melanocyte cultures and UV-irradiated dorsal skins of guinea pigs (Supplementary Figure S12 online). Finally, this study suggests a potential application of 4H3MC in the treatment of hyperpigmented skin disorders.

Animal experiments were carried out according to the protocols approved by Animal Experimentation Ethics Committee in CBNU institute.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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A Somatic Mutation of the *KEAP1* Gene in Malignant Melanoma Is Involved in Aberrant NRF2 Activation and an Increase in Intrinsic Drug Resistance

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TO THE EDITOR

Among the several characteristics of malignant melanoma, insensitivity to anti-cancer agents is a frequent clinical problem in the treatment of patients (Grossman and Altieri, 2001). In fact, the most commonly used chemotherapy agents cisplatin and dacarbazine for malignant melanoma elicit a response rate of only 10% (Flaherty, 2010).

The small-molecule inhibitor of BRAF, vemurafenib (also known as PLX4032), elicits potent tumor regression in patients with BRAF-positive stage IV melanoma (Huang *et al.*, 2012), and its use is expected in patients harboring the $BRAF^{V600E}$ mutation (Tsai *et al.*, 2008). However, vemurafenib is not effective against melanomas with wild-type BRAF protein (Joseph *et al.*, 2010).

Acral lentiginous melanoma (ALM) is one of the subtypes of cutaneous melanoma most frequent in colored races (Bradford *et al.*, 2009). In fact, only about 10% of ALM cases harbor the $BRAF^{V600E}$ mutation, compared with over 60% of cases of superficial spreading melanoma (SSM), which is most frequent in Caucasian populations (Saldanha *et al.*, 2006). Because these melanomas are insensitive to BRAF inhibitors (Joseph *et al.*, 2010), a search for molecular targets that would enhance sensitivity to standard treatment with cisplatin or dacarbazine would seem justified.

To address the genes responsible for drug resistance in melanoma, whole-exome sequencing was performed. We identified a single-nucleotide deletion in codon 507 from exon 4 of the *KEAP1* gene, common to MM-RU and PM-WK, as a candidate gene for drug resistance

Abbreviations: ALM, acral lentiginous melanoma; CDDP, cis-diamminedichloro-platinum (II); DTIC, 5-(3, 3-dimethyl-1-triazenyl) imidazole-4-carboxamide; FSM, frameshift mutant; ROS, reactive oxygen species; SSM, superficial spreading melanoma

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