Biochemical and Biophysical Research Communications 390 (2009) 500–505

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cardamonin suppresses melanogenesis by inhibition of Wnt/β-catenin signaling

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ARTICLE INFO

Article history:
Received 23 September 2009
Available online 1 October 2009

Keywords:
Melanogenesis
Cardamonin
Wnt/β-catenin signaling pathway
Protein degradation

ABSTRACT

Wnt/β-catenin signaling plays important roles in many developmental processes, including neural crest-derived melanocyte development. Here we show that cardamonin, a calchone from *Aplinia katsumadai* Hayata, inhibited pigmentation in melanocytes through suppression of Wnt/β-catenin signaling pathway. Cardamonin significantly suppressed the expression of microphthalmia-associated transcription factor (MITF) and tyrosinase, which are melanocyte differentiation-associated markers, in human normal melanocytes, thereby decreasing intracellular melanin production. In addition, cardamonin promoted the degradation of intracellular β-catenin that was accumulated by Wnt3a-conditioned medium (Wnt3a CM) or bromoindirubin-3′-oxime (BIO), a glycogen synthase kinase-3β (GSK-3β) inhibitor, in HEK293 reporter cells and human normal melanocytes. Our findings indicate that cardamonin may be a potential whitening agent for use in cosmetics and in the medical treatment of hyperpigmentation disorders.

Introduction

Melanocytes, which are found in the epidermis of the skin, the integument, and the choroids of the eye, are specialized cells that synthesize melanin, thereby determining eye, hair, and skin color [1]. Melanocytes are derived from neural crest cells, a multipotent pool of precursors that also differentiate into neurons, glial cells, and cartilage cells [2]. Melanocyte differentiation is accompanied by enhanced expression of microphthalmia-associated transcription factor (MITF), a master regulator of melanin synthesis [3,4]. MITF regulates the expression of tyrosinase and tyrosinase-related protein that catalyze the conversion of tyrosine into melanin pigments [5–7].

The Wnt/β-catenin pathway, which is activated by the interaction of Wnt1, Wnt3a, and Wnt8 with Frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5/6 co-receptors [8], controls cell differentiation, cell proliferation, and cell motility [9–12]. The key control in this pathway is the level of intracellular β-catenin, which activates its target genes, such as *c-myc*, *cyclin D1*, and *metalloproteinase-7* [13–16]. In the absence of a Wnt signal, N-terminal serine/threonine residues of β-catenin are sequentially phosphorylated by casein kinase 1 and glycogen synthase kinase-3β (GSK-3β) in a multiprotein complex containing adenomatous polyposis coli and axin [17,18]. Then, phosphorylated β-catenin is recognized by F-box β-transducin repeat-containing protein, a component of the ubiquitin ligase complex, resulting in the degradation of β-catenin via a ubiquitin-dependent mechanism [19]. Activation of the receptor by its Wnt ligands negatively regulates GSK-3β, leading to the accumulation of cytoplasmic β-catenin [20].

A link between Wnt/β-catenin signaling and melanocyte differentiation has been revealed by the finding that β-catenin, which accumulates with activation of Wnt/β-catenin signaling, forms a complex with lymphocyte enhancer factor-1 to up-regulate expression of the MITF gene [21]. Also, β-catenin directly interacts with the MITF protein itself and then activates MITF-specific target genes [22]. In addition to MITF, Wnt/β-catenin signaling is involved in neural crest formation, migration, proliferation, and differentiation [23]. Mice deficient in Wnt1 and Wnt3 lack pigment cells, and this phenotype is probably due to the failure of the early expansion of neural crest cells [24,25]. In the present study, we identified cardamonin as an inhibitor of melanocyte differentiation. Cardamonin promoted degradation of intracellular β-catenin while suppressing melanogenesis by down-regulating the expression of MITF and tyrosinase, resulting in decreased melanin content.

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doi:10.1016/j.bbrc.2009.09.124

Materials and methods

Cell culture, plasmid, transfection, and luciferase assay. Normal human melanocytes were obtained from Cascade Biologics Inc. and grown in melanocyte growth medium 254 supplemented with Human Melanocyte Growth Supplements (HMGS) (Cascade Biologics Inc.). Melanocytes from the third to fifth passage were used in these experiments. Wnt3a-secreting L cells were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 120 μg/ml penicillin, and 200 μg/ml streptomycin. Wnt3a-conditioned medium (Wnt3a CM) was prepared as previously described [26]. The HEK293 reporter and control cell lines were established as previously described [27]. BIO (6-bromoindirubin-3′-oxim) and MG-132 were purchased from Sigma–Aldrich.

Melanin content determination. The cells were seeded in 60 mm plates at a density of 2×10^5 cells and were allowed to attach overnight. Then the cells were incubated with Wnt3a or BIO and various concentration of cardamonin for 4 days. The cells were then washed twice with ice-cold PBS, lysed with RIPA buffer and centrifuged at 10,000*g* for 10 min. Supernatants were analyzed for protein concentration, and pellets were solubilized in 200 μl of 1 M NaOH. Following an incubation period of 2 h at 60 °C, the absorbance was measured spectrophotometrically at 405 nm using a plate reader. Standard curves using synthetic melanin (0–250 μg/ml) were prepared in duplicate for each experiment. Melanin production was calculated by normalizing the total melanin values with protein content (μg melanin/mg protein).

Cell viability assay. Human normal melanocytes were inoculated into 96-well plates and treated with cardamonin for 4 days. The cell viability from each treated sample was measured in triplicate using CellTiter-Glo assay kit (Promega) according to the manufacturer's instructions.

Western blotting. The cytosolic fraction was prepared as previously described [28]. Whole cell extracts were prepared with RIPA buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.2% sodium dodecyl sulfate (SDS), 5 mM NaF). Proteins were separated by 4–12% gradient SDS–PAGE (Invitrogen) and transferred to a nitrocellulose membrane (Amersham Biosciences). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin (BD Transduction Laboratories), anti-tyrosinase (Santa Cruz Biotechnology), anti-MITF (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology), and the bands were visualized using the ECL system (Santa Cruz Biotechnology).

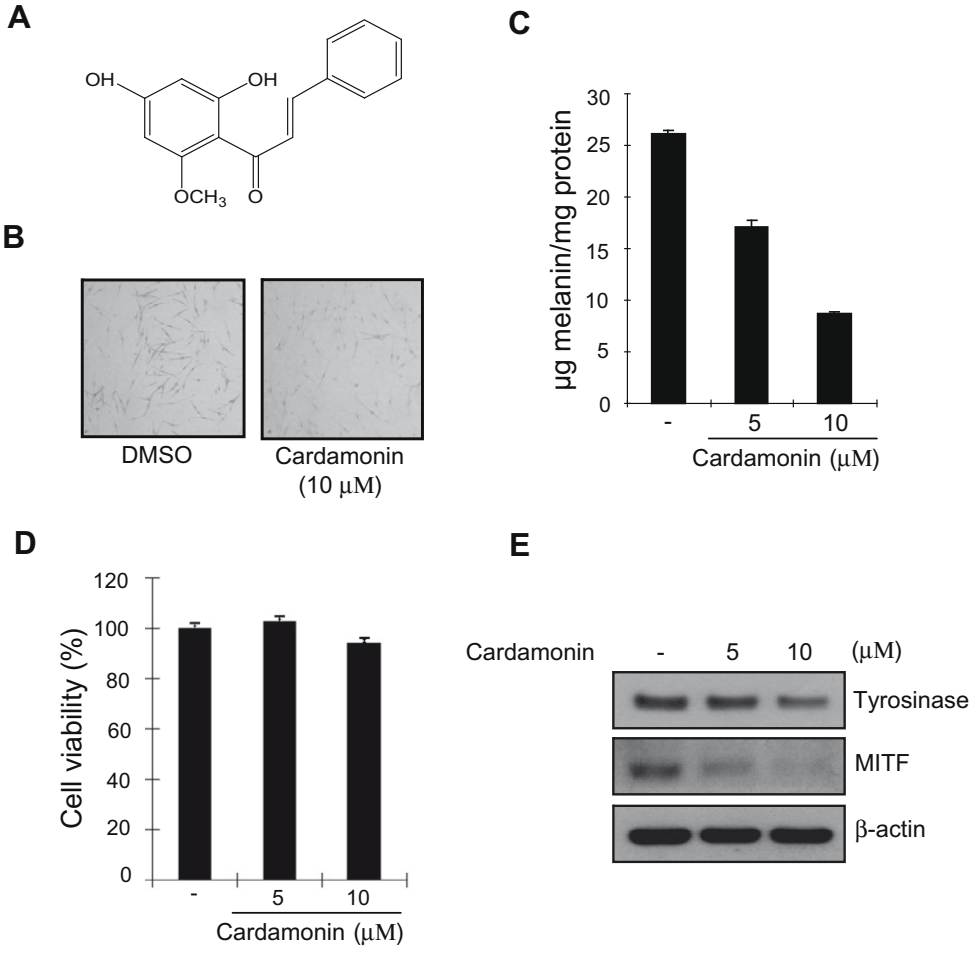

Fig. 1. Cardamonin decreases the pigmentation of human normal melanocytes. (A) Chemical structure of cardamonin. (B) Phase-contrast micrographs with DMSO or cardamonin (10 μM)-treated melanocytes. (C) Melanin production by DMSO or cardamonin-treated melanocytes was detected by the melanin assay as described in Materials and methods. (D) Growth of melanocytes treated with DMSO or cardamonin was measured by CellTiter-Glo assay as described in Materials and methods. In (C) and (D), the results are the average of three experiments, and the bars indicate standard deviations. (E) Cell extracts were prepared from human normal melanocytes treated with either DMSO or cardamonin (10 μM) for 4 days and then subjected to Western blotting with β-catenin, MITF, and tyrosinase antibodies. The blots were re-probed with anti-actin antibody as a loading control.